Induced mutation and somatic recombination as tools for genetic analysis and breeding of imperfect fungi

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Induced mutation and somatic recombination as tools for genetic analysis and breeding of imperfect fungi

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WAGENINGEN

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S t e l l i n g e n

1. Ávalos et al. (1985) komen op grond van hun experimenten met *Giberella fujikuroi* tot een aantal onjuiste beweringen en conclusies ten aanzien van het effect van preincubatie, het gebruik van hoge doses mutagens en het effect van NG in fungi.
   J.Ávalos, J.Casadesús, E.Cerdá-Olmedo, 1985. Giberella fujikuroi mutants obtained with UV radiation and N-methyl-N'-nitro-N-nitrosoguanidine. Appl. Environ. Microbiol. 49, 187-191.

   Dit proefschrift, hoofdstuk 2.

2. Bij *Colletotrichum lindemuthianum* speelt recombinatie waarschijnlijk een geringe rol bij het ontstaan van nieuwe fysio's.

3. Een heterokaryon is een dynamisch systeem gebaseerd op hyfen fusies en segregatie van homokaryotische hyfen van de oudertypes. Dientengevolge wordt de frekwentie van somatische karyogamie gewoonlijk sterk onderschat.

   Dit proefschrift, hoofdstuk 3.

4. Het onderscheiden van horizontale versus verticale resistentie heeft slechts descriptieve betekenis. Horizontale resistentie is vaak resistentie waarvan nog niet is aangetoond dat ze verticaal is.
   A.H.Ellingboe, 1981. Changing concepts in host-pathogen genetics. Ann. Rev. Phytopathol. 19, 125-143.
   R.Johnson, 1984. A critical analysis of durable resistance. Ann. Rev. Phytoplathol. 22, 309-330.
   J.E.Parlevliet en J.C.Zadoks, 1977. The integrated concept of disease resistance: a new view including horizontal and vertical resistance. Euphytica 26, 5-21.

5. Er zijn aanwijzingen dat mitotische overkruisingen in het bijzonder plaats vinden in specifieke cellen die daartoe in staat zijn. *Aspergillus niger* zou een zeer geschikt object zijn om dit te bestuderen.

   Dit proefschrift, hoofdstuk 4.

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6. Het verdient aanbeveling dat zowel veredelaar als fytopatholoog de notatie '+' gebruiken voor een compatibele reactie van een waardplant-parasiet combinatie.

7. De "resistentie veredeling" is een sprekend voorbeeld van 'man-guided' evolutie en verhoogt stellig de 'fitness' van de veredelaar.

8. De in de natuur voorkomende overeenkomsten en verschillen tussen organismen zeggen meer over de architectuur dan over de ontstaanswijze.

9. Het wetenschappelijk onderzoek kan zodanig grensverleggend zijn, dat bij toepassing het vermogen om verantwoorde beslissingen te nemen in essentie te kort schiet.

10. Als gevolg van de STC-operatie in het HBO dreigen de Middelbare Laboratoriumopleidingen (MLO) tussen wal en schip te raken vanwege de onomkeerbaarheid van de in gang gezette processen.

11. Het is kortzichtig om er van uit te gaan dat funkties op middelbaar niveau grotendeels door mensen met een hogere beroepsopleiding vervuld kunnen worden.

12. Het is een goede investering indien de plaatselijke overheid sociale activiteiten als sportverenigingen en muziekgezelschappen die zich in het bijzonder op de jeugd richten, in sterke mate financieel steunt.

13. Het afdwingen van deeltijdwerk c.q. werktijdverkorting beknot de persoonlijke vrijheid tot werkverdeling op grond van aanleg en voorkeur.

-.-

Stellingen bij het proefschrift van C.J.Bos: 'Induced mutation and somatic recombination as tools in genetic analysis and breeding of imperfect fungi'. Wageningen, 3 januari 1986.

Voorwoord

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Aan dit proefschrift liggen veel experimenten ten grondslag. Aan het onderzoek heeft menig student zijn steentje bijgedragen. Zonder voortdurende assistentie zou ik toch niet ver gekomen zijn: graag wil ik Nel Pieper-Brummelkamp, Marijke Slakhorst-Wandel, Gerda Kobus-Coops, Sjaak van Heusden, Edu Holub, Anja Huibers en Mw. Selbach bedanken voor hun inzet en voor de prettige samenwerking.

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Curriculum vitae

1. General introduction

1.0 Genetic mechanisms in fungi

In contrast to higher plants where the haploid phase (gametophyte) is restricted to only a few nuclear divisions before gametic fusion respectively karyogamy takes place, the fungal organism is haploid. At the sexual stage, karyogamy takes place in specialized binucleate hyphal cells and is immediately followed by meiosis of the resulting tetrad mother cell (see Fig.1.1). The meiotic mechanism of higher and lower eukaryotes is essentially similar.

Many fungal species, however, are 'imperfect', which means that they do not propagate sexually, or at least that a sexual stage is not known. Several species of the imperfect fungi are important in agriculture as plant pathogens, or in biotechnology as producers of amino acids, carboxylic acids, antibiotics and enzymes.

Although imperfect fungi have no meiotic recombination they have, like many perfect fungi, an effective somatic recombination mechanism known as the parasexual cycle (Pontecorvo, 1954) or better the parasexual sequence. In plant pathogenic fungi these processes confer genetic flexibility on the populations; in imperfect fungi of industrial interest somatic recombination provides possibilities for breeding production strains. In general somatic recombination enables genetic analyses of imperfect fungi.

The parasexual sequence has been extensively studied in Aspergillus nidulans, an ascomycete with a functional sexual stage (perfect stage). Its proper name is Emericella nidulans, but the fungus is generally known by its 'imperfect name'. In this orgamism the outcome of somatic recombination can be analysed with the help of meiotic products and the effects of the two recombination mechanisms can be compared. The basis for this work was laid in the fifties by Pontecorvo and co-workers

at Glasgow and has since been gradually extended, especially in Great Brittain. Recently A.nidulans received renewed attention by the development of protoplast techniques and host-vector systems for genetic manipulation. Figure 1.1 gives the generative and the vegetative life cycle of A.nidulans.

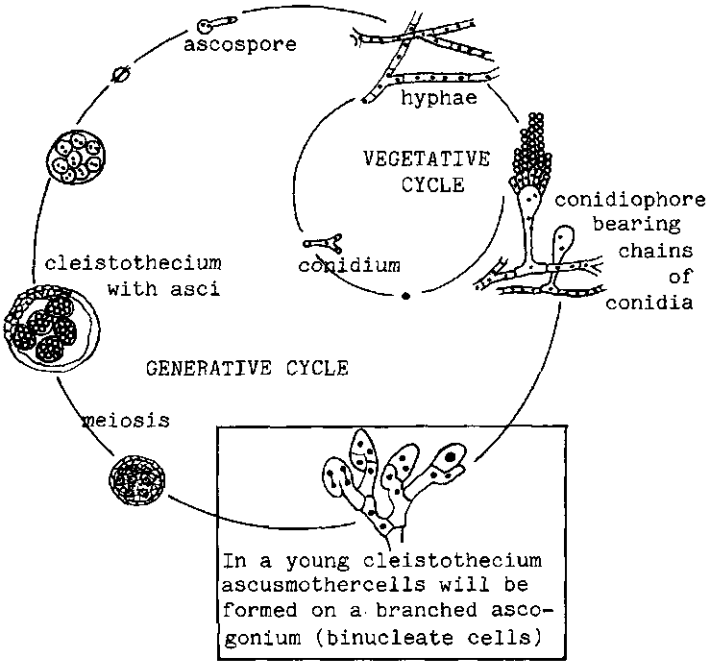


Figure 1.1 Life cycle of Aspergillus nidulans (Eidam) Wint.

On solid media these fungi grow as mycelial colonies. The cells of the branched mycelium are multinucleate and the septa have a pore allowing intercellular migration of nuclei. On specialized cells (conidiophores) vegetative spores (conidiospores or conidia) are formed. In A.nidulans the conidiospores are formed in chains each arising from one uninucleate sterigma. So the conidia of the same chain are genotypically identical, but chains may differ in genotype when the mycelium is heterokaryotic (i.e. when it contains nuclei of different genotype). Wildtype A.niger strains usually have long conidiophores, so that the spores readily disperse and lead to contaminations in

the laboratory. Wildtype A.nidulans strains have green, wildtype A.niger strains have black condiospores, so the colonies appear green resp. black. Mutant strains with other colours (yellow, white, pale, fawn, olive-green)) are useful for genetic studies.

The parasexual processes which may lead to somatic recombination consist of a number of steps. Here the basic aspects of these steps are briefly summarized.

Between two hyphae of the same strain or of compatible strains hyphal fusions (anastomoses) may occur, so that nuclei of different hyphae can migrate. If fusion is between hyphae of genotypically different mycelia, this gives rise to heterokaryons. In the conidiospores the two parental types will reappear. Sometimes somatic nuclei in the mycelium fuse and if fusion is between two genotypically different nuclei, a heterozygous diploid nucleus results. The heterokaryon will then produce diploid conidia and so heterozygous diploid colonies can be obtained. Details on heterokaryosis and somatic karyogamy are presented in Section 1.2.

In somatic diploid nuclei two recombination processes occur: mitotic crossing-over (between non-sisterchromatids of homologous chromosomes) and haploidization. Mitotic crossing-over results in recombinant chromatids. Haploidization results from mitotic non-disjunction of sisterchromatids which leads to aneuploid nuclei (2n-1, 2n-1-1, etc.) and by successive loss of chromosomes ultimately to haploid nuclei. During haploidization genes on the same chromosome segregate simultanously as linkage groups. Mitotic crossing-over enables recombination of linked genes. For details on these mechanisms for somatic recombination see Section 1.2.

1.1 Scope and outline of the present study

The imperfect fungus Aspergillus niger is of considerable biotechnological interest. Nevertheless hardly any systematic genetical research on it has been published so far.

Recently we started a genetic program with A.niger, the first step being the establishment of a collection of mutants with well defined markers to be used for the construction of master-strains. In this program we can profit from extensive experience with the imperfect fungus Colletotrichum lindemuthianum and the perfect fungus Aspergillus nidulans, which is one of the genetically best explored fungi. Aspergillus nidulans is used by us as a model organism for the development of specific techniques, besides being an ideal organism for the training of research students.

Much of the work was carried out as small research studies in which students participated and has not been published so far. However, in the course of time the different parts could be fitted together. Moreover, the obvious possibilities for application in the A.niger program justify presentation. It is an appropriate selection of the results with C.lindemuthianum, A.nidulans and A.niger.

In Chapter 2 methods for the optimalization of UV-mutagenesis procedures are explored, with emphasis on survival curves, mutant yield, and isolation and enrichment procedures, mainly with A.nidulans.

In Chapter 3 experiments on heterokaryosis by hyphal fusion in C.lindemuthianum are reported, which did not lead to unambiguous results. Another way to induce heterokaryosis, and ultimately somatic recombination, is protoplast fusion. Preference was given to conidial protoplasts and methods for protoplast isolation from conidiospores were developed. A.nidulans is used as a model to study protoplast fusion and karyogamy. Part of this work has been published earlier (Bos and Slakhorst, 1981; Bos et al., 1983; Bos, 1985). Protoplast fusion was applied to C.lindemuthianum to see whether heterokaryons could be established.

Chapter 4 deals with A.niger and gives the first results of the isolation of induced mutants suitable as genetic markers, and of a number of genetic analyses by somatic recombination. Special attention is paid to a number of complications which arise in such analyes.

1.2 Summary of somatic recombination processes

For a good understanding of somatic recombination, we summarize here the course of events and some details on the significance of the processes.

1.2.1 HETEROKARYOSIS

When a mycelium contains nuclei of different genotype it is called a heterokaryon. It can arise through mutation in a homokaryon, but in our study the main origin is the combination of existing genotypically different nuclei.

Between two hyphae of the same strain, or of compatible strains, hyphal fusions (anastomoses) may occur so that nuclei can migrate. Heterokaryons can be selected for by combining on minimal medium (MM) two strains with different auxotrophic markers, i.e. strains which are blocked in essential metabolic pathways. So in principle heterokaryons will grow on MM. If, in addition, the strains differ in colour, these heterokaryons show a dense mixture of the two conidial colours. This process is illustrated in Fig. 1.2. A heterokaryon can only be maintained if effective selection for the two complementary auxotrophies takes place, for on complete medium (CM) or supplemented medium (SM) one finds segregation (sorting out) of the two parental types. However, also on MM sorting out of nuclei occurs and depending on the types of deficiency in the parents some homokaryotic hyphae may grow among the otherwise heterokaryotic mycelium by cross-feeding. In general only part of a heterokaryon will be truly heterokaryotic.

In fungi with multinucleate conidiospores the heterokaryotic condition can be maintained by vegatative propagation.

Heterokaryons are useful for complementation studies. Two phenotypically similar mutants usually complement in a heterokaryon if different genes are involved. Because in some cases intragenic complementation can take place, only when two mutants do not complement they are considered as alleles of the same gene. However, some types of non-allelic mutants only complement in diploid nuclei (Roberts, 1964).

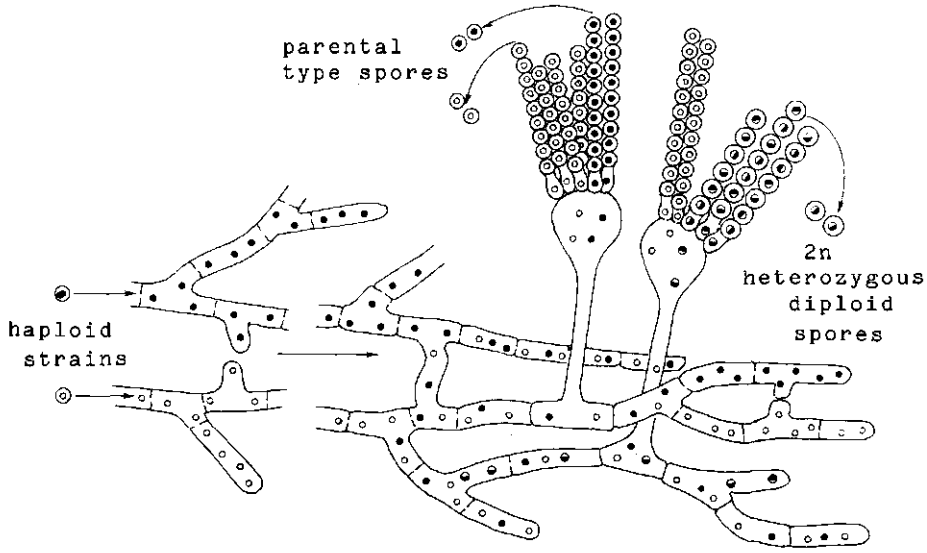


Figure 1.2 Heterokaryosis in A.nidulans.
Balanced heterokaryons produce conidia with parental genotypes and at low frequency also heterozygous diploid conidia.

1.2.2 SOMATIC KARYOGAMY

In the vegetative mycelium diploid nuclei can arise by fusion of two nuclei or by restitution of all chromosomes in one nucleus during mitosis. Somatic karyogamy can only be recorded when different nuclei in a heterokaryon fuse to give heterozygous diploid nuclei. The resulting heterozygous diploid conidia can be identified by their capacity to grow on MM. The process is illustrated in Fig 1.2. Selection of heterozygous diploids is usually performed in a sandwich of MM as shown in Fig 1.3.

The frequency of heterozygous diploid conidia is both species and strain dependent. For A.nidulans frequencies of about 10^{-6} are found. Clutterbuck and Roper (1966) mentioned that diploid nuclei are twice as frequent in hyphae as in conidia. However it is difficult to obtain a reliable estimate of the frequency

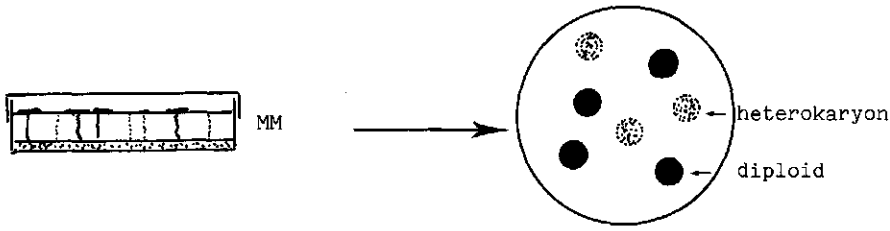


Figure 1.3 Isolation of heterozygous diploid colonies.
Conidiospores collected from heterokaryon are plated in a MM layer (bottom) and covered with another layer MM. After 4-6 days incubation at 37°C diploid colonies (and also some heterokaryons) appear.

of karyogamy. Due to sorting out variable parts of a heterokaryotic mycelium become homokaryotic. Moreover, diploid sectors of varying size can be present in a heterokaryon. As will be shown in Chapter 3, protoplast fusion opens a way for a more realistic estimation of the frequency of somatic karyogamy. Naturally occurring diploid strains have been found in plantpathogenic fungi (Caten and Day, 1977) as well as in biotechnology by important fungi. In some fungal species (e.g. Cladosporium cucumerinum, Van Tuyl, 1977) diploids have been found while no balanced heterokaryons could be established.

1.2.3 MITOTIC CROSSING-OVER

Mitotic crossing-over was first observed by Stern (1936) in somatic cells of Drosophila from so called twin spots. The phenomenon has been studied extensively in A.nidulans, which is very suitable for the purpose. The frequency of mitotic crossing over is high (approximately 10^{-3} per diploid nucleus), collections of several hundreds of genetic markers are available and heterozygous diploids can be easily selected for.

The phenomenon has been observed in several other fungal species (see e.g. reviews by Bradley, 1962; Caten, 1981), where the frequency of mitotic crossing-over might be somewhat higher or lower.

Essentially mitotic crossing-over may take place either in the G1-phase between homologous chromosomes or after DNA-replication in the G2-phase, i.e. between non-sisterchromatids of homologous chromosomes. From experimental data it is concluded that mitotic crossing-over occurs predominantly or exclusively in the G2-phase (Roper and Pritchard, 1955; Käfer, 1961, 1977).

In a heterozygote, mitotic crossing-over between non-sister-chromatids can result in homozygosity of all markers distal of the point of exchange. Homozygous daughter cells result only in one of the two possible anaphase assortments of the chromatids (1+3 and 2+4 in Fig. 1.4). As mitotic crossing-over is fairly rare, in general only one cross-over will take place between locus and centromere.

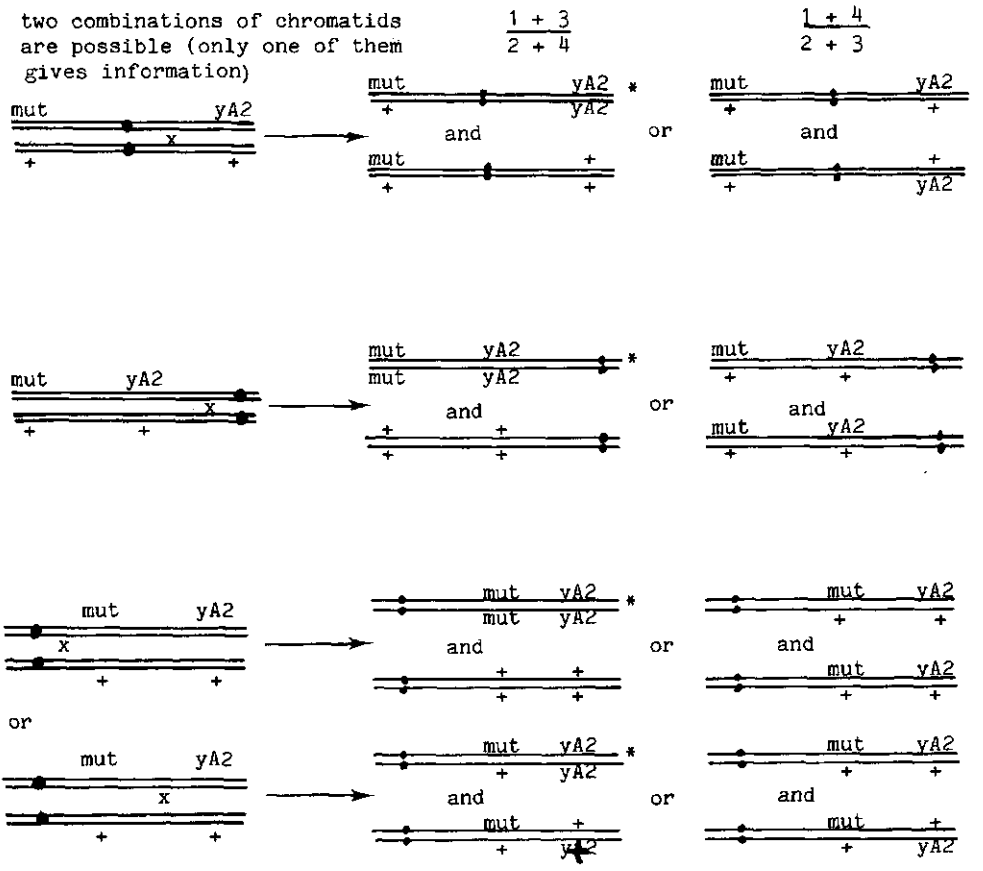


Figure 1.4 Consequences of mitotic crossing-over.
In this example homozygous yellow (yA2) diploids (*) are selected and tested for another mutant phenotype (mut). If e.g. all yellow diploids have the mut-phenotype then the linear arrangement is: mut-------yA2-------centromere (second situation).
Note that a cross-over must occur between yA2 and the centromere, otherwise no yellow diploids will be obtained.

Mitotic crossing-over provides a tool for the determination of the relative distances of genes with respect to the centromere (mitotic mapping). Note that pairs of recessive markers must be in cis-position (i.e. on the same chromosome) because otherwise homozygous recombinants for both recessive genes do not arise. In Fig. 1.4, if mut is located further from the centromere than yA2, the yA2 (yellow) homozygotes (which can be isolated upon visual selection) are always also homozygous for mut (recessive mutant allele on a given locus). If mut is on the same chromosome arm and closer to the centromere a certain ratio of mut and mut^+ phenotypes will be found.

In general the availability of good selection markers terminal on the chromosome arm is a prerequisite. On the basis of different types of markers a number of selection systems have been developed (Pontecorvo et al, 1953). Genetic analysis of A.niger wholly depends on somatic recombination and has been hampered by the absense of suitable selection markers (Pontecorvo, Roper and Forbes, 1953; Lhoas, 1967).

1.2.4 HAPLOIDIZATION

A.nidulans has eight very small chromosomes. During mitosis sister-chromatids sometimes fail to separate. In diploid A.nidulans, as a result of mitotic non-disjunction, aneuploid nuclei arise at a frequency of about 2.10^{-2} (Käfer, 1961, 1977). These aneuploids (2n+1 and 2n-1) have impaired growth as a consequence of genome inbalance. A monosomic cell (2n-1) will, by successive loss of other chromosomes, give rise to a haploid cell with a complete set of chromosomes and normal growth. So, on complete medium haploid segregants are present in addition to homozygous diploid mitotic crossing-over products. They can be observed visually if the diploid is heterozygous for suitable colour markers. The process of haploidization is illustrated in Fig. 1.5.

The frequency of non-disjunction can be enhanched by addition to the medium of substances which interfere with the formation of the spindle, such as p-fluorophenylalanine, benomyl, and to a lesser extent chloralhydrate. On these plates not only the

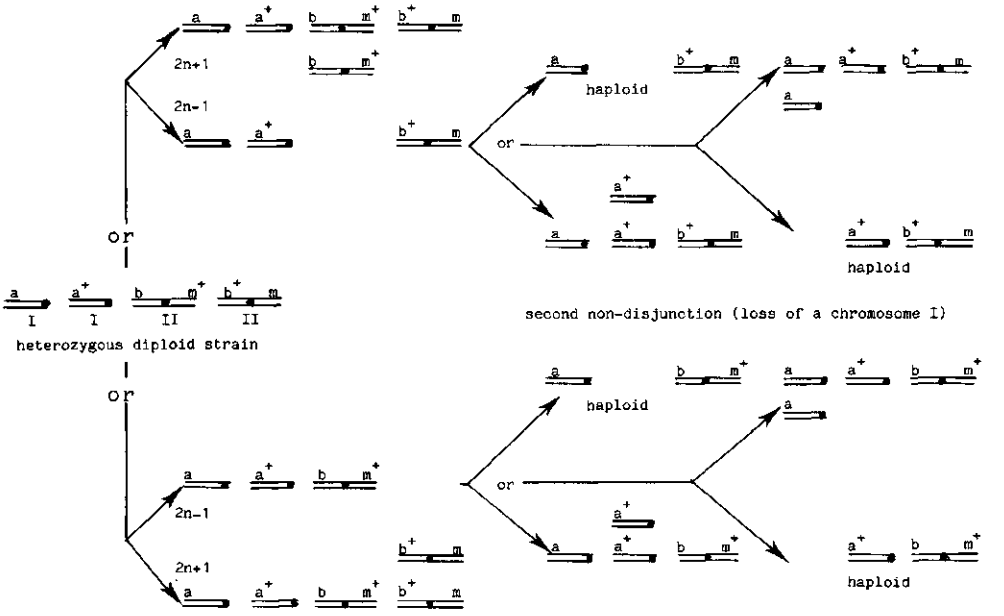


Figure 1.5 The process of haploidization in A.nidulans for 2n = 4. Two modes of non-disjunction for one chromosome II (with m resp. m^+) and subsequent haploidization are illustrated . Only aneuploids and haploids are shown.

aneuploid hyphae grow poorly, but also the diploids have reduced growth. So haploid segregants can be isolated.

The segregants are purified by a number of transfers to fresh plates and tested for the presence of the various markers. Two types of segregants (haploids and homozygous diploids) can be distinghuished by means of certain markers. Moreover, diploid conidia have about twice the volume of haploid conidia, which facilitates the identification of diploid segregants.

2. Induction and isolation of mutants

2.0 Introduction

2.0.0 GENERAL ASPECTS

Mutations arise spontaneously at low frequencies. Their frequency can be highly enhanced by mutagenic treatment. Some of these mutations have little or no effect on the phenotype, but others cause a failure of essential metabolic functions. It is also possible that the secondary gene products (enzymes) are changed in such a way that they have new potentialities (e.g. other or new substrate specificity).

As the geneticist requires mutants for analyzing a variety of processes, and in general also a collection of strains with suitable marker genes, and because in industrial microbiology many production strains have been and are still being obtained by mutation breeding, optimalization of procedures of mutation induction deserves special attention.

A mutational lesion may occur in only one of the two DNA strands, and after DNA replication it should give a mutant and a non-mutant double helix. Consequently, upon mitosis only one of the two daughter nuclei is expected to carry the mutation. However, upon mutagenic treatment of uninucleate cells predominantly both daughter nuclei carry the same mutation, since in general uniform, i.e. non-heterokaryotic, colonies arise. This means that mutations either directly involve both strands, or that single strand mutations by some mechanism lead to a mutation in the complementary strand (Kimball, 1964; Haefner, 1967; Nasim and Auerbach, 1967; James et al., 1978; Kilbey, 1984).

Resting spores are generally in the G1 phase (situation before DNA-replication). A mutation arising in G2 (post DNA-replication) will again be carried in only one of the two chromatids respectively only one of the two daughter nuclei.

In A.nidulans the conidia are uninucleate. Other fungal species may have binucleate or multinucleate conidiospores. Here a mutation gives rise to a heterokaryon. Unless the mutant

allele is dominant, the mutant can only be found after sorting out of nuclei or upon vegetative propagation.

2.0.1 CHOICE OF MUTAGEN

Apart from point mutations, mutagenic treatment can induce larger deletions or chromosomal aberations, especially with ionizing radiations like X-rays. Such disturbances in the genetic background occur to a far lesser extent with UV. Moreover, UV equipment can be easely installed in any laboratory. These are the reasons why we restricted mutation induction mainly to UV.

Alkylating agents are an alternative. Especially nitrosoguanidines (NG) are very effective mutagens (e.g. Gichner and Veleminsky, 1982) and are applied in fungi by several workers. Apart from requiring very careful handling in view of their carcinogenicity, NG-compounds often produce a sequence of mutations, i.e. very closely linked mutations in the progressing DNA-replication fork (Carter and Daves, 1978; Cerdá-Olmedo and Reau, 1970; Calderón and Cerdá-Olmedo, 1982). Significantly, Van Tuyl (1977) observed that various auxotrophic mutants of A.niger obtained by NG-treatment showed slow growth and poor sporulation, in contrast to similar mutants obtained by UV-treatment. For these reasons we did not make use of these compounds.

Using UV-mutagenesis some specificity may exist for attacking pyrimidine nucleotides and that certain genes may be less sensitive to UV than others (Prakash and Sherman, 1973). However, UV can in principle induce mutations in any gene, causing substitution, insertion and deletion of basepairs and also a small amount of somewhat larger intragenic deletions (Kilbey et al., 1971). In general mutation induction depends more on DNA-repair mechanisms than on specificity of action of the mutagen (Ishii and Kondo, 1975; Balbinder et al., 1983).

2.0.2 THE PROBLEM OF MUTAGEN DOSE

For the induction of mutants mostly high mutagen doses are applied, which result in low survival of the treated spores, but in relatively high frequencies of desired mutants among survivors. Often linear relationships between the frequency of

mutants among survivors and (the logarithm of) the mutagen dose are presented. This leads to the notion that it is profitable to isolate mutants at low levels of survival.

For obtaining high yielding production strains in industrial practice, cells are treated with a mutagenic agent until a certain 'desired' kill is obtained and the survivors are tested for production characteristics. Survival levels of 0.1% to 5 % seem to be common practice (e.g. Das and Ilczuk, 1978; Nevalainen, 1981).

However, high doses of mutagen can result in chromosomal aberrations (Käfer, 1977) and in general disturb the genetic background by an enhanced load of undesirable mutations especially when recurrent mutagenic treatment is given, as is often the case with strains of industrial interest. If mutants can be crossed to wildtype this can be traced and cured. When the progeny consist of two homogeneous classes (wildtype and mutant type), this is an indication that the mutant is 'clean'. Imperfect fungi, however, cannot be outcrossed to remove (accumulated) background damage. Upshall and co-workers go another way: they use only spontaneous mutants under the assumption that these will be unlikely to carry multiple aberrations (Upshall et al., 1979; Teow and Upshall, 1983).

The question can be raised whether such high mutagen doses are necessary at all, for appropriate selection procedures and enrichment techniques can considerably enhance the mutant yield and thus compensate for the lower frequency at lower dose. In this connection, attention will be paid to mutagenic treatment and its effect on survival (Section 2.2), mutant yield (2.3) and to enrichment procedures which can be applied to filamentous fungi (2.4).

2.1 Material and methods

2.1.0 ORGANISMS AND STRAINS

The following Aspergillus nidulans strains descending from Glasgow strains were used. The A.nidulans strains in our collection have a WG number. Gene symbols are according to Clutterbuck (1984); markers on different chromosomes are separated by a semicolon, if not they are on the same chromosome.

WG015 biA1; pyroA4; pA1 (biotine and pyridoxin requiring; pale conidia)

WG076 yA2; nicA2, riboD5 (yellow conidia; nicotinamide and riboflavin)

WG094 pabaA1; pyroA4 (p-aminobenzoic acid; pyridoxin)

WG096 pabaA1, yA2 (p-aminobenzoic acid, yellow conidia)

WG136 biA1; wA3; pycB4 (biotin; white conidia; pyruvatecarboxylase)

WG145 wA3; pyroA4 (white; pyridoxin)

WG176 biA1; uvsD53 (biotin; UV-repair deficient) (uvsD53 from a cross with UT517 kindly provided by Dr.G.J.O.Jansen, Utrecht)

WG179 yA2; nicA2, riboD5 (yellow; nicotinamide, riboflavin).

WG282 pabaA1, yA2; acrA1; metG1; lacA1; choA1 (p-aminobezoic acid, yellow; acriflavin; methionine; lactose; choline).

An Aspergillus niger wildtype strain was obtained from CBS (Baarn, The Netherlands): N400 (= CBS 120-49). From this strain morphological and auxotrophic mutants were derived by UV-mutagenesis as will be described in Section 2.2. The following strains were used:

N402 cspA1: a strain with low conidiophores descending from N400

N410 cspA1; fwnA1 (a fawn mutant from N402)

N408 cspA1; argA1 (an arginine requiring mutant from N402)

N420 cspA1; lysA1 (a lysine requiring mutant from N402)

An Aspergillus oryzae wild type strain was also obtained from CBS (Baarn, The Netherlands) (= CBS 574.65).

For long term preservation of strains (cf. Perkins, 1962), conidia were collected from a conidial suspension (see below) by centrifugation or filtration, and resuspended in a mixture of

skimmed milk (5%) and sodium glutaminate (4%) Aliquots of 0.1 ml were added to small ice-cooled screw cap vials containing 0.5 g sterile silicagel (without indicator), immediately stirred on a vortex mixer and put back on ice for about ten minutes. The vials were stored in the refrigerator (4°C).

For short term storage of strains, 3 day old cultures were stored in a refrigerator, prior to experimental use during mostly 3 (-5) days.

2.1.1 MEDIA

The fungi were grown on minimal medium (MM), supplemented MM (SM), complete medium (CM) (composition according to Pontecorvo et al., 1953) or malt extract agar (ME).

MM: contains per litre demineralized water

6.0 g $NaNO_3$,
0.5 g KCl,
0.5 g $MgSO_4.7H_2O$,
1.5 g KH_2PO_4,
Traces (one crystal) $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$.

SM: MM supplemented with growth factors (amino acids 20 mg/ml; vitamins (0.2 mg/ml).

CM: contained in addition to MM per litre:

2 g neopeptone,
1 g casamino acids,
1 g yeast extract,
0.3 g ribonucleic acid hydrolysate (sodium salt),
2 ml vitamin stock solution.

The vitamin stock solution contained per 100 ml:

10 mg p-aminobenzoic acid,
10 mg thiamin,
100 mg riboflavin,
100 mg nicotinamide,
10 mg pantothenic acid,
50 mg pyridoxin.HCl,
0.2 mg biotin.

CMT: CM containing 0.01% Triton-X100 in order to reduce colony size on count-plates (Cf. Maleszka and Pieniazek, 1981).

ME: contains per litre:

20 g maltextract,
4 g sucrose,
1 g neopepton,
1 g casamino acids,
1 g yeast extract,
0.2 g ribonucleic acid hydrolysate (sodium salt),
2 ml vitamin solution.
Traces (one crystal) $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$.

Solid media contained 1.5 % agar and soft-agar media 0.8%. The pH of the media was adjusted to 6.0 with NaOH before autoclaving for 20 minutes at 120°C. The carbon source, mostly 0.05 M glucose, was added as a sterile solution (1:20).

2.1.2 CONIDIAL SUSPENSIONS

Conidial suspensions were made from 3 day old cultures of A.nidulans (grown on agar at 37°C), or 4 day old cultures of A.niger (30°C) and A.oryzae (24°C) with additional short term storage at 4°C for 3 to 7 day. Suspensions were made in saline-Tween (0.005 % w/v Tween-80), vigorously shaken during 10 min in order to break conidial chains, and then filtered through a cotton wool plug to remove mycelial fragments. The spore-concentrations were calculated by means of a Coulter counter and usually adjusted to approx. 10^7 conidia / ml. In general suspensions were stored overnight at 4°C prior to experimental use. Viability of spores was 65-75% unless otherwise stated.

2.1.3 MUTAGENIC TREATMENT AND SURVIVAL

For UV-treatment 10 - 12 ml of the suspension stored at 4°C was transferred to a glass Petridish which was placed, lid removed, under a Philips TUV-tube (30W) at a dose rate of about 120 $J/m^2/min$. The UV-fluence was determined with a short wave UV-meter (Blak Ray Ultraviolet Sensitivity meter J225). Upon irradiation (at room temperature) the suspension was transferred to a 30 ml screw cap flask, or in case of smaller samples to test tubes, placed in ice.

When applying a single dose, the fraction of surviving spores could be calculated from a sample taken from the original suspension and a sample from the suspension after treatment. To this end the samples were diluted to give upon estimation approx. 50-150 colonies per plate (after plating of 0.1 ml aliquots in duplo or triplo). Often more than one dilution was plated especially when it was difficult to predict the results. The resulting colonies were counted.

When applying a dose range, samples of 0.5 ml (added to 0.5 ml cooled saline) were taken in duplicate from the same Petridish at time 0, followed by time intervals corresponding to the different doses. Taking the samples from Petridishes, which can not be shaken, involves fluctuations in spore concentrations up to 25%. To correct for such unequal sampling the number of conidia in each sample is determined with the Coulter counter.

2.2 Survival curves

Examination of dose-response survival curves provide information on the process of cell killing itself and also indirectly on what mutagen dose can be used best for obtaining mutants without too heavy a load of genetic background damage.

Since A.nidulans conidiospores are unicellular and uninucleate, a suspension may behave like any population of single uninucleate cells. Typical for such cell populations are sigmoid curves when survival is plotted against irradiation dose. Such curves become linear on a log survival scale. Fig 2.1 (solid line) gives a linear log survival curve for UV-irradiation of microconidia of Neurospora crassa as found by Giles (1951). Norman (1954) found likewise.

However, upon UV-treatment of haploid A.nidulans conidia, an initial shoulder is found at lower doses, followed by a linear decline (Fig 2.1 dashed line). At higher doses a small fraction (e.g. 0.5 %) seems to be more resistent against killing by UV, probably due to experimental conditions. Experiments with N.crassa macroconidia also lead to shouldered curves (e.g. Atwood and Norman, 1949; Schroeder, 1970; Furukawa and Hasunuma, 1984), contrary to what is usually found for microconidia. Chang and Tuveson (1967) however found a shoulder also for a wildtype microconidial strain. In fact the same must have been found by Atwood and Norman (1949) as they mention that usually an extrapolation number (see later) of 1.5 has been found for N.crassa microconidia. Significally, Chang and Tuveson (l.c.) found no initial shoulder with microconidia from an UV-repair deficient strain (see 2.2.4).

Initial shoulders can only be convincingly demonstrated when lower doses are included, otherwise they may go unnoticed. Such shoulders can be generated by several causes: multiplicity of hits or multiplicity of targets (2.2.0), and natural repair processes (2.2.1).

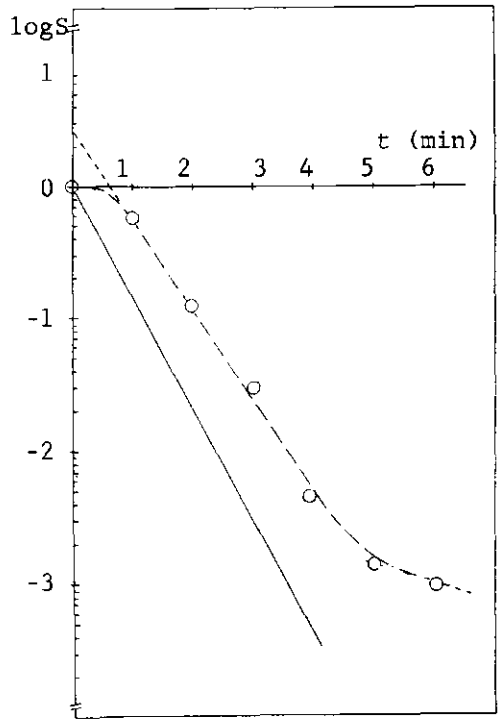


Figure 2.1 Survival curves.
——— UV-survival of Neurospora crassa microconidia based on Giles (1951); data converted to a dose rate of 100 $J/m^2/min$.
—O— UV-survival of conidiospores of A.nidulans strains WG096 (own data).

2.2.0 MULTI-HIT AND MULTI-TARGET CURVES

The term number of targets can best be understood as number of genomes per cell. So a conidiospore which contains one haploid nucleus in the G1-phase is a one target cell. A hit is an event producing a lesion in a target.

In the formula for survival curves used in this section the following symbols are used:

S is the fraction of spores among viable spores surviving mutagenic treatment. The number of viable spores is measured at dose zero, and in general it is 65 to 75% of the total number of spores.

kt is the effective mutagen dose, where

t is the duration of the treatment (i.c. irradiation), usually in minutes, and

k is a compound constant, being the product of the (inherent) sensitivity of the cells, the dose rate received in the cells, and a multiplier to transform kt (or kt.^{10}loge with ^{10}logS-graphs) to the t-scale.

n is the number of targets in the cell,

h is the number of hits per individual target to achieve target "killing" if no repair takes place. Cell-killing is supposed to occur when all targets in the cell are "killed".

It is assumed that the hits are randomly distributed both over the cells and over the targets within the cell. So the number of hits per target follows a Poisson distribution and the fraction of targets that receives no hits equals e^{-kt}. For h = 1 and n = 1, this is also the fraction of surviving cells.

Formulae for dose-response relationships in which either the target number or the hit number is taken into account, have already been discussed by Atwood and Norman (1949). These formulae have been used earlier to describe physioligical processes. The survival curve for a multi-target process is:

$$S = 1 - (1 - e^{-kt})^{n} \qquad \text{(Eq.2.1)}$$

Fig.2.2a shows a number of multi-target curves.

For the single target process (n=1) Eq.2.1 becomes

$$S = e^{-kt},$$

an exponential decay, which is linear on a logS- scale since

$$\log S = -kt \log e$$

For a multi-target process (n>1) the curves of (Eq.2.1) show an initial shoulder. Upon expansion it is seen that for somewhat higher doses one obtains the approximation

$$S = n.e^{-kt}, \quad \text{or} \quad \log S = \log n - kt \log e,$$

Now , n can be obtained by solving this equation for kt = 0. This means that the intercept with the S-axis of the extrapolated linear part of a multi-target survival curve corresponds to the target number, n.

The other mathematical model for the explanation of initial shoulders in log-survival curves is the so called multi-hit hypothesis. Here it is postulated that each cell has only one target and that at least h hits per target are required for cell killing. For the multi-hit process the survival curve (at n = 1) is given by

$$S = e^{-kt} \sum_{j=0}^{h-1} \frac{(kt)^j}{j!} \qquad \text{(Eq.2.2)}$$

In a semi-logarithmic plot, these curves (Fig.2.2b) are of similar general shape as those for a multi-target process (Fig 2.2a). Note, however, that an increase in hit number has a much larger effect on the logS-intercept than a similar increase in target number.

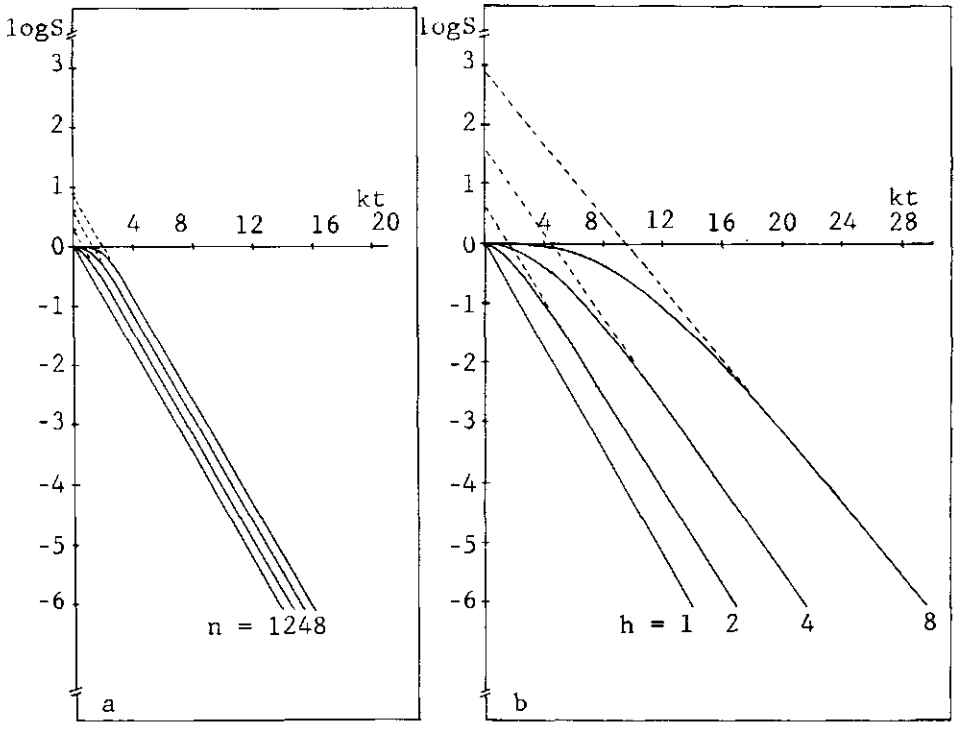


Figure 2.2 Survival curves according to two models.
a. Survival curves for different target numbers (n) (Eq 2.1).
b. Survival curves for different hit numbers (h) (Eq.2.2).

A natural extension of the above models is to incorporate both target number and hit number. This postulates that a cell has one or more targets (n) and killing of an individual target requires h hits.

The general formula for survival curves in such a process reads

$$S = 1 - \left(1 - e^{-kt} \sum_{j=0}^{h-1} \frac{(kt)^j}{j!}\right)^n, \text{ or for short}$$

$$S \approx 1 - \; 1 - e^{-kt}.Q^{\,n} \qquad \text{(Eq.2.3)}$$

where $Q = \sum_{j=0}^{h-1} \frac{(kt)^j}{j!} = 1 + kt + \frac{(kt)^2}{2!} + \ldots\ldots + \frac{(kt)^{h-1}}{(h-1)!}$

Equations (2.1) and (2.2) are special cases of Eq.2.3 by taking h = 1 and n = 1 respectively.

Graphs for logS at h = 1, 2, 4 and n = 1, 2, 4 are given in Fig.2.3. The curves are of the same general shape, all showing an initial shoulder except for h = 1, n = 1, which gives

$$S = e^{-kt} \quad \text{or} \quad \log S = -kt.\log e,$$

which is a straight line through the origin.

Now, except for the lowest doses (i.e. the lowest values of kt) Eq.2.3 is fairly well approximinated by

$$S = n.e^{-kt}.Q \qquad \text{or}$$

$$\log S = \log n - kt.\log e + \log Q \qquad \text{(Eq.2.4)}$$

For h = 1, i.e. log Q = 0 one obtains

$$\log S = \log n - kt.\log e \qquad \text{(Eq.2.5)}$$

This linear function of t is approximated relatively rapidly as can be seen from Fig.2.2a and 2.3 (h = 1). The dashed lines give the extrapolations of this linear part of the curves. They represent Eq.2.5. The intercept with the logS-axis (at t = o) is logS = logn, from which n can be calculated. For h > 1 Eq.2.4 is not a linear function of t and the convergence to linearity is very slow (Fig.2.3).

From Fig.2.3 it is seen that all extrapolation lines tend to run parallel. In fact they should do so, as can be shown as follows, but for low values of kt the extrapolation lines for different values of h are still not parallel.

For very high values of kt, the last term in Q becomes much larger than the sum of the preceeding terms, so that we may put

$$Q = \frac{(kt)^{h-1}}{(h-1)!}$$

Then differentiating Eq.2.4 one obtains

$$\frac{d(\log S)}{d(kt)} = -\log e + \frac{h-1}{kt}, \text{ or for high kt: } \frac{d(\log S)}{d(kt)} = -\log e$$

So for all values of n and h the extrapolation lines have the same slope (run parallel).

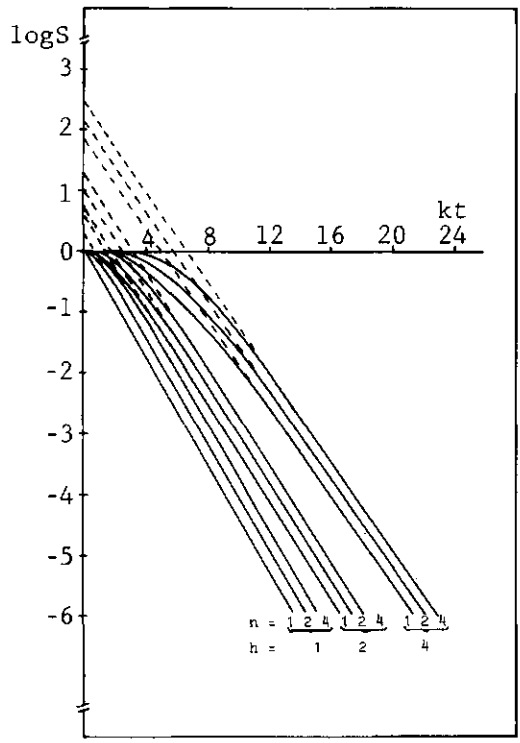


Figure 2.3 Survival curves for combinations of n (1, 2, 4) and h (1, 2, 4). See Eq. 2.3

As can be seen from Fig.2.3 for $h > 1$ convergence to linearity is only at higher doses. However, at such high kt-values survival fractions are so low that they fall outside the dose-range, or rather are well below the point where the decline levels off (see e.g. Fig.2.1). So, in contrast to $h = 1$, it is with $h > 1$ an uncertain procedure to construct correct linear extrapolation lines from experimental data. The linear extrapolation lines for $h > 1$ in Fig.2.2b and 2.3 were eye fitted.

It is further seen from Fig.2.3 that hit number (h) has a much larger impact on the width of a shoulder than the number of targets (n) has. For example a fourfold increase of n always leads to a fourfold increase of the S-intercept (logS-intercept converted to a linear scale), whereas a fourfold increase of h results in aproximately a sixtyfold increase. The latter is far beyond the range of existing target numbers in spores.

This quantitative difference between the effects of n and h on the shape and location of survival curves seems not to have been recognized previously (e.g. Atwood and Norman, 1949; Alper, 1979). This possibly is why the terms multi-hit and multi-target curves often have been used indiscriminately (e.g. Meynell and Meynell, 1970). Similary, the terms hit number and target number have both been used in connection with the logS-intercept of extrapolated lines from the linear part of the experimental data. It is clear that whilst at $h = 1$, $\log S = \log n$, no such simple relationship exist between S and h, since except for $h = 1$ the relationship between Q and h is complex. Note that extrapolation to $t = 0$ yields $\log S = \log n + \log Q$.

Already in 1960 Alper et al. pointed out that the S-intercept of a survival curve may not at all reflect some kind of 'multiplicity' in the cell. They therefore proposed the neutral term 'extrapolation number' instead of the terms target number or hit number.

Differences in k may result from differences in inherent sensitivity of the cell populations compared (e.g. age of spores) or from differences in dose rate received in the cell (e.g. as a consequence of spore wall colour). At equal S, doubling of k mimics halving of irradiation time. So, while k affects the slope of the curve, and correspondingly the width of the shoulder, it does not change the logS-intercept of the extrapolation lines. This holds for any combination of n and h. Fig.2.4 gives the effect of k for h = 1 and n = 4. It implies a.o. that mixing spores which only differ in sensitivity, e.g. mixing older and younger spores from a colony, will not influence the logS-intercept.

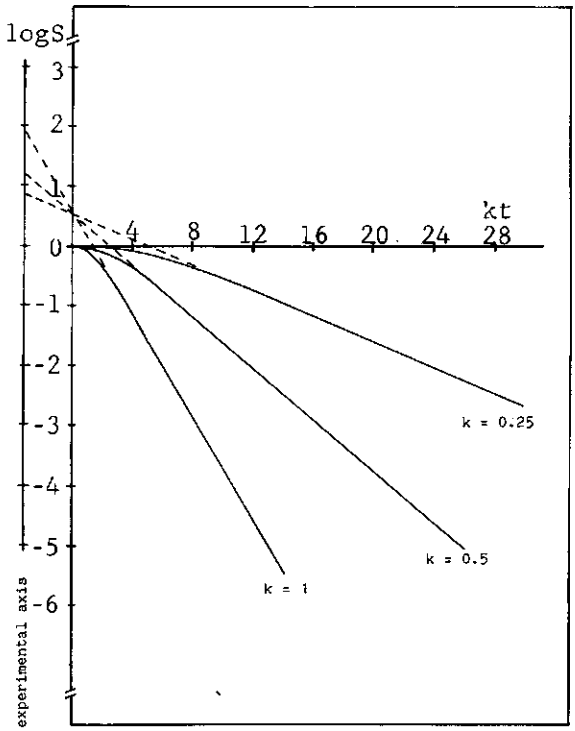


Figure 2.4 Effect of effective dose (kt) on the log-survival curve for n = 4 (resulting in an initial shoulder) and h = 1.
Scale at left: shift of origin when repair is allowed for (see 2.2.1).

2.2.1 THE EFFECT OF REPAIR

For the explanation of initial shoulders only target and hit multiplicity have been discussed so far.

Bridges and Munson (1968) mentioned repair as a third explanation for initial shoulders in logS curves. In this hypothesis, proposed by Haynes (1964), effective natural repair mechanims can at lower doses cope with UV-induced lesions, but at higher doses they become saturated or inhibited. This holds for a dark repair system. It differs from the (light dependent) photo-repair, which eliminates a constant fraction of potentially lethal photo-products throughout the dose range (Harm, 1980). The prevailing dark-repair system probably is the excision-resynthesis repair, or excision repair for short.

The effect of excision repair is to elongate the shoulder till the dose of saturation. From this point the curve proceeds as from dose 0 (Figs. 2.2 - 2.4). Notably, for n = 1 and h = 1 decline follows immediately at the dose of saturation. Thus in the graphs constructed for the situation without repair, dose zero is in reality the dose where the repair capacity is saturated. This means that in experiments with repair the dose zero correspondents to a negative dose in Fig.2.2 - 2.4. Such a logS-axis for the experimental situation where repair takes place has been added in Fig.2.4.

This is of course a simplified model, but it can very well illustrate the effect of repair on the level of the logS-intercept from extrapolation lines. Thus in Fig 2.4 (h = 1, n = 4) it is seen that, when repair is allowed for ('experimental axis'), the logS-intercept found from experiments overestimates logn, the more so the higher the repair capacity is. Secondly a change in k, e.g. due to a difference in sensitivity, will not leave the experimental logS-intercept unaffected. A high sensitivity will in fact give a more pronounced overestimation of logn than a low sensitivity does.

2.2.2 INFERENCES FROM EXPERIMENTS

In the analysis of survival curves, the initial shoulders and the causes of it play a central role. Experiments can be constructed where different internal conditions of the spores or different experimental conditions are compared. These conditions specifically should relate to a change in k (inherent sensitivity or dose rate received in the cell), or to a change in n (diploidy or change of G1 to G2 by preincubation), or to repair capacity (e.g. using excision repair deficient strains).

Summarizing, in the interpretation of such experiments, a number of points should be kept in mind (see Figs. 2.2 till 2.5).

1. With a given k the extrapolation lines for different values of n and h run parallel at high doses.
2. The slope of the extrapolation lines can only change as a result of change in k.
3. Both repair and values of $h > 1$ can give overestimates of target number when simply putting $\log S = \log n$ at $t = 0$.
4. Even in the absence of repair, $h > 1$ gives extrapolation numbers which exceed the number of targets (n) expected in the material. Conversely, larger extrapolation numbers far exceeding the expected n indicate repair and/or a multi-hit process.
5. In the presence of repair, a change of k can mimic a change in n.

The choice of points to be used for the construction of the linear extrapolation line, remains subjective even with a theoretical curve. Inclusion of a higher or a lower point may influence the slope of the line and the level of the logS-intercept. This is illustrated in Fig.2.5. In practice, the normal experimental point to point fluctuations introduce additional uncertainty on what points to include in the regression.

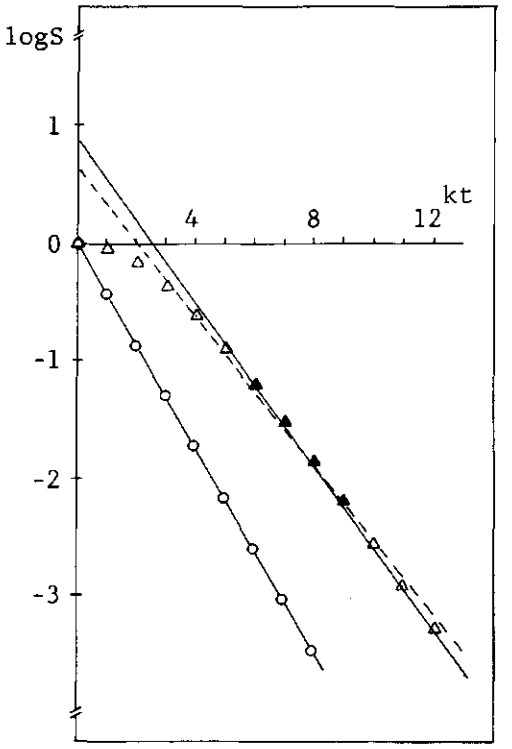


Figure 2.5 Effect of choice of points.
Regression lines are drawn based on six points: four black points and two higher (– –) or two lower points (——). The width of the points can be taken to represent the experimental variation. Δ for n=1, h=3; O for n=1, h=1.

Since uncontrolled photorepair can interfere with the factors to be studied, the samples of the spore suspension were placed on ice immediately after UV-irradiation. An experiment was done to see whether photorepair was excluded under the experimental conditions used.

Conidiospores of the yellow A.nidulans strain WG096 were irradiated with UV for 30 sec and half of the suspension was placed on ice for 1 h under the usual dimmed light conditions. Subsequently this suspension was treated further in the same way as the control half (Fig.2.6). The experiment was repeated with a UV dose of 1 min followed by 1 h on ice and further treatment. In both experiments no difference in survival was found, indicating that photorepair did not occur under these expermental conditions. In a control experiment complete repair was found after 1 h incubation at roomtemperature.

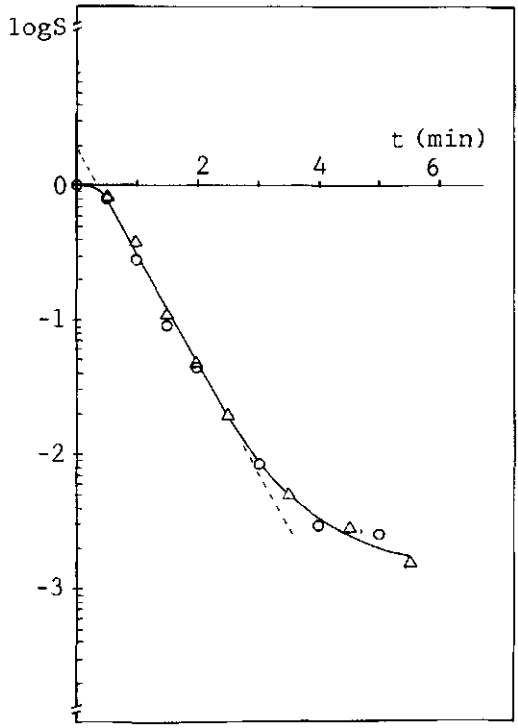


Figure 2.6 Absence of photorepair in A.nidulans under the experimental conditions used.
o Survival curve of WG096 under standard conditions
△ Survival of WG096 conidia after 0.5 min irradiation subsequently stored in ice for 1 h, upon which the procedure was continued.

2.2.3 EXPERIMENTS ON FACTORS MODIFYING SENSITIVITY (k)

The factor k depends on the effective dose rate (the fraction of the dose rate given which is recieved in the cells) and on the inherent sensitivity of the cells.

It can be expected that the wall-colour of the conidiospores influences the effective dose rate, as a result of differences in UV-absorption. Secondly, spores at the bottom of the irradiated Petridish can be sheltered by the spores higher in the suspension. Conidiospores of different age may have different inherent sensitivities.

It will be assumed that the uninucleate haploid conidiospores of A.nidulans and A.niger strains are single target cells (n=1), i.e. that the nuclei are in the G1-phase (see Section 2.0.0).

2.2.3.1 SPORE COLOUR AND SHELTER EFFECTS

In Fig 2.7 logS-curves are plotted for a white strain (WG145) and a yellow strain (WG096) of A.nidulans. As expected the white strain is the more sensitive one, as can be seen from the steeper decline of the linear extrapolation line, which implies a smaller k value. From the survival at doses of 1 min till 3 min the extrapolation numbers are 3.3 and 2.7 respectively. If for the white strain also the survival at a dose of 0.5 min is included for regression then an extrapolation number of 2.7 is obtained too (black triangles). Assuming n = 1, these extrapolation numbers could either reflect h > 1 (e.g. h = 2; see Fig 2.3) or repair with a saturation dose of about 0.3 min.

In a comparable experiment, except that the cultures were stored for 3 days at 4°C, about the same extrapolation numbers have been obtained (3.4 and 2.6 respectively). The yellow strain WG096 has also been used in the photorepair experiment (Fig.2.6).

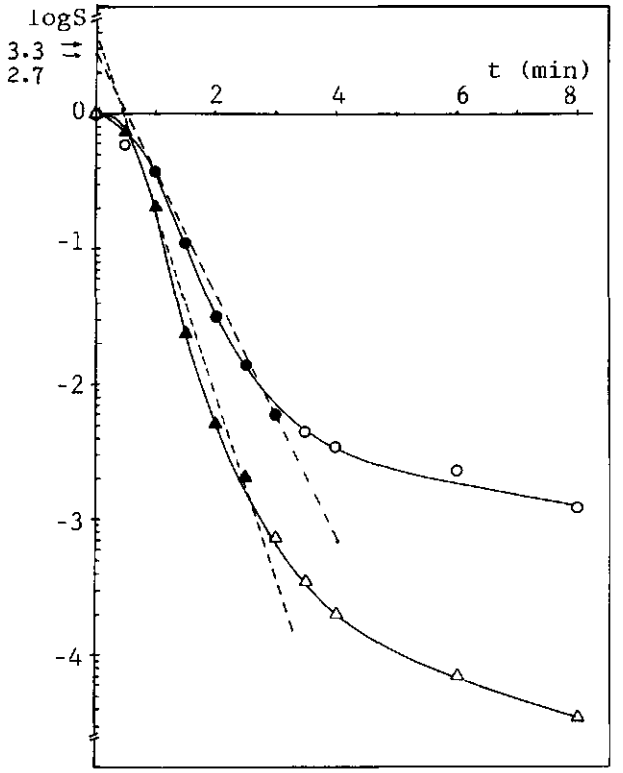


Figure 2.7 Effect of spore colour on survival in A.nidulans.
o yellow (WG096); △ white (WG145); ● , ▲ used for regression.
Conidia from 3 day old cultures (37°C) and stored for 1 day (4°C) instead of 3 days as usual.

The yellow strain WG096 has also been used in the photorepair experiment (Fig.2.6). The logS-curves in Figs.2.6 and 2.7 show virtually identical slope and shoulder.

In still another experiment, a green A.nidulans strain (WG094) was used under the same conditions (see spores from 3 day old cultures in Fig.2.9). The green strains had about the same sensitivity as the yellow strain. See e.g. the level of 1% survival which in all three cases is reached at about 3 min irradiation.

Conidiospores of A.niger are far more UV resistant than those of A.nidulans. As can be seen from Fig.2.8 the log-survival curve is of the same shape as that of A.nidulans (note the shortened t-scale in the A.niger graphs). Thus at 3 min A.niger survival is still higher than 50%. The lower sensitivity is probably for a large part due to the black spore colour. At least, colour

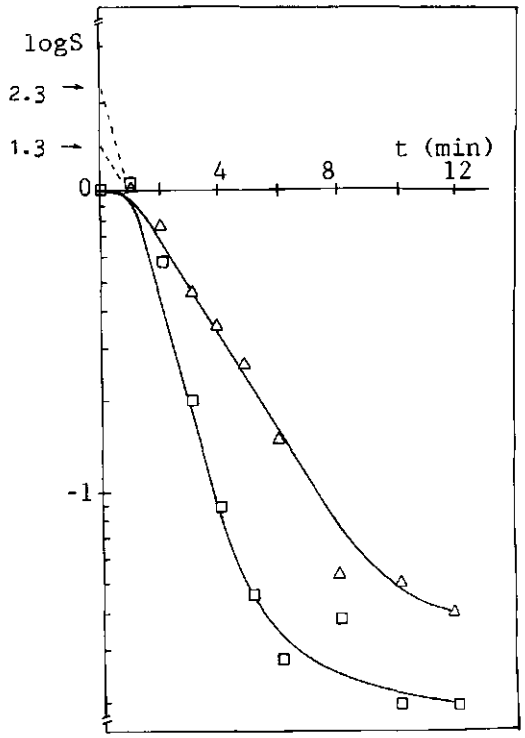


Figure 2.8 Shelter effect.
LogS-curves for conidiospores of A.niger strain N408.
o normal spore concentration (10^7/ml).
△ diluted suspension (10^4/ml).

mutants from this strain, e.g white (gene whi), olive green (olv) and fawn (fwn) have much steeper survival curves (not presented), which resemble those of A.nidulans strains.

In Fig.2.8 (A.niger, strain N408) a normal spore concentration (10^7/ml) and a diluted suspension (10^4/ml) were compared. The sheltering effect of the higher spore concentration is very pronounced. The diluted sample has a much steeper decline of the linear extrapolation line, implying a much smaller k-value.

For the diluted and the normal suspension the log-S intercepts are 2.3 and 1.3 respectively. For a given value of h, a change in k is not expected to generate a difference in logS-intercept (compare Fig.2.4). In fact the extrapolation lines intersect at the level logS = 0. Assuming n = 1, this happens to be precisely what is expected when the shoulder is only due to repair (with saturation at about 1 min).

2.2.3.2 AGE OF THE SPORES

The internal physiological condition of the spores may also influence their sensitivity. It fits best with the time schedule in the laboratory to collect spores from 3 day old cultures, which are subsequently stored in the dark for about three days at 4°C prior to use in experiments. Unless stated otherwise this is the normal procedure.

Fig.2.9 gives the result of two experiments, viz. Exp.I where the age of the culture was varied (40 h and 7 days) at fixed storage time (1 day), and Exp.II where the duration of storage was varied (none and 3 days) at fixed age of culture (3 days). It is seen that "very young" spores are far more sensitive than "very old" spores. With the 7 day old spores the results are irregular so no attempt was made to construct a linear extrapolation line. Moreover the viability of the spores was as low as about 20% (in this experiment 22%), which makes them less suitable for the present purpose.

For spores from 3 day old cultures subsequent storage has little effect down to 1% survival (i.e. little effect on the slope of the linear part of the curve and on the shoulder).

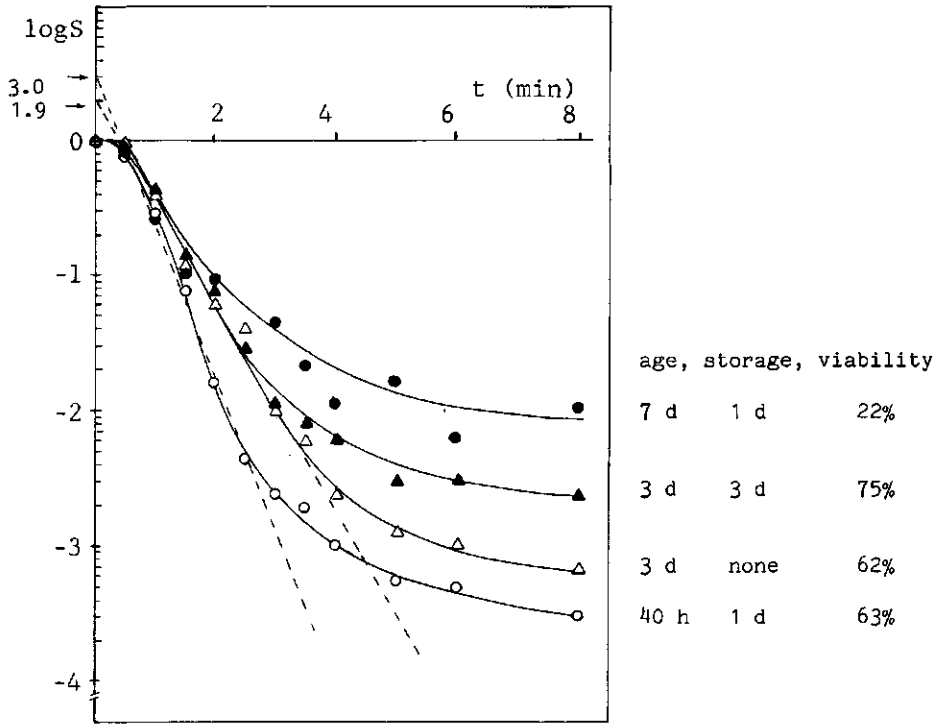


Figure 2.9 Age of cultures and duration of storage.
LogS-curves for the green A.nidulans strain WG094 grown at 37°C and stored at 4°C.

Obviously age of culture has a main effect on sensitivity. For practical reasons three day old cultures were preferred over younger cultures. It should be noted that harvesting from 3 day old cultures implies a certain range of spore ages. However, this only affects k (the slope of the linear extrapolation lines), and not the logS-intercept.

In Fig.2.9 the extrapolations for 3 day old cultures virtually conincide. The lines intersect at the level of logS = 0 as expected for n = 1 when repair is considered as the cause of the shoulder.

2.2.4 EFFECT OF REPAIR DEFICIENCY ON LOG-SURVIVAL CURVES

From experiments with haploid conidiospores, which are generally thought to be one target cells, it can be concluded that the logS-survival curves always have about the same general shape. This becomes especially clear when, by adjusting the t-scale, the sensitivities at 1% survival are made to coincide. So e.g. in the experiment on the shelter effect (Fig 2.8) after correction for the difference in effective dose at 1% survival, the extrapolation lines are found to coincide. This correction makes it possible to compare strains with different sensitivity.

If the initial shoulder is the result of an effective repair mechanism it will probably not be found with UV-repair deficient strains.

Such uvs-mutants are characterized by their increased sensitivity to inactivation by UV (Shanfield and Käfer, 1969; Jansen 1970; Fortuin, 1971). The uvsD53 mutant used in the following experiment was obtained from Dr.Jansen and has in addition reduced meiotic recombination and an enhanced frequency of mitotic recombination. By comparing certain characteristics of uvs-mutants Jansen and Fortuin inferred that the uvsD53 mutant is excision-defective.

In Fig.2.10 the survival curves of A.nidulans strains WG176 (uvsD53) and WG096 (uvsD+) are shown. As expected, with the uvsD-mutant strain an immidiate linear decline was found without an intial shoulder. When a regression line is drawn based on the points for 15, 30, 45 60 and 90 seconds UV, the extrapolation number is -0,14. In two other experiments values of -0,18 and -0,10 were found. It can be concluded that A.nidulans has a repair mechanism, which becomes saturated after a low dose UV-irradiation.

The initial repair is typical for UV-lesions as the shoulder was not found with X-ray survival curves. This is shown in Fig.2.11 for A.nidulans strain WG096, where the level of survival at 300 Gy X-ray is about equal to that at 3 min UV.

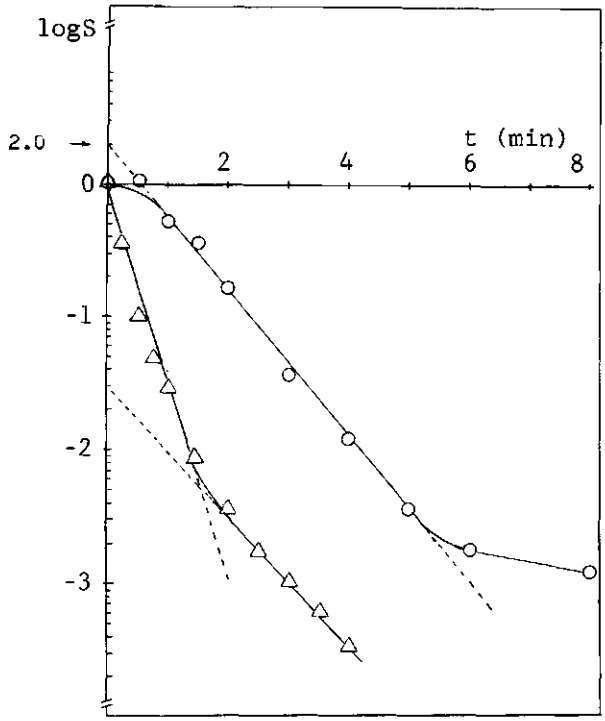


Figure 2.10 Effect of repair deficiency on the logS-survival curve in A.nidulans.
o strain with wildtype UV-repair (WG096).
Δ UV-repair deficient strain uvsD53 (WG176).

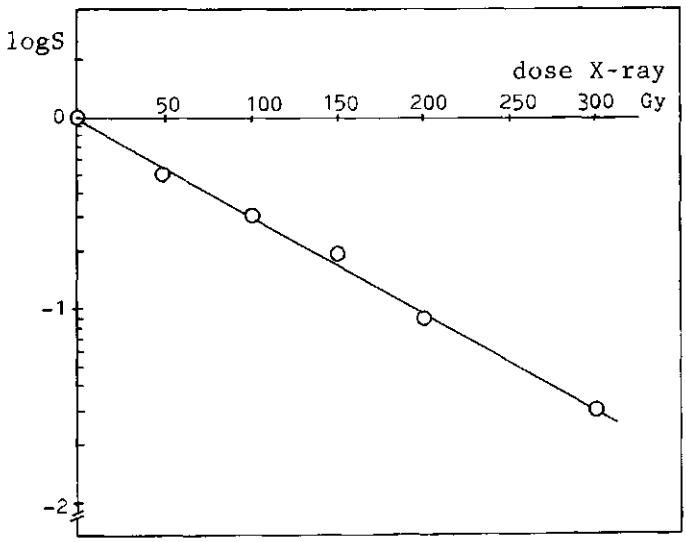


Figure 2.11 Survival curve for X-irradiation in A.nidulans (WG096).

2.2.5 EXPERIMENTS ON MODIFICATION OF n

When conidia of haploid A.nidulans are one-target cells (n = 1), then those of diploid A.nidulans are two-target cells (n = 2). A change from n=1 to n=2 is expected to result in a one unit increase in extrapolation number, regardless the value of h (although it is not expected that h will change), and provided the repair capacity does not change from haploid to diploid. Moreover, if repair is involved a change in sensitivity (k) may also affect the extrapolation number, but if repair is not involved a change in k has no influence on the log S-intercept.

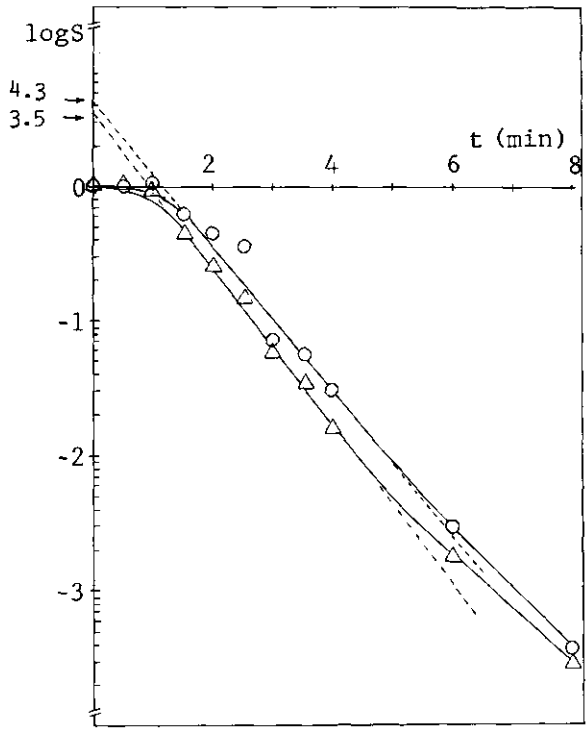


Figure 2.12 Effect of increase in target number.
Survival of conidiospores of a diploid green A.nidulans strain WG015//WG096 (obtained from somatic karyogamy between the strains WG015 and WG096).
○ Exp.1; △ Exp.2 (two subsequent experiments).

With conidiospores of a green diploid A.nidulans strain (WG015//WG096) an extrapolation number of about 4 has been found (3.5 and 4.3 respectively; see Fig.2.12). With the haploid green strain (WG094) an extrapolation number of about 2 was obtained (Fig.2.9). The slope of the logS-survival curves was about the same for haploid and diploid A.nidulans.

At first sight this increase in extrapolation number seems to be the result of the doubling of the target number (n = 2). In the discussion it will be shown that the situation is somewhat more complex. However, the results justify the conslusion that the extrapolation number increases with increase in target number.

A second approach to study the effect of a change in n is to include a preincubation before UV-irradiation. In our experiments on the isolation of protoplasts from conidiospores (Section 3.2) it was found that upon incubation of conidiospores mitosis takes place between three and five hours of incubation. This agrees with the observations of Fiddy and Trinci (1976) that mitosis preceeds germination of conidiospores. So if the conidiospores are in G1-phase the chromosomal material should be duplicated during preincubation and the extrapolation number should increase.

A suspension of A.nidulans WG096 conidiospores in saline was divided into two portions of 12 ml. The first was used directly and the spores of the other portion were collected and resuspended in 30 ml liquid SM and preincubated in a reciprocal incubator at 37°C for three hours. Then the spores were collected again and resuspended in 12 ml saline and irradiated as the first portion. The results are shown in Fig.2.13.

Without preincubation the extrapolation number was 1.9 and with preincubation it was 3.5. The slope of the extrapolation line becomes steeper with preincubation, indicating increased sensitivity. Note that the two extrapolation lines intersect below the level logS = 0. When one corrects for the difference in sensitivity at the 1 % survival level, the extrapolation lines coincide.

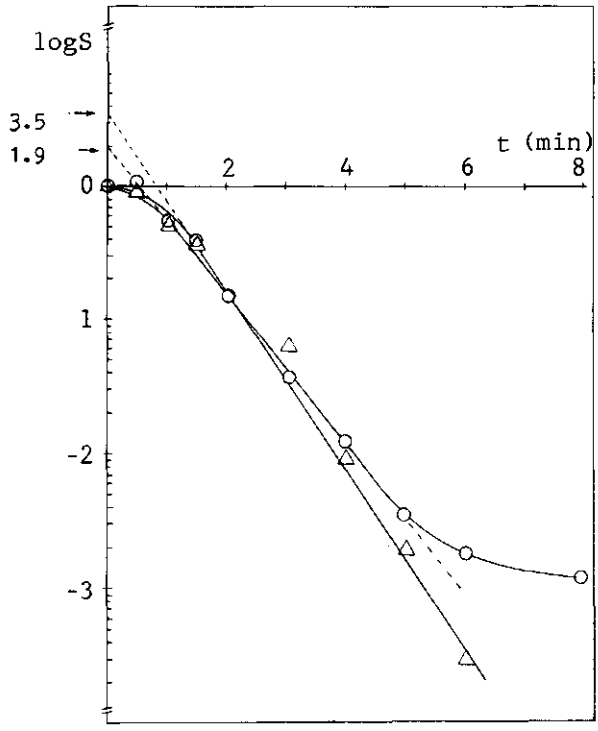


Figure 2.13 Effect of preincubation of conididospores on survival. Conidia of the green A.nidulans strain WG094 were UV-irradiated.
o without preincubation.
△ with preincubation for 3 h at 37°C in liquid SM.

The preincubation experiment was repeated with the yellow A.nidulans strain WG282. This strain was used later to study mutation induction. Preincubation was extended to 4 and 5 hours. Extrapolation numbers of 2 (without preincubation), 4 (3h), 9 (4h) and 15 (5h) were found (Fig.2.14). For clarity, only the regression lines are presented in Fig.2.14. With prolonged incubation the slope of the extrapolation line becomes steeper, which indicates increased sensitivity. As before, the extrapolation lines intersect well below the level logS = 0.

From the marked increase in extrapolation number it can be concluded that the conidia are indeed in the G1-phase. The increase exceeds, however, far above what is expected from a doubling of the target number.

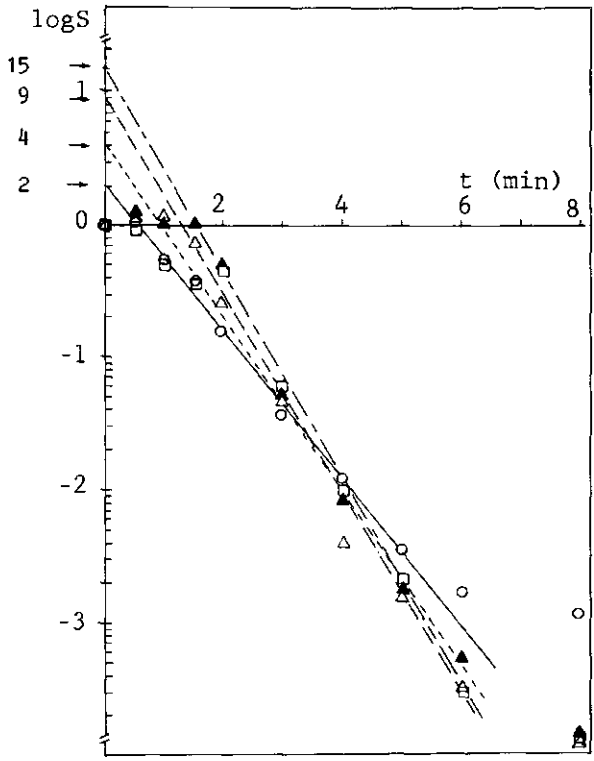


Figure 2.14 Effect of preincubation on extrapolation number.
Conidia of the yellow A.nidulans strain WG282 were preincubated in liquid MM at 37°C.
o without preincubation,
△ 3h preincubation; ▲ 4h and □ 5h preincubation.

When in this graph the extrapolation lines are corrected for sensitivity at the level of 1% survival ('without preincubation' as a standard) even more extreme extrapolation numbers are obtained (Fig.2.15): for 3, 4 and 5 h preincubation the extraplation numbers become 6, 11 and 20 respectively.

The changing slope of the curves indicates increasing sensitivity and this causes a disproportionate increase of extrapolation numbers (cf. Fig.2.4). In Fig.2.15 a second logS-axis is drawn at the level where the repair capacity of normal (not preincubated) conidia is saturated. This logS-axis intersects the t-axis at 0.6 minutes and here extrapolation numbers of 2.5 (3h), 3.5 (4h) and 8 (5h) are found.

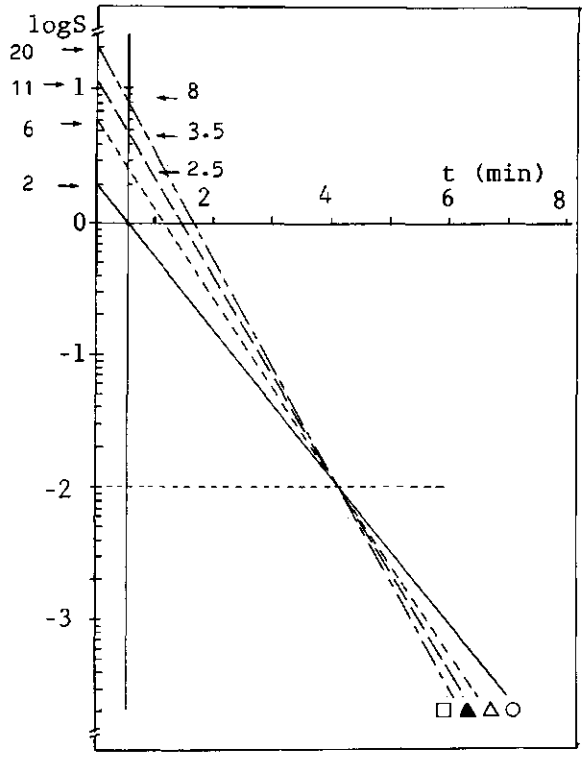


Figure 2.15 Effect of preincubation after correction for k.
The data of Fig.2.14 have been corrected for differences in sensitivity (k) at 1 % survival level, taking the survival without preincubation as standard.
A second logS-axis is drawn at the point where the repair capacity of normal cells is saturated (t = 0.6 min).
Extrapolation numbers are inserted.
o without preincubation;
Δ 3h preincubation; ▲ 4h and □ 5h preincubation.

The contineous increase in extrapolation number, notably from 3 to 5 h preincubation, may partly reflect non-synchronous division of nuclei. However, one is led to conclude that during extended preincubation the repair capacity increases considerably, which progressively extends the shoulder of the curves. The increase in extrapolation number cannot solely be accounted for by the increase in target number. It can only be explained by an increase of repair capacity.

A.oryzae strains are known to have multinucleate conidiospores (Yuil, 1950). An A.oryzae strain which had on the average 6 nuclei per conidiospore was used as an illustration of a high target number. The survival curve (Fig.2.16) has a much more extended shoulder than the curves obtained for diploid of preincubated haploid A.nidulans conidiospores.

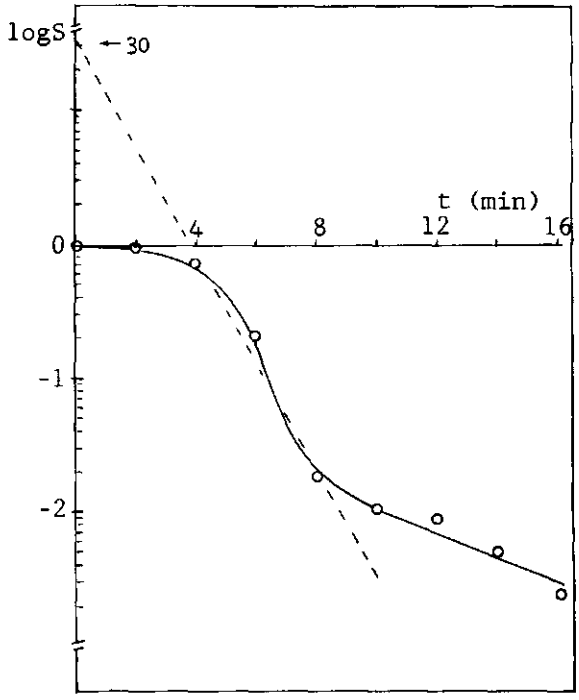


Figure 2.16 Survival of multinucleate A.oryzae conidiospores.
Note the shortened t-scale.
There are insuffient data for an exact extrapolation line. A minimum estimate for the extrapolation number seems to be 30.

The extrapolation number of at least 30 cannot be wholly accounted for by a pure multi-target process, since at most 10 nuclei (average 6) are present in conidiospores of this A.oryzae strain. There are too few data for an exact extrapolation line, but two parallel experiments gave similar results. The slope of the extrapolation line with A.oryzae is steeper than with A.nidulans, indicating a higher sensitivity of the former. As such, a change in sensitivity does not affect the extrapolation number.

So the high extrapolation number and the very extended shoulder of A.oryzae indicate a high repair capacity.

In summary the following prelimary conclusions can be drawn:

- Repair mechanisms cause initial shoulders in logS-survival curves.
- A higher target number is reflected by a higher extrapolation number (logS-intercept).
- Increase in repair capacity may coincide with increase in target number.
- The extrapolation number cannot be used as an estimator of target number.

2.2.6 DISCUSSION

When plotting log-survival against irradiation dose, the curves show an initial shoulder. This is generally explained as resulting from either a multi-target process (n > 1) or a multi-hit process (h > 1). The two models are used rather indiscriminately in litterature.

Fig.2.3 compares the effect of increasing target number with that of increasing hit number. The graphs are based on Eq.2.3, which is the general formula covering all combinations of n and h. It is seen that doubling of hit number has a much larger effect on the width of the shoulder than doubling of target number has. Correspondingly, the intercepts of the linear extrapolation lines with the logS-axis increase much more with an increase in h than with an increase in n. For h = 1, these intercepts give extrapolation numbers equal to the number of targets. So, when extrapolation numbers are found which are clearly larger than expected on the basis of target number, one is inclined to conclude to a multi-hit process, provided no other factors are involved in the generation of initial shoulders.

A.nidulans conidiospores are haploid and uninucleate. Moreover, they can be assumed to be in the G1-phase, which is generally the case with resting cells. In work on cell division and mitosis in fungi, including A.nidulans, a doubling of DNA

content is observed prior to germination of the conidiospores (Bainbridge, 1971; Fiddy and Trinci, 1976; Bergen and Morris, 1983). This indicates that the conidiospores are in G1-phase rather than in the G2-phase, which is a short transient state. The results of our experiments with preincubation of conidiospores are in agreement with the statement that the spores are in G1-phase. Preincubation results in an immediate increase in extrapolation number. In conclusion, the target number of resting conidiospores can be put at unity (n = 1). So one is the more inclined to ascribe initial shoulders, respectively intercepts of logS > 0 to a multi-hit process. Provided, as before, that no other factors are involved.

Now (still if no other factors are involved), a change in k, i.e. a change in the dose received in the cells and/or a change in the inherent sensitivity of the cells, should have no effect on the logS-intercept, regardless the value of n and h. This is illustrated in Fig.2.4.

However, in our experiments on factors modifying k, viz. on spore colour and shelter effect (2.2.3.1) and on the age of the spores (2.2.3.2), a change in k results in a change of the logS-intercept. This strongly suggests the involvement of another shoulder generating factor. It is clear that a shoulder can be generated by an inherent repair capacity of the spores, which becomes saturated around a particular UV-dose. It is significant that in our graph for varying k-values, the linear extrapolation lines intersect at the X-axis, or near it. In other words they intersect at the level logS = 0, which is precisely what is expected for n = 1 in the case of an effective repair mechanism. Fig.2.4 shows for n = 4 a point of intersection well above the X-axis, and when repair is included also a shift of the origin which corresponds to the effect of repair.

If repair is the predominant factor causing an initial shoulder when resting conidiospores (n = 1) are irradiated, then a repair deficient strain is expected to show no shoulder. This is confirmed by our experiments with an uvs-strain (2.2.4) which gives straight lines running through the origin, or rather intersects the logS-axis even somewhat below the origin. It should

be noted that the line of the uvs-strain is much steeper than that of the wildtype strain (Fig.2.10), which indicates a higher UV-sensitivity. Obviously uvs-strains are selected predominantly on the basis of a higher overall UV-sensitivity and in addition the present uvs-strain is devoid of the initial repair capacity. Finally, the change in slope of the uvs-strain at a point below 1% survival is probably due to reversion to UV-insensitivity, represented by a small fraction of the spores.

Turning to our experiments on preincubation (2.2.5), it is seen that the relationships are complex. One expects an increase in target number, when the cells go from G1 (n = 1) to G2 (n = 2). However, the sensitivity of the cells appeares to increase at the same time. This results in a disproportionate increase in extrapolation number (Fig.2.14). The increased sensitivity can be expected when the treated cells approach the S-phase (Bainbridge, 1981; Davis et al., 1978; Jansen, 1970; Fortuin, 1971). The most outstanding feature is that the extrapolation lines intersect well below the X-axis (see also Fig.2.15). This can only be explained when along with increasing sensitivity also the repair capacity increases with prolonged incubation.

Finally, two points should be discussed here which have not been mentioned so far.

In several of the logS-graphs a point is found in the low dose region, which is slightly above the X-axis. This means that here viability of the spores is somewhat higher than at dose zero. There is no unanimity in literature on a stimulating effect of UV on spore germination (Griffin, 1984), but for A.niger light stimulation has been observed by Kahn (1977) and UV-stimulation by Golubtsova et al. (1976).

In diploid spores, complementation may play a role. The same holds for spores in G2 (upon preincubation). It is not likely that the majority of lethal lesions is dominant, so a certain amount of lesions does not come to expression due to the presence of the homologous wildtype alleles. In diploids the shoulders are more extended than expected from the target number (n = 2).

This extra extension can originate both from extra repair capacity and from complementation. Of course, complementation can be understood as 'repair' on the level of expression. So, in general the extrapolation number would give an overestimation of the target number as illustrated with A.oryzae.

2.3 Frequency of mutants

2.3.0 INTRODUCTION

Since the yield of mutants per surviving cell in general increases with the dose of mutagen, it has often been concluded in literature that it is efficient to apply high mutagen doses where most spores are killed.

Since conidiospores of haploid A.nidulans and A.niger are one-target cells (see 2.2) it is reasonable to expect that there is no need for the application of high mutagen doses in procedures for isolation of recessive mutants. In this Section it is studied whether low doses UV can be used in general for the induction of different types of recessive mutants.

In general one is more interested in the frequency of mutants among survivors ("mutant fraction") than in the frequency of mutants among total spores ("mutant yield"). When mutants are selected by testing survivors, the frequency of mutants among survivors determines how much work is involved in the isolation of a certain amount of mutants. However, in this study special attention is also paid to the frequency of mutants among total spores.

For studies on mutation induction often a selection system for revertants of auxotrophic mutants is used. E.g. Giles (1951) working with Neurospora crassa plotted the frequency of reverse mutations of two inositol-less mutants against the X-ray dose and found a linear relationship. Ashwood-Smith and Bridges (1966) used a tryptophane-less strain of E.coli and found that the frequency of mutants (revertants) induced by low doses of UV was proportional to the square of the dose. However, the majority of their revertants were suppressor mutants and the frequency of true revertants proved to be linear with the dose (Bridges and Munson, 1968). One of the most suitable systems to study mutation induction is the red-white adenine system in yeast. Forward mutation to adenine-deficiency can be recorded by visual inspection. James and Kilbey (1977) used this system to analyze whether mutations were single or double stranded. From their data it can be inferred that the dose-effect relationship is fitted

best by a cubic function. In these experiments mostly low doses were used and when the frequency of mutants was plotted against the logarithm of the surviving fraction straight lines were obtained. Witkin (1956), already pointed out that there was a saturation of the mutant yield at higher doses.

2.3.1 RESULTS

Different types of A.nidulans mutants were used to study mutation induction, firstly resistance mutations since these are easy to score.

From an UV-irradiated suspension of conidiospores from the green A.nidulans strain WG094, undiluted samples of 0.1 ml were plated in duplo on CM+acriflavin (100 ug/ml) and on CM+benomyl (10 ug/ml) in order to score for resistance mutants. Diluted samples were plated on CMT for survival.

In the figures the relative frequency of mutants is expressed in two ways:

a. "mutant yield", i.e. number of mutants as a fraction of the total number of spores. See closed symbols and solid lines.

b. "mutant fraction", i.e. the number of mutants as a fraction of the number of surviving spores. See open symbols and dashed lines.

For both acriflavin and benomyl resistent mutants the highest yield (solid lines in Fig.2.17) was already obtained at a dose corresponding to more than 10% survival. The curves for mutant fraction, especially that for acriflavine resistant mutants were of a complex nature, but both tend to soon reach a plateau at a relative frequency (mutants among survivors) of about 0.1 %. The complexicity of these curves becomes even more obvious when the relative frequency of resistant mutants is plotted against log-survival as is usually done in literature (Cf. Fig.2.19). Note that the maximum yield of mutants was obtained at relatively high survival level.

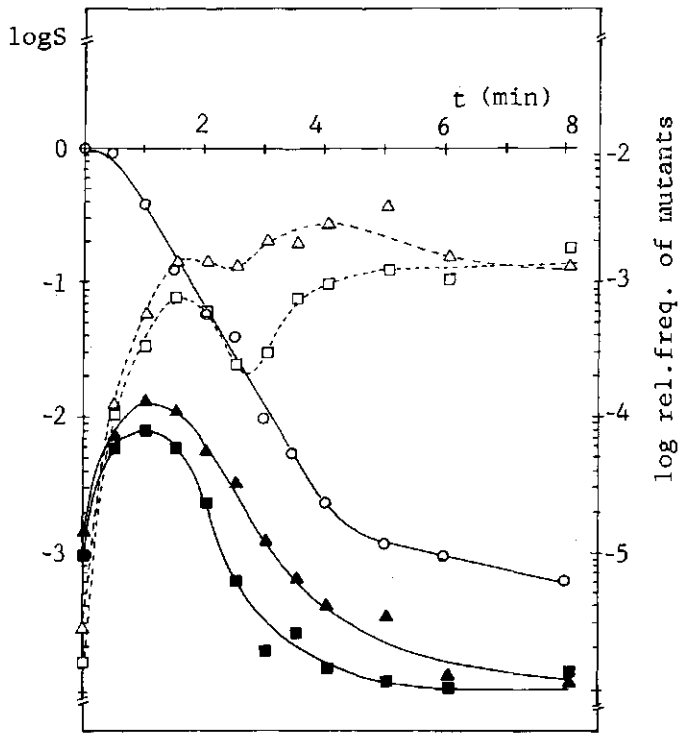


Figure 2.17 Frequency of mutants in A.nidulans WG094.
Relative frequency of induced acriflavine (100 ug/ml) (■) and benomyl (10 ug/ml) (▲) resistant mutants among total spores ("mutant yield") and the log relative frequency of mutants among the survivors ("mutant fraction") (□ resp. △).
The survival curve has also been plotted (○).

A similar experiment was done with the paba-deficient yellow A.nidulans strain WG096. Acriflavin resistant mutants were scored on CM + 100 ug/ml acriflavin (as before) and on CM + 150 ug/ml acriflavin. Again, notably for resistant mutants on 100 ug acriflavin, a complex dose-response relationship was found (Fig.2.18a). For the frequency of mutants among survivors, curves $\log y = a.t^b$ were fitted (dashed lines) with b = 1.04 (100 ug/ml and b = 0.70 (150 ug/ml) respectively and $a = 3.1 \times 10^{-6}$ and 3.7×10^{-6} respectively.

In this experiment also pabaAl-revertants were scored, but their frequency turned out to be too low for reliable estimates. Therefore an experiment with a somewhat different procedure was carried out. At each UV-dose a separate 12 ml sample from the

spore suspension was irradiated. From each sample 10 ml was concentrated 10 times (by centrifugation and resuspension in 1 ml), and plated in duplicate on MM. From the remaining non-concentrated suspension 0.5 ml was also plated in duplicate on MM. Diluted samples were plated on CMT for survival count.
So at each UV-dose a choice between the concentrated and non-concentrated platings could be made, to avoid less satisfactory counts due to overcrowding on the one hand and too low numbers of revertants on the other hand. Among revertants always slow and fast growing mutants are found, and therefore in these experiments plating numbers asked special attention.

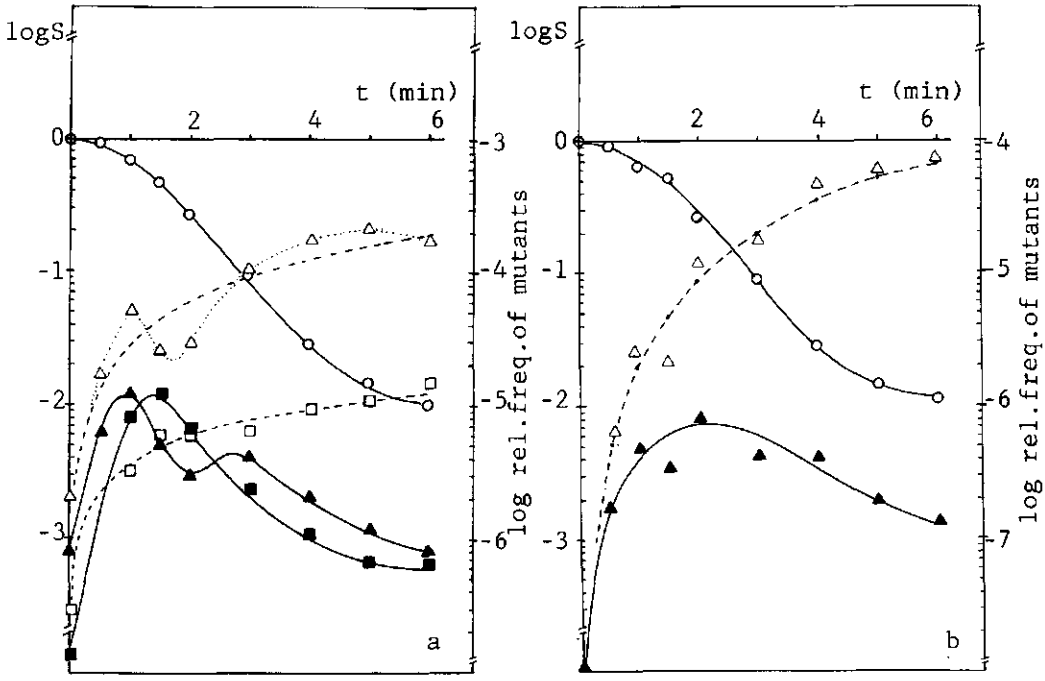


Figure 2.18 Resistant mutants and pabaA1-revertants in A.nidulans WG096.
a. Experiment in which acriflavine resistant mutants were scored.
On plates with 100 ug/ml: ▲ mutants among total spores;
△ mutants among survivors;
On plates with 150 ug/ml: ■ mutants among totals spores;
□ mutants among survivors.
b. Experiment in which pabaA1-revertants were scored.
▲ mutants among total spores; △ mutants among survivors.
For both experiments the survival curves have been added (o).

The results are presented in Fig 2.18b. At low doses (1-3 min) pabaA1-revertants with nearly wildtype colony growth were found in addition to small colonies of irregular shape. At higher doses only small and thinly growing colonies were found. The revertants with irregular or thin growth are perhaps suppressor mutants with poor growth characteristics. At higher doses probably also double mutants were present. Three well growing revertants were analyzed (not presented) and turned out to be at three non-linked pabaA1-suppressor loci.

The function $y = at^b$ (with $b = 2.1$ and $a = 20.7$) fits well to the log rel. frequency of pabaA1-revertants among survivors (dashed line in Fig.2.17b).

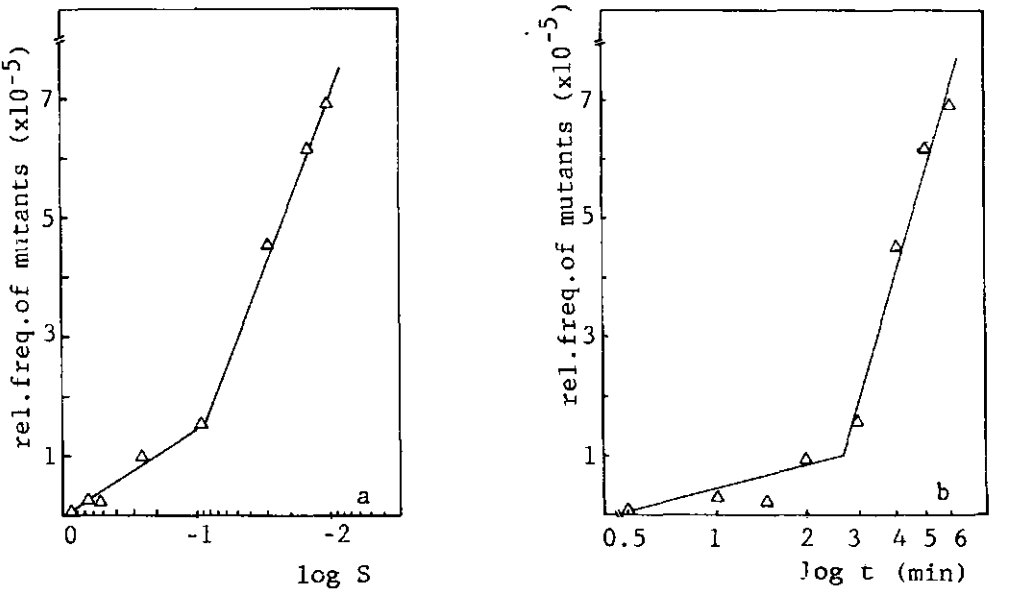


Figure 2.19 PabaA1-revertants in A.nidulans WG096.
a. Data from Fig.2.18, frequency of mutants among survivors was plotted against log S.
b. Same data, but now plotted against log t.

Graphs in which the relative frequency of mutants is plotted against logS (Fig.2.19a) or against logt. (Fig.2.19b) show a steep rise as a result of a scaling effect. This might suggest that only at higher doses (or at low survival) it is profitable to screen for the wanted mutants. However, the curves in Fig.2.18, where log frequency of mutants among survivors is plotted against the dose (on linear scale), give a more balanced picture for deciding what dose to choose.

In literature on short dose-ranges it is often found that the frequency of mutants among survivors is linear with the duration of mutagenic treatment (e.g. Kølmark and Kilbey, 1968; Kilbey et al., 1978), but often (e.g. in textbooks) the relationship of mutant frequency with dose is represented by plotting the untransformed relative mutant frequency among survivors against logS or against logt. The curves presented are mostly linear (e.g. Bridges and Munson, 1968; Munson and Goodhead, 1977; Lawrence and Christensen, 1978). This is at variance with the results of the present experiments on the pabaA1 revertants where no simple linear function is found. (See Fig.2.19a and b for the relative mutant frequency among survivors as functions of logS and logt respectively.)

The metG1-system described by Lilly (1965) is often used to study mutation induction in A.nidulans (e.g. Scott and Alderson, 1971; Boschloo, 1985). Therefore we included an experiment with this system (strain WG282) to widen the range of mutant types studied. In a parallel experiment the conidia were preincubated in liquid SM for 3 hours to see whether this influenced the frequency of mutants. The two experiments started from the same conidial suspension and were carried out in duplicate.

The results (Fig.2.20) are similar to those of the foregoing experiments. Again the maximum number of mutants was obtained at a low UV-dose (survival level of about 50%). With preincubation the cells are more sensitive than without (see logS curves in Fig.2.20) and the effect is that the mutant-curve has shifted somewhat to the left. However, the relative frequency of mutants

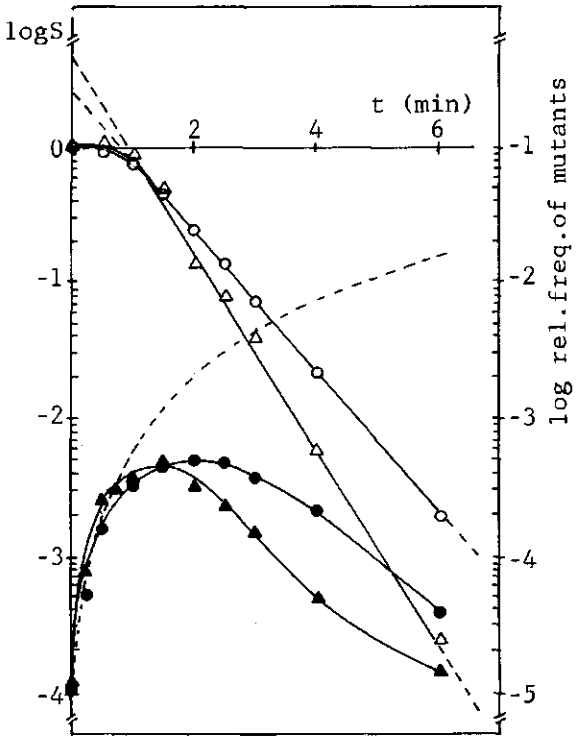


Figure 2.20 MetG1-revertants in A.nidulans strain WG282.
○ , △ Survival curves for conidiospores without resp. with 3h preincubation in liquid SM.
● , ▲ Relative frequency of mutants among total spores (viable at t = 0).
- - - - Curve for the function $y = at^b$ which fits for the relative frequency of mutants among survivors both without and with preincubation.

among survivors is about the same for with and without preincubation. A good fit is given by the function $y = a.t^b$ (dashed line in Fig.2.20), with b = 1.8 and a = 62 for non-preincubated spores, and b = 1.7 and a = 64 for preincubated spores. It can be concluded that 3 h preincubation has no effect on the relative frequency of mutants among survivors.

2.3.2 DISCUSSION

The experiments described in the previous section show that it is very well possible to isolate recessive mutants at low mutagen doses. Among the acriflavine and perhaps also among the benomyl resistant colonies there could be dominant mutants, but from the work of Van Tuyl (1977) it is known that benomyl resistant mutants mostly are recessive. Also some acriflavin mutants are known to be recessive (see Clutterbuck, 1984). For the pabaA1-revertants it is shown that the three revertants analyzed were recessive. The frequency of pabaA1 revertants was low, much lower than the frequency of resistent mutants. The reason probably is that only very specific mutations lead to revertants whereas mutations towards resistance are possible at many sites in different genes (cf. Roper and Käfer, 1957; Van Arkel, 1958; Van Tuyl, 1977). For the metG1-system it is known that most of the revertants belong to several classes of suppressor mutants (Lilly, 1965; Scott and Alderson, 1971).

In our experiments no simple relationship between the frequency of mutants among survivors and the mutagen dose or the logarithm of the survival was found. The linear relationships reported in literature (discussed by Munson and Goodhead, 1977; Chadwick and Leenhouts, 1976; Balbinder et al.,1983) were probably found because only small dose ranges were studied or because the curves are based on only few data. As the revertants are at different loci, complex relationships may be expected, for from our experiments it is seen that the maximum yield is not at the same UV-dose with different types of mutants (for complex relationship see also Alderson and Hartley, 1969). Furthermore, the relative frequency of acriflavine resistent mutants seems to be about linear with the effective dose, whereas the frequency of pabaA1 revertants among survivors is proportional to the square of the effective dose. This is in agreement with the value found for N.crassa ad3-revertants following diepoxybutane treatment of conidia (Kølmark and Kilbey, 1968). The yield of methG1-revertants in our experiments was proportional to or somewhat less than the square of the effective dose.

At higher doses the curve for the relative frequency of mutants among survivors tends to level off or even to decrease. This has also been found in Schizosaccharomyces pombe for certain chemical mutagens by Heslot (1962; cited by Fincham et al.,1979).

Graphs in which the relative frequency of mutants is plotted against logS (as in Fig.2.19a) or against logt (Fig.2.19b) show a steep rise as result of scaling effects. The value of plotting the frequency of mutants among survivors against logS or logt and especially of plotting its logarithm against logS or logt is very questionable. Nevertheless such graphs appear in textbooks (Burnett, 1975; Fincham et al., 1979). These transformations wrongly suggest that the yield of mutants will always increase with the dose and that only at higher doses it is profitable to screen for the wanted mutants.

In conclusion it can be argued that for the induction of mutants low doses mutagen are optimal. Maximum numbers of mutants were obtained at survival levels above 20% say, but when the harmfull effects of high mutagen doses on the genetic back-ground are considered, the optimal conditions for mutation induction will be at still higher survival levels. In practice we prefer to aim at about 80% survival level. When direct selection is possible, as is the case with resistance mutants, the advantage of low doses is obvious. For auxotrophic mutants the situation may be somewhat less pronounced as the frequency of mutants among the survivors is a little higher at high doses of mutagen. But this advantage does not offset the chance of unnoticed double mutations or chromosome rearrangments. The fact that at higher doses only small slow growing revertants were found in the experiment with WG096, and that a similar tendency was observed with metG1-revertants in strain WG282, is a clear indication of a distorted genetic background.

In order to compensate for the low frequencies of mutants among the survivors, appropriate enrichment procedures will be very useful. These will be discussed in the next section.

2.4 Enrichment of mutants

2.4.0 INTRODUCTION

A number of distinct enrichment methods have been reported for the isolation of auxotrophic mutants of filamentous fungi. The most classical one is the filtration enrichment procedure developed for Ophiostoma multiannulatum by Fries (1947, 1948) and adapted to Neurospora crassa by Woodward et al.(1954) and also to several other fungi including A.nidulans (e.g. Roberts, 1963). In A.nidulans the starvation method has been used too (Pontecorvo et al., 1953). Upon the discovery of the penicillin technique for E.coli, several suggestions for enrichment of fungal mutants by antibiotics were made (Snow, 1966; Bal et al., 1974; Ditchburn and MacDonald, 1971). When techniques for the isolation of protoplast had become available, these protoplast were proposed as tools for the isolation of auxotrophic mutants (Piedra and Herrera, 1976; Sipiczki and Ferenczy, 1978).

Several of these procedures have been tested by us for the isolation of A.nidulans auxotrophs and other biochemical mutants (e.g. mutants unable to use specific carbon sources). In our hands none of these procedures gave better results than the filtration enrichment procedure.

In the filtration enrichment procedure the original strain is allowed to grow in liquid MM supplemented with the essential growth factors. After successive incubation periods the mycelium and the germinated conidia are removed by filtering through a plug of cotton wool. The spores that pass the cotton woll plug are reincubated in fresh medium if desired. Finally the conidia are collected and resuspended in a small volume and plated for rescue. The resulting colonies are tested for mutant phenotype (in addition to the markers in the original strain).

In order to test this procedure some model experiments with A.nidulans and A.niger have been performed. In these model experiments, a known quantity (e.g. 0.5%) of spores from a mutant strain was added to the spores of a "wildtype" strain, which carried a marker so both types can be counted upon plating.

2.4.1 RESULTS

In prelimary experiments it was found that the quality of the cotton wool plug used for filtration is very important. In a funnel (diameter 7 cm) 50 mg cotton wool was applied. The filters were sterilized in a pressure cooker, because they become felted upon dry sterilization. These filters were compared with layers of small-mesh gauze and Miracloth and proved to be the most satisfactory. Incubation was in a reciprocal shaker at about 180-200 strokes per minute.

A representative model experiment is shown in Fig. 2.21. Here conidial suspensions of three A.nidulans strains were mixed in order to test the enrichment of two mutant types. A pabaA1 deficient strain (WG096) served as prototrophic strain and as

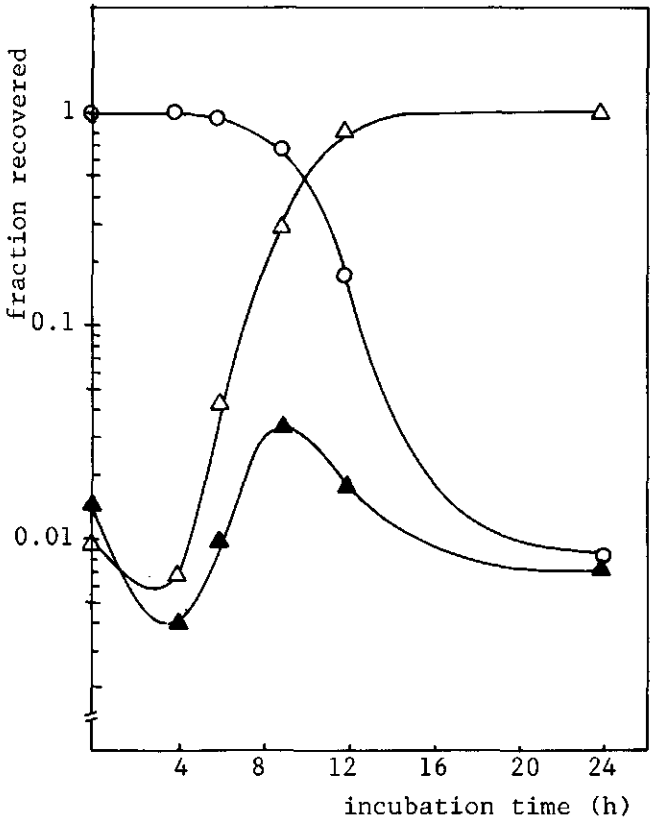


Figure 2.21. Filtration enrichment in a model experiment with A.nidulans. WG096 served as prototrophic strain (o) and WG136 (pyc-deficient: △) and WG176 (ribo-deficient: ▲) as mutants.
Incubation in liquid SM with glucose as carbon source at 37°C in a reciprocal shakerbath.

mutants we used a pycB4, biA1 strain (WG136) and a riboD5, nicA2 strain (WG179). Conidiospores of the mutant strains (about 0.5 %) were added to the prototrophic strain and incubated in liquid MM supplemented for the paba-deficiency of WG096. Upon plating the frequency of each of the "mutants" proved to be about 1% (of the viable spores). The suspensions were incubated at 37°C. At successive time intervals the suspensions were filtered through a cotton wool plug. Diluted samples were plated on medium supplemented for one of the strains. The conidia of the remaining suspension were collected and resuspened in fresh medium and incubated for another time interval.

From Fig. 2.21 it is seen that a mutant which is blocked in a major metabolic pathway (i.e. pyruvate carboxylase; WG136) can be enriched very efficiently. When only riboflavin was ommitted from the incubation medium the frequency of the mutant increased first, but later on the relative frequency of the mutant decreases, probably due to cross-feeding as only little amounts of vitamin are necessary to allow growth.

In Fig. 2.22 the results of an enrichment experiment with A.niger are shown. This fungus has slower growth and an optimal temperature of 30°C. In this model experiment an arg-less strain was mixed with 0.1% of a lys-deficient strain. Incubation was in MM supplemented with arginine. At successive intervals the suspension was filtered through a cotton wool plug, which was washed with saline because otherwise part of the conidia stay behind. The conidiospores of these A.niger strains had more difficulty in passing the cotton wool plug than A.nidulans conidiospores. The conidia were collected on a membrane filter and resuspended in fresh medium. Then a sample was plated in appropriate dilutions for viable count. Fig. 2.22 shows that after prolonged incubation (>36h) the frequency of mutant type conidia decreases somewhat, but after 24-36 hours a high yield is obtained.

It was also studied whether the filtration enrichment procedure was applicable to multi-target cells. Recessive mutations will not be expressed in the resulting heterokaryon and often this is met by very high mutagenic treatments in order to

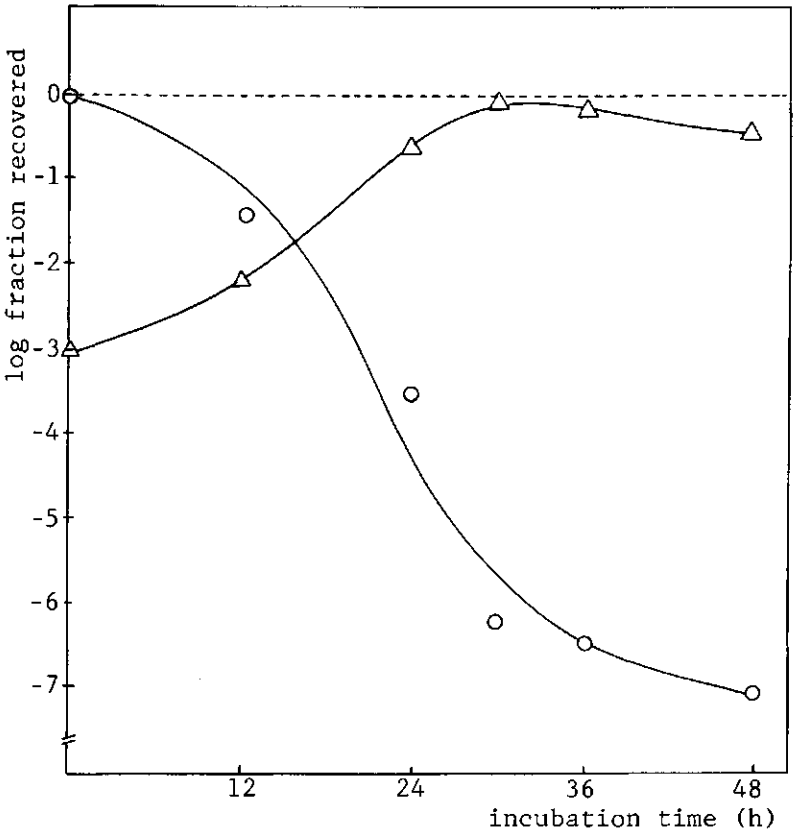


Figure 2.22 Filtration enrichment in A.niger.
Simulation in a model experiment with N408 (arg-deficient) as prototrophic (O) and N420 (lys-deficient) as auxotrophic component (Δ). Incubation in liquid MM supplemented with arginine at 30°C in a reciprocal shakerbath.

inactivate all other nuclei of a cell (e.g. Bergman et al., 1973). Conidia of A.oryzae were UV-treated and incubated for enrichment as usual for A.niger. No mutants were obtained. In a similar experiment a segregation step was included. After UV-irradiation the spore-suspension was divided into 10 portions of 1 ml. These were each transferred to 30 ml culture flasks with solidified CM. These cultures were incubated at 30°C for 4 days and spores were collected from each culture separately. The spore suspensions were treated for enrichment and plated on CMT for rescue as usual. From one such experiment three different auxotrophic mutants could be obtained.

2.4.2 DISCUSSION

Model experiments with A.nidulans and A.niger showed that the filtration enrichment method is very suitable for the isolation of auxotrophic mutants. The efficiency of the method depends to some extent on the type of mutants, but it proved to be very useful for the isolation of mutants with a deficiency in the carbon metabolism (Bos et al, 1981; Uitzetter, 1982). Roberts and coworkers used a comparable enrichment procedure for the isolation of carbon metabolism mutants (Roberts, 1959; Armit et al, 1976; McCullough and Roberts, 1974; Payton et al., 1976).

For the isolation of auxotrophic mutants which are more sensitive to cross-feeding the medium has to be refreshed at shorter intervals and the procedure can be continued for a shorter time only. As will be shown in Chapter 4 a great variety of auxotrophic mutants of A.niger have been isolated by us in this way.

For certain types of mutants the rescue medium may be critical: Some essential substance may not be present in CM (e.g. tryptophan). It is also possible that certain mutants need extra supplementation of CM (e.g. some arg-less and pyr-less mutants).

With somatic recombination experiments too, the filtration enrichment method is very suitable for the isolation of homozygous diploid recombinants (from mitotic crossing-over) in addition to haploid recombinants. For genetic analysis of A.nidulans (Bos et al., 1981) and also for A.niger (Chapter 4) this method could be profitably used.

The isolation of auxotrophic mutants of A.oryzae was not successful if the enrichment procedure immediately followed the mutagenic treatment. This can only be expected, because the conidia are multinucleate. After introduction of a segregation step, however, recessive auxotrophic mutants have been obtained. So, there is no need for the application of a high dose mutagen to kill nuclei till only one viable nucleus is left.

2.5 Conclusions

ad 2.2

- Semi-logarithmic dose response curves for survival of UV-irradiated conidiospores of A.nidulans have an intial shoulder (at low doses) followed by a decline in survival which is linear with the dose.
- Resting conidiospores of haploid A.nidulans and A.niger strains are single target cells, so one is inclined to explain the initial shoulder by a multi-hit process.
- However, experiments on the modification of k, viz. or differences in inherent sensitivity of the spores or differences in dose rate received in the spores, clearly indicate the involvement of another factor.
- This factor is the repair capacity of the cell, which becomes saturated around a certain dose. This is confirmed by irradiation of repair deficient strains, which do not show an initial shoulder.
- In diploid conidia, or conidia which are in G2-phase, or multinucleate conidia, the extended shoulder results only for a small part from an increased target number and mainly from both repair and complementation, which two factors reinforce each other.
- In general the logS-intercept of the linear extrapolation lines cannot be used to estimate the target number.

ad 2.3

- Studies on mutants frequency showed that for different types of mutants the maximum yield is obtained at rather high survival levels (20-50%).
- The relative frequency of mutants among survivors increases with the mutagen dose, but levels off at higher doses, or even declines somewhat. Plotting the frequency of mutants among survivors against log t or log S, as is often done in literature, wrongly suggests that high mutagen doses are to be preferred.

- Since at higher doses the mutations looked for do not come by themselves, but are present in a disturbed genetic background, it is advisable to use doses giving high survival levels (e.g. 70-80%), and to limit the number of rounds of mutations induction in a strain to a minimum.
- For combining different mutant genes in a strain recombination procedures are to be preferred.

ad 2.4

- To compensate for the lower frequency of mutants among survivors, appropriate filtration enrichment procedures can be succesfully used for different types of mutants.
- With fungi with multinulceate spores a segregation step can be introduced, which will uncover the recessives present. This can be followed by enrichment procedures.

Acknowledgements: I wish to thank Prof. J.H. van der Veen and Dr.P.Stam for their valuable suggestions and contributions.

3. Somatic recombination

3.0 Introduction

In phytopathogenic imperfect fungi somatic recombination is important for the evolution of physiological races. Mutations in virulence and pathogenicity can be maintained and recombined, thus providing genetic flexibility. Mitotic crossing-over allows recombination even of tightly linked genes.

Prerequisites for somatic recombination are heterokaryosis and the formation of heterozygous diploid nuclei. In fact heterokaryosis is the first phase of somatic recombination because in a multinucleate organism it allows combination and recombination of whole nuclei. Heterokaryons are formed by hyphal fusions (anastomoses) followed by exchange of nuclei from genetically different strains. When heterokaryons only produce uninucleate conidiospores, the heterokaryotic condition can not be maintained via these spores, as they give the parental types only. More details of the process of somatic recombination have been given in Chapter 1.

In several fungi heterokaryosis and karyogamy have been observed. First in A.nidulans and A.niger (Roper, 1952; Pontecorvo et al., 1953) and later also in Aspergillus oryzae (Ishitani and Sakaguchi, 1956), A.flavus (Papa, 1973, 1976), A.parasiticus (Papa, 1978), Verticillium albo-atrum (Hastie, 1964, 1967), Fusarium oxysporum (Buxton, 1956; Garber et al., 1961), Penicillium chrysogenum (Pontecorvo and Sermonti, 1953, 1954; MacDonald et al., 1963), Cochiobolus sativus (Tinline, 1962). Heterokaryosis is also well known in Neurospora crassa (Garnjobst 1953, 1955). Since the review on fungal flexibility by Hansen (1938), several reviews on heterokaryosis and parasexuality in fungi have been published (Bradley, 1962; Parmeter et al., 1963; Tinline and MacNeill, 1969; Caten, 1981).

Jinks (1952) pointed out that heterokaryosis is a system for adaptation in wild fungi, and it was recognized that it might be important in plant pathology as an adaptive system by Buxton (1960) and Johnson (1960).

Köhler (1930) was perhaps the first to study anastomosis in imperfect fungi (e.g. Botrytis, Fusarium). Since then anastomoses have been observed in many imperfect fungi. In Fusarium anastomoses occur only at a certain distance from the border of the colony (Dickinson, 1932). In Verticillium on the other hand also fusion between germ tubes of conidia has been observed (Schreiber and Green, 1966). In Helminthosporium fusion has been observed just behind the hyphal tips (Hrushovetz, 1956). Hoffmann (196) studied the frequency of anastomosis between different types of hyphae in Fusarium oxysporum. About two third of the fusions was between older parallel situated and less than 4% between younger hyphae near the tip.

Although the possibility of hyphal fusions does not depend on generative compatibility systems, notably in Aspergillus nidulans, several heterokaryon compatibility groups have been found (Caten and Jinks, 1966). Sometimes anastomosis can proceed, but is followed by an incompatibility reaction resulting in cell death. This phenomenon has been mainly studied in Neurospora crassa (Garnjobst, 1955; Garnjobst and Wilson, 1956). In these cases anastomosis took place but no plasmogamy followed. Vegetatively incompatible A.nidulans strains might produce heterokaryons upon prolonged incubation under selective conditions (Dales et al., 1983).

Heterokaryosis sometimes is also possible between species. Hansen and Smith (1932) found interspecific fusions between Botrytis allii and B.ricini, Uchida et al. (1958) between Aspergillus oryzae and A.sojae and Hastie (1973) between Verticillium albo-atrum and V.dahliae.

For studying heterokaryosis, auxotrophic mutants are essential, and the fungus should grow on simple synthetic media (MM). For analyzing somatic recombination the conidiospores should be unicellular and uninucleate.

The imperfect fungus Colletotrichum lindemuthianum met these requirements. It is the causal organism of bean anthracnose, and therefore it was attractive to study the genetic basis of its phytopathogenicity. There was ample information on its races and on resistance in beans (e.g. Barrus, 1918; Bannerot, 1965; Hubbeling, 1961; Krüger et al, 1977). Physiological races could be obtained from the collection at Wageningen (Hubbeling, Instituut voor Plantenziektenkundig Onderzoek, Wageningen, The Netherlands).

Much effort was spent to study somatic recombination of C. lindemuthianum in vitro. However, it turned out that a number of features of this fungus interfered with the interpretation of the experimental results. The conidiospores are large and contain much reserve material, so auxotrophic strains may have some growth on MM. On the ohter hand germination percentage of the spores is low, even on CM, and the viability of cultures decreases rapidly with time.

It was difficult to distinghuish between heterokaryosis and cross-feeding. Of course the isolation of heterozygous diploids would provide proof for heterokaryosis, but such diploids could not be found with certainty either (see further 3.2).

Therefore we resorted to Aspergillus nidulans as a model organism.. Its genetics is well established (Pontecorvo et al., 1953; Käfer, 1958,1977), it has both mitotic and meiotic recombination, and many markers are available in the extensive strain collection at Glasgow University and in the Fungal Genetic Stock Centre (FGSC, Humbold State University Foundation, Arcata). A.nidulans is known to form balanced heterokaryons (Jinks et al., 1966). Between different wildtype isolates heterokaryon incompatibility is found (Jinks and Grindle, 1963; Grindle, 1963; Jinks et al., 1966). This type of incompatibility is found generally (Esser and Blaich, 1973).

The problem of crossfeeding can be avoided by making heterokaryons via protoplast fusion. Fusion between two protoplasts from genetically different strains will unambiguously lead to heterokaryons. Moreover, by using protoplasts, natural heterokaryon incompatibility between unrelated strains may be

bypassed (Dales and Croft, 1979; Kevei and Peberdy, 1983) and even fusion of protoplasts from different (related) species may lead to heterokaryons (Ferenczy et al., 1977; Kevei and Peberdy, 1977; Mellon et al., 1983). Protoplast fusion between unrelated species may be usefull for exchange of mitochondria or for virus transmission (Ferenczy, 1981; Kaiying and Pingyan, 1984).

Methods for protoplasts isolation and fusion in A.nidulans are presented in the Sections 3.3. and 3.4, respectively. Section 3.5 gives the application of this method to C.lindemuthianum.

3.1 Materials and methods

3.1.0 ORGANISMS AND STRAINS

The following A. nidulans strains descending from Glasgow strains were used. Gene symbols are according to Clutterbuck (1984); markers on different chromosomes separated by a semicolon.

WG019 biA1; AcrA1; phenA2 (biotin; phenylalanine requiring, acriflavin resistant)

WG021 yA2; AcrA1; lysB5 (yellow conidia; acriflavin; lysine)

WG076 yA2; nicA2, riboD5 (yellow; nicotinamide, riboflavin)

WG093 pabaA1, yA2; AcrA1; pyroA4 (p-aminobenzoic acid, yellow; acriflavin; pyridoxin)

WG132 pabaA1; wA3, AcrA1; pyroA4 (p-aminobenzoic acid; white, acriflavin; pyridoxin)

WG179 yA2; nicA2, riboD5 (yellow; nicotinamide, riboflavin).

Colletotrichum lindemuthianum strains were kindly provided by Dr. N. Hubbeling (IPO, Wageningen). They represented different physiological races, C409: alpha and C420: gamma. From these wildtypes auxotrophic mutants were isolated by UV-mutagenesis at about 0.1-1% survival. At that time, the experiments described in Chapter 2 had not been carried out yet, so we still used survival levels customary in literature.

C409-20 pro (proline requiring) from C409

C409-36 ade (adenine) from C409

C409-41 pro, thr (proline, threonine) from C409-20

C409-45 arg, lys (arginine, lysine) in two steps from C409

C409-47 arg, lys, azg (arginine, lysine, azguanine-resistant) from C409-45

C420-08 arg (arginin) from C420

C420-10 pro, cys (proline, cysteine) in two steps from C420

C420-12 pro, cys, azu (proline, cysteine, azauracil-res.) from C420-10

Strains of Cladosporium cumerinum, Aspergillus carbonarius, A.niger, Fusarium culmorum and Penicillium expansum were provided by the Department of Phytopathology (Agric. Univ., Wageningen).

For the production of lytic enzymes Oerskovia xanthineolytica (ATCC 27402) was used.

3.1.1 MEDIA

The fungi were grown on complete medium (CM), minimal medium (MM), supplemented MM (SM) and malt extract agar (ME),the compositions of which are given in Section 2.1.1. In addition a liquid synthetic medium (SG) was used that was suitable for spore germination. For C.lindemuthianum a peptone medium (M) advised by Mathur et al. (1950) and a medium with ureum as nitrogen source (C3) were used. The compositions of these media are per litre demineralized water:

SG: 0.05 g $MgSO_4.7H_2O$
0.15 g KH_2PO_4
0.05 g KCl
0.04 g NH_4NO_3,the salt solution was adjusted to pH 6.0
0.6 g glucose (added as filter sterilized solution).

M: 1.23 g $MgSO_4.7H_2O$
2.72 g KH_2PO_4
2.0 g neopeptone (Difco)
2.8 g glucose.

C3: 1.23 g $MgSO_4.7H_2O$
2.72 g KH_2PO_4
1.0 g NaCl
0.75 g ureum
trace $FeSO_4$ and $ZnSO_4$
1.0 g inositol
1.0 g sorbose
4.0 g glucose.

The filter sterilized carbon source was added separately; solid media contained 1.5 % agar, soft agar media 0.8%. The media were adjusted to pH 6.0 and sterilized by autoclaving for 20 minutes.

Aspergillus nidulans cultures were grown for 3 days at 37°C, A.niger 4 days at 30°C, C.lindemuthianum 12-14 days at 22°C and Oerskovia xanthineolytica at 30°C.

Aspergillus cultures could be stored at 4°C for months but usually the cultures were kept at 4°C for 3 days prior to experimental use. . The strains were preserved by storage of conidia on silicagel as described in 2.1.0.

Oerskovia cells were preserved by lyophilisation and from these fresh start plates on nutrient agar were made. Details are given in 3.1.5.

With the maintainance of C. lindemuthianum cultures several difficulties arose. The viability of cultures diminishes strongly after 3-4 weeks, also upon storage at 4°C C.lindemuthianum conidia did not survive storage on silicagel. A method for lyophilization could be developed which maintained viability: Spores from 12-14 day old cultures are suspended in a lyophilization medium containing 75% (w/w) egg albumin solution (50 g powdered soluble egg albumin (Merck) in 75 ml demineralized water and decontaminated by centrifugation at 15.000 rpm for 20 min), 10 % glucose, 10 % glycerol and 5 % sodium glutamate. See further 3.2.0.

In general conidial suspensions were made in 0.8% (w/v) saline containing 0.005% (w/v) Tween-80. For Aspergillus strains the suspensions were vigorously shaken during 10 min to break conidial chains. Suspensions were subsequently filtered through a cotton wool plug to remove mycelial debris.

Prior to experimental use conidial suspensions of A. nidulans and A.niger usually were kept in saline overnight at 4°C. With the other fungi always fresh suspensions were prepared.

Each series of experiments started from monospore cultures which had been tested for genetic markers previously.

3.1.2 HETEROKARYONS

Aspergillus nidulans heterokaryons were made following Pontecorvo et al. (1953). Conidia of two strains with different auxotrophic markers were mixed in 2 ml liquid complete medium and incubated at 37°C for one day. Pieces of the resulting mycelial material were transferred to MM plates and incubated at 37°C for 4 to 5 days. The usually poorly growing mycelium incidentally produced vigorously growing sectors. Uniformly growing balanced heterokaryons were obtained by transfer of pieces of such sectors to MM plates. For A.niger a longer incubation period at 30°C was used and the MM plates were incubated at 30°C too.

Attempts to establish heterokaryons in C.lindemuthianum were made by combining conidia from two auxotrophic strains in liquid CM at 22°C for 4 days. The mycelium was then collected on a membrane filter (0.45 um) to remove nutrients and washed with saline. Pieces of mycelium were transferred to MM plates and incubated at 22° C for 14 to 20 days. As a control the parental strains were treated separately in the same way.

3.2.3 ISOLATION OF DIPLOIDS

For the isolation of heterozygous diploids from heterokaryons a suspension was made of conidia from a heterokaryon in saline/ Tween as described earlier. Viability counts were done by plating a diluted suspension on CM. Diploids were isolated by plating a concentrated suspension in MM with a thick MM toplayer as described by Pontecorvo et al. (1953). In some experiments with A.niger 0.5 or 1 ml aliquots of a concentrated suspension were mixed with 10 ml soft MM-agar (0.8 % agar; 45°C) and plated in a toplayer on MM bottoms. In this way diploid colonies will appear well before heterkayons arise due to the dense plating.

3.1.4 MICROSCOPICAL EXAMINATION OF ANASTOMOSIS

Several methods were tried for microscopical examination of anastomosis (the onset of heterokaryosis). Two of these were satisfactory.

a. Microscopic slides. Slides covered with a thin layer of MM were inoculated with spores by means of a thin cotton thread soaked in a spore suspension. The two strains were inoculated at a distance of about 3 mm. The slides were incubated in a Petri dish on two glass rods with some sterile water on the bottom preventing drying.

b. A Moist chamber method. Anastomosis can also be observed in a hanging drop. On a microscopic slide a perspex ring is mounted with silicone grease. A cover glass with a hanging drop inoculated at opposite sites is placed on the ring. At the bottom of the chamber some water is present.

3.1.5 LYTIC ENZYMES

For the isolation of protoplasts from conidiospores the lytic system used was a combination lytic enzymes excreted by O. xanthineolytica (Mann et al., 1972) grown on A. nidulans cell wall material and glucanases, either glucanases containing autolytic enzymes from Aspergillus (Zonneveld, 1972) as used by Van den Broek et al. (1979), or commercial glucanases. Portions (5 ml) of the enzyme preparations were either frozen and kept at -80 °C, or freeze-dried, which gave no noticeable loss of activity over a period of 2 years.

The Oerskovia enzymes were obtained from shake cultures by precipitation with ammonium sulphate; the yield was 20-25 ml of enzyme from 1.8 l of culture filtrate at a concentration of 0.5-1 mg protein/ml determined by the Lowry reaction. The Aspergillus glucanases were isolated from plate-grown Aspergillus and the yield per plate (9-cm diameter) was 6-8 ml. In later experiments the Aspergillus glucanases were replaced by β-glucanases from Penicillium emersonii purchased from British Drug Houses Ltd.

3.1.6 PREPARATION OF PROTOPLASTS FROM CONIDIOSPORES

Protoplasts were prepared from conidia that were pre-incubated in liquid minimal medium (pH 6.0) supplemented with the essential growth factors and 2-deoxy-D-glucose (25 ug/ml). This minimal medium (CP) was essentially the same as the minimal medium of Pontecorvo et al. (1953), except that now 5.9 g/l sodium citrate and 1.0 g/l sodium pyruvate were added. Occasionally also a medium (SG) very suitable for spore germination was used.

Preincubation of the conidia (10^6/ml) was at 37°C for 3 h in a reciprocal shaking bath (180 oscillations/min). Shaking is necessary to avoid clumping of conidia. After this period the conidiospores from 50 ml suspension were collected on a membrane filter (0.45 um) and resuspended in 2 ml buffer (pH 6.5) containing 10% (v/v) Oerskovia enzyme and either Aspergillus glucanases (10%, v/v) or commercial Penicillium glucanase (0.1 mg ml). The buffer consisted of 0.2 M KH_2PO_4, 0.4 M $(NH_4)_2SO_4$ and 0.5 mM $CaCl_2 .2H_2O$, adjusted to pH 6.5 with 1 M KOH. Addition of $(NH_4)_2SO_4$ to the mixture prevented the disruption of protoplasts.

After 3 h of incubation at 30°C in a shaker bath (120 oscillations / min) most of the conidiospores were converted into protoplasts.

3.1.7 PURIFICATION OF CONIDIAL PROTOPLASTS

In order to remove conidia and cell wall fragments, approximately 1 ml of the protoplast suspension was layered on 7 ml of a 30% or 35% (w/v) sucrose solution in a glass tube and centrifuged in a swing-out rotor at 1200 rpm for 20 min. The conidia and cell wall fragments sedimented, while the protoplasts banded at the interface between buffer and sucrose. They could easily be withdrawn with a Pasteur pipette, and stabilizing buffer (usually 0.6 M KCl according to Ferenczy et al., 1976) was added stepwise up to a final volume of 5 ml. After 20 min the protoplasts were recollected by centrifugation at 4000 rpm and resuspended in fresh stabilizing buffer.

3.1.8 COUNTING AND MEASUREMENT OF PROTOPLASTS AND CONIDIA

The counting of conidia and protoplasts was carried out with a Coulter counter (model ZF with channelyzer), using 0.6 M KCl as electrolyte in order to stabilize the protoplasts. The distribution patterns were based on the volume of the particles. For calibration, latex particles of 8.07 um diameter were used. The process of protoplast formation was followed by microscopical inspection of samples in a haemocytometer, thus allowing a rough estimate of the number of protoplasts released. The viable protoplasts and conidia were determined by plating on complete medium, with or without 0.4 M KCl as stabilizer.

3.1.9 STAINING OF NUCLEI IN PROTOPLASTS

Protoplasts were stabilized with 0.6 M KCl and fixed with a solution of 0.25% (w/v) glutaraldehyde (Miegeville and Morin, 1977) in 0.6 M KCl for 20 min. The fixative was removed by centrifugation and the protoplasts were washed with demineralized water. A drop of the protoplast suspension was placed on a slide and freeze-dried. Then the dried protoplasts were stained with lacto-acetic orcein (2% w/v orcein in a 2:1 mixture of acetic acid and lactic acid).

3.2 Heterokaryosis in Colletotrichum lindemuthianum

3.2.0 EXPERIMENTS TO IMPROVE VIABILITY OF STRAINS

Genetical and physiological experiments with Colletotrichum lindemuthianum were hampered both by problems in maintaining the strains and by the low germination frequencies of the conidiospores.

When stock cultures are maintained by monthly transfer of conidiospores there is a strong selection favouring the production of conidia, but other characteristics like auxotrophic markers or virulence may be lost. Germination percentage was as low as 10% (sometimes 20%) and when cultures of C.lindemuthianum were stored for four weeks at 15°C germination decreased to 10% of the original germination frequency. Storage at 4°C resulted in even lower viabilities. C.lindemuthianum did not survive storage on silicagel, the standard procedure for many fungi. Lyophilization as used for fungi (cf Hesseltine et al., 1960)

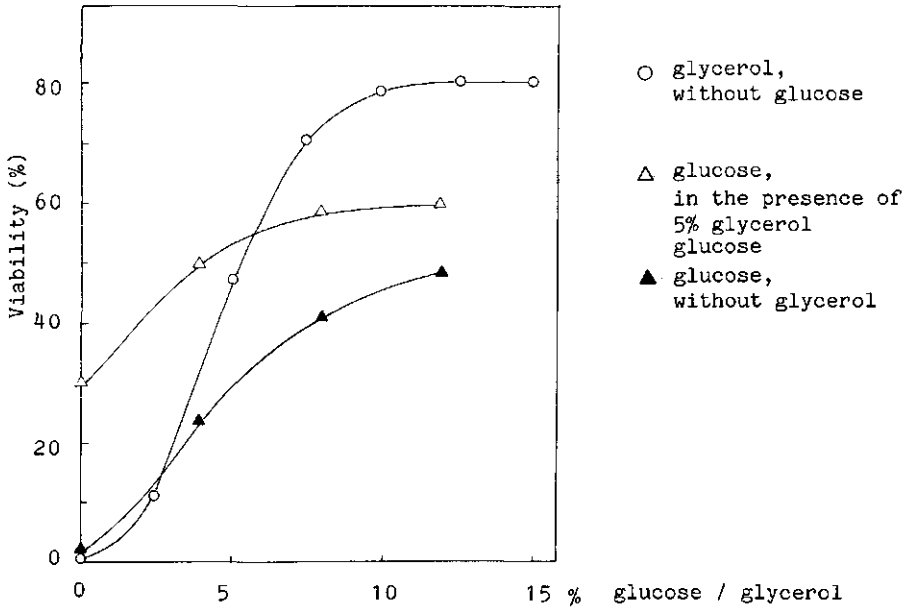


Figure 3.1 Effect of glycerol and glucose on lyophilization of C.lindemuthianum. Conidia of C.lindemuthianum (strain C409) were lyophilized in albumin-glutaminate medium with varying concentrations of glucose and/or glycerol.

was not possible either. Therefore we looked for a method of lyophilization in which a certain amount of bound water was maintained.

Empirically we found that lyophilization of a suspension of conidia in a concentrated egg-albumin solution containing 10% glucose, 5% sodium glutaminate and 10% glycerol resulted in high survival. The experiments on the composition of this preservation medium will be published in detail elsewhere. Here the effects of glycerol and glucose are shown (Fig.3.1). When small samples of 0.04 ml were dryed, freezing was not necessary. Routinely some samples were used for the determination of survival after a few days or a week. Usualy a survival of 50-80 % was found. These batches could be stored for more than 15 years without loss of viability.

During the search for a medium that allowed higher plating efficiency the medium used for Aspergillus nidulans (Section 2.1) proved to be superior to that proposed for C.lindemuthianum by Mathur et al.(1950). Especially on MM the colonies sporulated much better than on Mathur's medium, and also the viability of conidia harvested after some weeks was better. A modified complete medium based on that from Mathur et al.(1950) was used too.

In Fig. 3.2 the results of a growth experiment with different media are shown. In this experiment the fungus was grown in 30 ml vials with 10 ml of medium. After some time of incubation at 22°C conidia were collected in saline-Tween and plated on CM for viable count, as on this medium the germination of conidia was the highest and the colonies could be counted very well. On CM the colonies could be counted after two days; on MM a day later. Colonies varied in size due to asynchronous germination.

Germination in liquid media was studied too. Besides saline and liquid MM, a medium (SG) with a lower salt concentration and less nitrogen, including different glucose concentrations were tested. After a few hours of incubation samples taken and 150 - 200 conidia were scored microscopically for germination. Conidia with a germination tube of half the width of the conidium were scored as germinated. The results are summarized in Fig.3.3.

The low salt medium (SG) (see Fig.3.3 C,D) gave the best results. This liquid medium was used for incubations of spores in the experiments to follow.

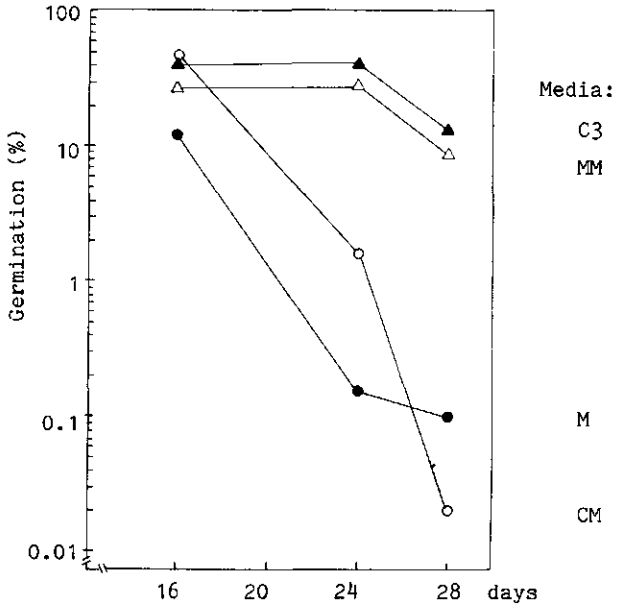


Figure 3.2 Viability of conidia frown on different media.
C.lindemuthianum (strain C409) was grown on different media for 16-28 days at 22°C. Conidiospores were harvested at successive times and plated on CM for viability count.

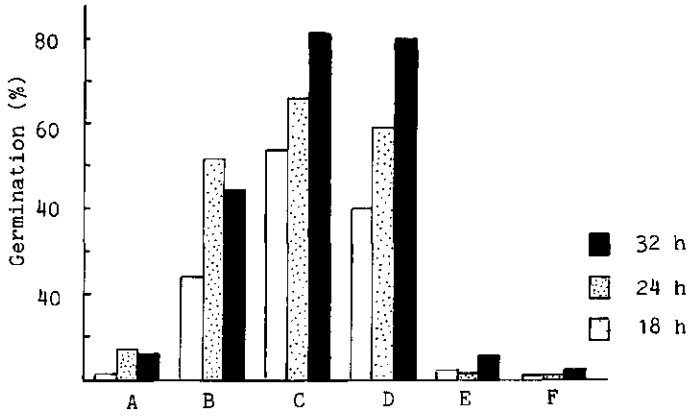


Figure 3.3 Germination of C.lindemuthianum conidia in liquid media.
Conidiospores wre harvested from 12 days old cultures grown at 22°C.
Conidiospores were incubated in liquid media (10 ml) in a shakinhg waterbath at 22°C and scored for germination after 18, 24 and 32 h.
Incubation media:

A. MM + 0.06% glucose	C. SG + 0.06% glucose	E. Saline
B. MM + 0.6% glucose	D. SG + 0.6% glucose	F. Saline without vitamins

All media except F contained 0.5% (v/v) vitamin solution (as used in CM).

3.2.1 ATTEMPT TO INDUCE HETEROKARYOSIS

A combination of two double auxotrophic strains of C.lindemuthianum C409-45 (arg, lys) and C420-10 (pro, cys) was inoculated in liquid CM (i.e. without agar) and after four days at 22°C the mycelium was collected on a membrane filter and thoroughly washed with saline. Pieces of mycelium were transferred to MM plates and incubated at 22°C for 14 days. Growth was as good as that of wildtype mycelium. In a control experiment with the separate strains no growth was observed. (Fig.3.4).

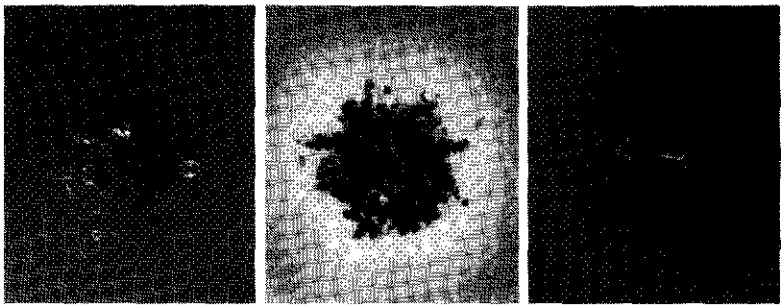


Figure 3.4 Attempt to force heterokaryosis of auxotrophic strains of C.lindemuthianum on MM.
In the middle: a combination of strains C409-45 and C420-10; left strain C409-45; right C420-10
Pieces of mycelium transferred to MM; incubation for 14 days at 22°C.

It now had to be tested whether the good growth is due to heterokaryon formation or to cross-feeding. Heterozygous diploids could provide proof for heterokaryosis.

From the putative heterokaryon conidia were collected in saline and 10^6 conidia were plated in a double layer MM as for Aspergillus nidulans (cf 3.1.3). After incubation for 11 days at 22°C only a few very slowly growing colonies could be observed. These colonies sporulated very badly, only a few spores were found after incubation for 3 weeks. These had according to Coulter counter determination a slightly larger volume than those of the parental strains.

In a similar experiment with the strains C409-47 (arg, lys, azg) and 420-12 (cys, pro, azu) putative heterokaryons were also obtained, whereas mycelium of separate strains showed no growth.

3.2.2 MICROSCOPIC CHEQUE ON ANASTOMOSIS

In the preceeding experiments it was not possible to prove whether the presumed heterokaryons were real heterokaryons or mimicked by cross-feeding. Therefore the proces of anastomosis was studied by microscopical examination. The moist-chamber method was more satisfactory than growth on microscopic slides. Moist-chambers were inoculated at opposite points with strain C409-20 and C409-36 respectively. These two strains differ in type of mycelial growth so that the hyphae could be recognized at the border of contact. Within strain C409-20 hyphal fusions were observed very frequently, also between younger hyphae. In strain C409-36 hyphal fusions were only observed between older hyphae, i.e. in a rather dense network of mycelium. Also their type of fusion bridges differed as is illustrated in Fig 3.5.

Figure 3.5 Types of intra-strain anastomoses between hyphae of C.lindemuthianum. Left: strain C409-20; right: strain C409-36.

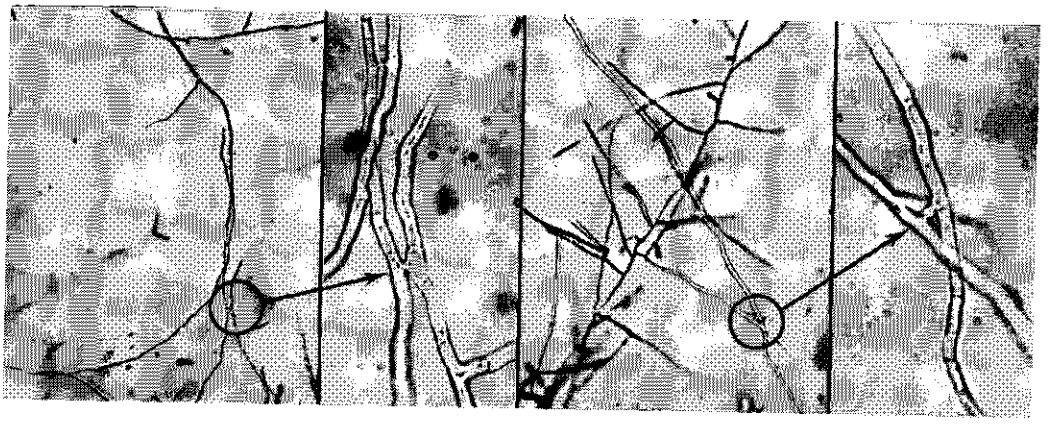


Figure 3.6 Inter-strain anastomosis between C.lindemuthianum strains C409-20 and C409-36.
Two observations in moist chamber. Left: the overall picture; right (see arrow) the anastomosis in detail.

Microscopic examinations revealed only few cases of anastomosis between the two strains. They can only be seen during the time that the strains make contact with each other and the mycelia are not yet interwoven. Fig.3.6 shows the short bridges observed between two strains (races).

Within strain C409-20 the process of anastomosis could be followed more closely. In Fig.3.7 two events of anastomosis are shown. At a given moment two neighbouring hyphae form branches which approach each other. When the hyphae change their position a little, the branches seek each other again. In mycelium of a few days old many anastomoses connect the different hyphae giving a solid net-work.

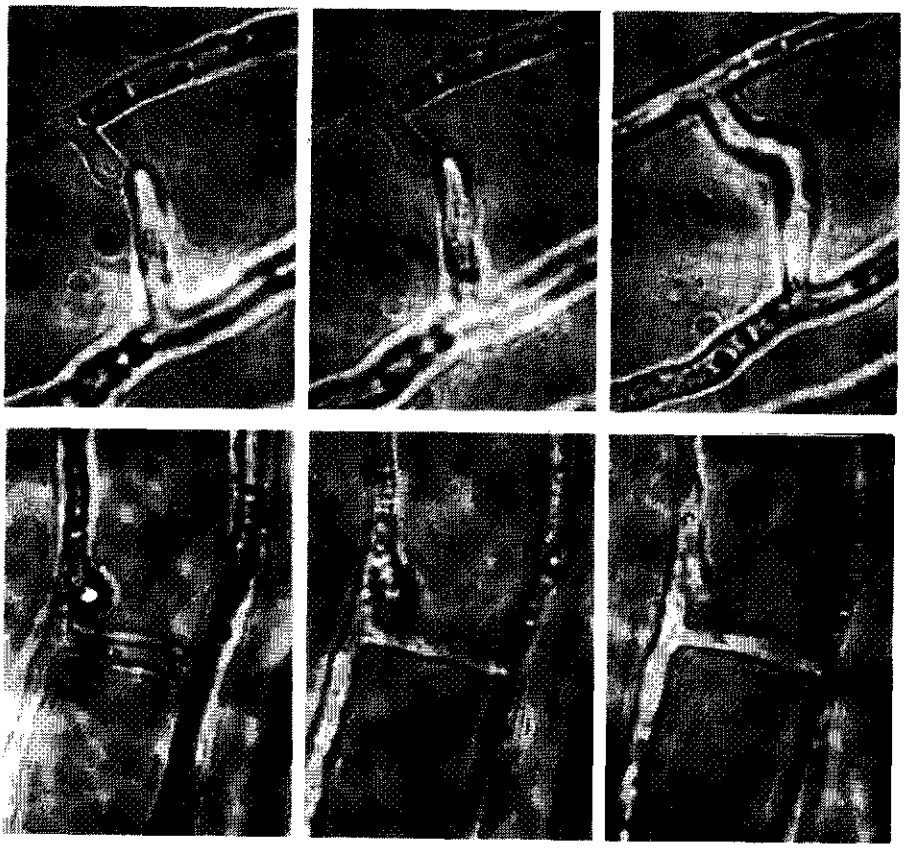


Figure 3.7 The process of anastomosis in C.lindemuthianum (C409-20). Anastomosis in a moist chamber in a drop liquid CM at 22 °C. Upper and lower row: two different modes of the development of anastomoses. Pictures at time intervals of about 1 h.

3.2.3 RENEWED ATTEMPT TO INDUCE HETEROKARYOSIS

As hyphal fusions could be observed indeed, it was attractive to study once more the possibility of karyogamy and the distinction of heterokaryosis and cross-feeding. This was now done by combining the strains C409-47 (arg, lys, azg) and C420-12 (cys, pro, azu). These strains each have a marker for resistance against an antimetabiolite in addition to two auxotrophic markers (see 3.1.0). The resistance markers however were not satisfactory for selection. The two strains were derived from two different wildtype isolates (races).

After 30 days of incubation at 22°C the presumed heterokaryons had sufficient conidia. The conidia were collected and plated in a MM toplayer on a MM bottom-layer and not in a double layer as in the preceeding experiment (3.2.1) because of the slow growth of colonies in the double layer. After 30 days colonies arose on MM, with a frequency of 0.001% while the germination on CM was 8% (Table 3.1, Exp.1). From one presumed diploid colony on MM spores were collected and plated on different media (between brackets the percentage viable count): MM (3.3%), MM+arg+lys (3.6%), MM+pro+cys (3.0%) and on SM (2.0%) (see Table 3.1). All spores seem to grow on MM as may be expected from a heterozygous diploid. However, from determination with the Coulter Counter, the size of the conida of the 'diploid' was intermediate between that of the parental strains, instead of much larger as expected for diploid cells (diameter conidia C409-47: 120 um^3; C420-12:100 um^3; presumed diploid: 115 um^3).

From the MM plates mycelia of 95 presumed diploid colonies were transferred to CM+fpa (2mg p-fluorophenylalanine/ml CM to induce non-disjunction). As no colour markers were present the segregants could not be purified in the usual way of manual transfer. Therefore from 10 sectors (i.e. from 10 different colonies with segregation sectors) small samples of spores were each plated on media supplemented for each of the parental strains (SMA resp. SMB), on MM and on complete SM. None of them gave colonies on MM and on the other media about the same frequencies were found. The average results were (between

brackets the frequency of colonies on the basis of number of spores plated): MM (0.0%), MM+arg+lys (0.08%), MM+pro+cys (0.05%) and on complete SM (0.10%) (see Table 3.1: "from CM+fpa").

In total 100 colonies on complete SM (10 from each segregating sector) were tested for auxotrophic markers with as (average) result 35 of one parental type (C409-47), 60 of the other (C420-12) and 5 gave no growth at all (probably due to poor transfer) (see Table 3.1: from SMAB). Only parental types were recovered, whereas it is unlikely that all four markers are on the same chromosome.

Table 3.1. Analysis of possible diploids of C.lindemuthianum.

Exp.	Strains	% heterokaryon		% growth on different media with conidiospores from 'diploid'				CM+fpa				SMAB*)	
		MM	CM	MM	SMA	SMB	SMAB	MM	SMA	SMB	SMAB	A	B
1	C409-47 C420-12	0.001	8	3.3	3.6	3.0	2.0	0.0	0.08	0.05	0.10	35	60
2	C409-47 C409-41	0.0001	12	0.6	0.3	15.8	14.5	0.1	0.8	0.1	0.8	4	96

*) by transfer; other tests by plating conidia.
SMA = MM + arg + lys;
SMB = MM + pro + cys (Exp.1) resp. thr (Exp.2);
SMAB (complete SM) = MM + arg + lys + pro + cys (Exp.1) resp. thr (Exp.2).

In a parallel experiment C409-47 was combined with C409-41 (mutants derived from the same wildtype isolate). The results were more or less similar (Table 3.1: Exp.2). The frequency of presumed diploids was even lower than in Exp.1 where strains of different origin were used, and one parental type was in great excess. It was, also here, not possible to isolate recombinants.

3.2.4 COMPARISON WITH A.NIDULANS HETEROKARYONS

As in the C .lindemuthianum heterokaryons several contradictory features were observed, A. nidulans heterokaryons were studied for comparison. Auxotrophic strains of this fungus are known to form very homogeneous balanced heterokaryons on MM.

Strains with strong amino acid deficiencies (lysB5 and phenA2 respectively) were used in order to avoid cross-feeding. Heterokaryons from the A.nidulans strains WG019 and WG021 can be

maintained by transfer of pieces of mycelium from the border of a heterokaryon. However, when hyphal tips of about 2 mm were cut off and transferred to MM only 10% of these hyphal tips produced colonies. For control, hyphal tips from the same regions were also transferred to CM with as result that 90% were viable. This experiment shows that also in a homogeneous heterokaryon of A. nidulans there is a continuous segregation of parental hyphae. As the colonies can be maintained as balanced heterokaryons, even by transfer of pieces mycelium from the border of the colony, it is assumed that new heterokaryotic hyphae are currently formed by anastomosis. So, it can be assumed that even a balanced A.nidulans heterokaryon consists only partly of heterokaryotic hyphae.

From these heterokaryons heterozygous diploids were isolated. The frequency of heterozygous diploid conidia was 5.10^{-6}. If a heterokaryon contains many homokaryotic hyphae, the frequency of somatic karyogamy will be much higher than is deducted from the frequency of heterozygous diploid conidia.

This experiment shows that it may be difficult to prove the excistance of heterokaryons in C.lindemuthianum by analysis of colony growth on MM.

3.2.5 DISCUSSION

In section 3.2.2 the term presumed heterokaryon was used for simplicity since it had to be proven that the colonies concerned were real heterokaryons. The best proof for the existance of a heterokaryon is the isolation of heterozygous diploid conidia. Although in these experiments no unambiguous proof was obtained for the occurrence of somatic recombination in C.lindemuthianum, it is probable that at least heterokaryosis occurs. When two complementary double auxotrophic strains were grown on MM, colonies were formed with a phenotype as expected for a heterokaryon. As no good colour markers could be included, it could not be visually assessed how homogeneous these colonies were. It was significant that tranfers from the border of the colony did not show growth on MM, which may indicate sorting out of parental types.

The first prerequisite for heterokaryosis, anastomosis, was observed microscopically. In our observations hyphal fusions occurred almost exclusively between somewhat older parallel hyphae. These (still young) hyphae are apparently old enough to form anastomoses. Therefore it is somewhat surprising that punched pieces from the border of a heterokaryon did not grow on MM. Sorting out was, however, supported by analysis of balanced A.nidulans heterokaryons.

The initiation of hyphal contact was bilateral and between the side branches of strain C409-20 attraction was observed. In strain C409-36 the type of anastomosis was different. Different types of fusion bridges were also seen by Flentje and Stretton (1964) between isolates of Thanatephorus.

Although we observed plasmogamy between hyphae of C. lindemuthianum we did not study migration of nuclei. Only Hoffmann (1967) demonstrated with certainty nuclei in hyphal bridges in Fusarium (34 out of 509), but several other authors working with different fungi did not come to a conclusion.

The moist-chamber method was good for observing anastomosis, but here staining of nuclei is not possible. The observation of a nucleus in a hyphal bridge is still not a proof for migration of nuclei from one to the other hypha. The isolation of hybrid conidia and recombinants is in fact the only positive proof.

In our experiments we could isolate prototrophic colonies starting with a presumed heterokaryon of two double auxotrophic strains of C. lindemuthianum, but we did not find any new combination of auxotrophic markers.

It can be concluded that probably heterokaryons had been induced. They could be maintained by transfer of pieces of mycelium from near the centre of the colony, but transfer of punched pieces from the border of the heterokaryons to fresh MM plates did not result in growth. Furthermore, it is still not clear whether heterozygous diploids have been obtained. Although the presumed diploids grew on MM (i.e. complementation), their colonies produced conidiospores of the same size as the parental strains and segregated the two parental types only. With

p-fluorophenylalanine-treatment one would certainly expect recombinants. So it is still uncertain whether these colonies were diploid.

Stephan (1966, 1967) stated that he could isolate a prototrophic recombinant from a heterokaryon of Colletotrichum gloesporoides. When a segregant is found in which an auxotrophic marker is replaced by the prototrophic allele, it is possible that revertants are involved. He might have isolated a revertant, especially since he started from hyphal tips. In our experiments hyphal tips appeared to be homokaryotic.

It is unlikely that the growth on MM in our experiments was due to reverse mutations (two reverse mutations required, which later are lost again). Cross-feeding is still a possible explanation. Perhaps some conidia have sufficient reserve material to start initial growth on MM selection plates and since plating density is high, colonies may appear owing to cross-feeding. But also in this case heterokaryotic hyphae may be expected among homokaryotic hyphae. This is supported by the observation that only pieces of mycelium from older parts of the presumed heterokaryon could grow upon transfer to fresh MM.

Although conidia of C.lindemuthianum are uninucleate (as confirmed by microscopic examination after staining with lacto-acetic orcein) it cannot be excluded that a very low percentage of spores has two nuclei. This may be an explanation for the 'presumed diploids'.

3.3 Isolation of protoplasts from A.nidulans conidiospores

3.3.0 INTRODUCTION

Protoplast fusion may be an alternative way to establish heterokaryons. We resorted to A.nidulans to develop procedures for isolation of protoplasts from conidiospores and for protoplast fusion.

Chapter 3.3 describes the isolation of protoplasts from A.nidulans conidiospores. The main aspects and some details have been published earlier (Bos and Slakhorst, 1981; Bos, 1985).

Many aspects of fungal protoplasts have been reviewed by Peberdy (1979). Several procedures for the isolation of fungal protoplasts apply to Aspergillus nidulans mycelium, and these mycelial protoplasts have been used in fusion and transformation experiments (e.g., Anné and Peberdy, 1976; Ferenczy et al., 1976; Dales and Croft, 1977; resp. Ballance et al., 1983; Kelly and Hynes, 1985; Tilburn et al., 1983; Yelton et al., 1983).

However, protoplasts isolated from mycelium vary in size and in number of nuclei. In terms of organelle constitution and biochemical functions they can be expected to show a marked heterogeneity (Peberdy, 1976). Such heterogeneous protoplast suspensions from filamentous fungi are in fact less suitable for quantitative recombination experiments.

As conidiospores of A. nidulans are very uniform in size and are mononucleate, these difficulties can be overcome by the isolation of protoplasts from conidiospores. Also, for regeneration studies it can be preferable to isolate protoplasts from conidiospores. Only a few reports describe procedures for the preparation of protoplasts from conidia and they mention poor yields (see Bos, 1985). Moore and Peberdy (1976) isolated protoplasts from Aspergillus flavus conidia produced in liquid culture; however, agar-grown conidia of A. flavus gave only a very poor release of protoplasts.

Our first studies on the application of lytic enzymes showed that germinating conidia of A. nidulans respond well to the lytic system described by Van den Broek et al. (1979) for the isolation

of organelles from A. nidulans hyphae. Therefore, this lytic enzyme mixture was used for the isolation of protoplasts from conidiospores. A preincubation step was included to activate the conidia to synthesize new cell wall material during swelling. Since autolytic enzymes are involved in cell wall synthesis, the wall of a swollen conidiospore will in a sense resemble a hyphal tip. Preincubation was in the presence of 2-deoxy-D-glucose, for Birnboim (1971) and Foury and Goffeau (1973) found that this facilitates the digestion of cell walls of Schizosaccharomyces pombe. A similar effect was found by Van den Broek et al. (1979) for A. nidulans mycelium grown in the presence of low concentrations of 2-deoxy-D-glucose. This agrees well with the inhibitory effect of 2-deoxy-D-glucose on α-1,3-glucan synthesis in Aspergillus cell walls as found by Zonneveld (1973).

Since polyoxin D inhibits chitin synthetase (Endo et al., 1970) and active chitin synthetase is predominantly found in membrane preparations of protoplasts derived from hyphal tips (Aspergillus fumigatus, Archer, 1977), it is likely that polyoxin D makes swollen conidia more sensitive to lytic enzymes. It was tested whether including polyoxin D in the preincubation facilitated protoplast isolation.

The isolation procedure was adapted by us to conidiospores of some other (imperfect) fungi.

3.3.1 PREPARATION OF PROTOPLASTS

The first attempts to prepare protoplasts from agar-grown Aspergillus conidiospores showed that preincubation of the conidia was a prerequisite for obtaining protoplasts. Preincubation should be stopped after about 3 hours, since spores forming a germ tube (after 4 h of incubation) tend to aggregate (see below). When the growth factors (necessary for the auxotrophic strains) were omitted from the preincubation medium (see 3.1.6), protoplasts could be prepared in the same space of time, though with slightly lower yields.

The procedure for the isolation of protoplasts and the process of regeneration are outlined in Fig.3.8.

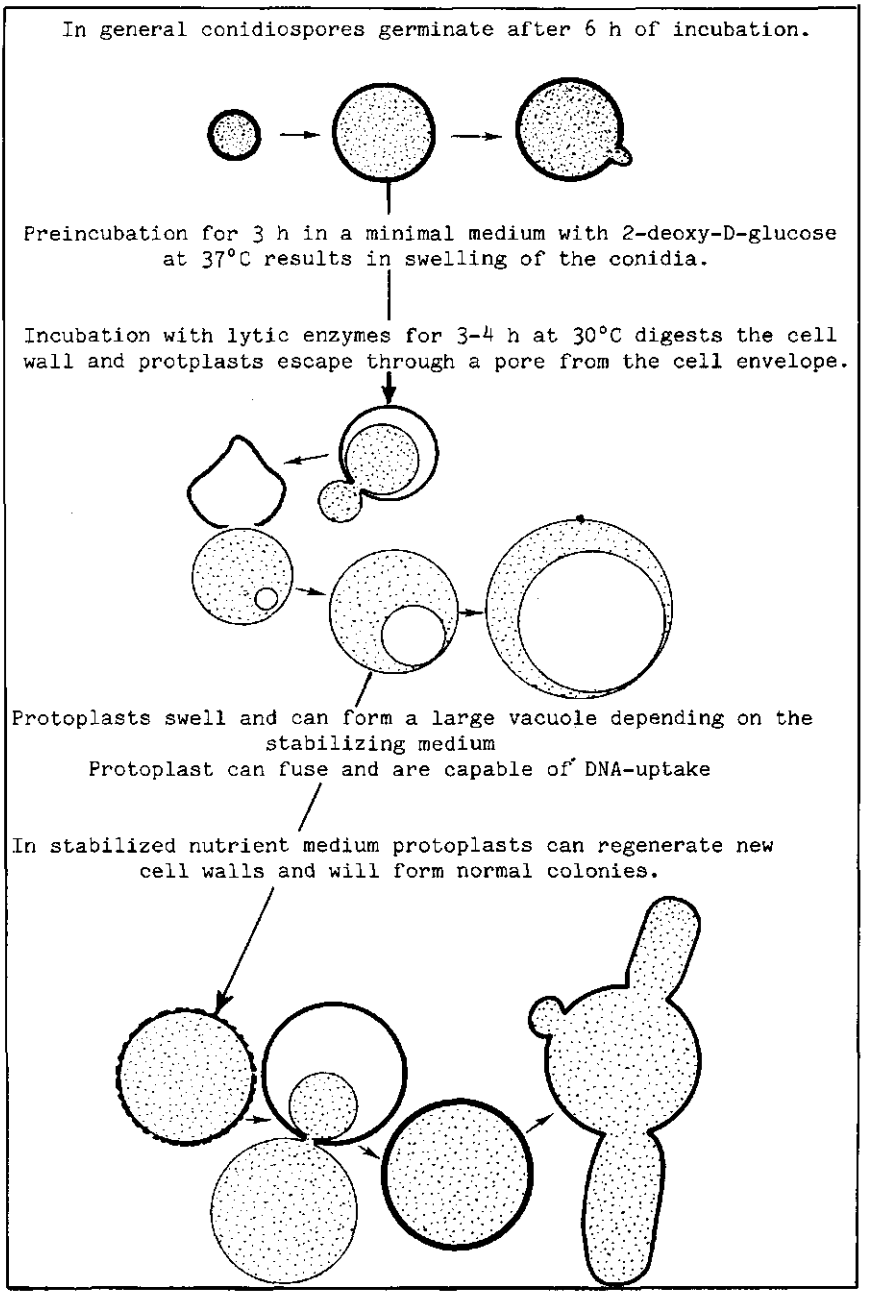


Figure 3.8 Outline of the isolation and regeneration of protoplasts from A.nidulans conidiospores.

The presence of 25 ug 2-deoxy-D-glucose / ml medium increased the final yield of protoplasts by a factor of five, with the additional advantage that subsequent incubation with lytic enzymes could be reduced from 5 to 3 h.

On the basis of these preliminary results the following standard conditions for protoplast preparation were chosen: preincubation of conidiospores for 3 h at 37°C in the presence of 25 ug 2-deoxy-D-glucose/ml, followed by incubation with lytic enzymes for 3 h at 30°C with 0.4 M $(NH_4)_2SO_4$ as stabilizer.

Table 3.2 Effects of various conditions on the conversion of A. nidulans (strain WG093) conidiospores into protoplasts

Exp. no.	Variable *)	Protoplast yield: Protoplasts counted/ml	Protoplast yield: as % of conidia	% intact conidia as determined in a haemocytometer
1	Preincubation time (h)			
	1.5	Very low		100
	2	8.0×10^6	32	50
	2.5	1.6×10^7	64	5
	→ 3	1.7×10^7	68	5
2	2-Deoxy-D-glucose during preincubation (ug/ml)			
	0	2.3×10^6	9	90
	10	1.5×10^7	58	5
	→ 25	1.7×10^7	68	5
3	Incubation with lytic enzymes (h)			
	2	8.4×10^6	33	50
	→ 3	1.3×10^7	52	5
	3.5	1.6×10^7	64	5
4	Presence of ammonium sulphate during incubation with lytic enzymes (M)			
	0.1	3.8×10^6	15	25
	0.2	5.6×10^6	22	25
	0.3	1.1×10^7	44	5-10
	→ 0.4	1.7×10^7	68	5
	0.4 (3.5 h incubation)	2.1×10^7	83	5

* When varying one parameter the others were kept constant, as under standard conditions. Standard conditions (see →) were preincubation of conidia (2.5×10^7 /25 ml in 100 ml bottle) for 3 h at 37°C in citrate-pyruvate medium containing 25 ug/ml of 2-deoxy-D-glucose, followed by incubation with the lytic enzymes for 3 h at 30°C in the presence of 0.4 M $(NH_4)_2SO_4$ as stabilizer.

The conditions for 3 and 3.5 h of incubation with lytic enzymes in Exp.3 are strictly comparable to those in Exp.4 and the results show the experimental variation.

Subsequent experiments on the effect of variation of each of these factors are summarized in Table 3.2. It was found that incubation with lytic enzymes for 3.5 h instead of 3 h gave higher yields of protoplasts, but that the other standard conditions were suitable. Polyoxin D had a stimulating effect too, but not additive to that of 2-deoxy-D-glucose and since it is more expensive, it was not included in the standard procedure.

During preincubation the conidia swell and the increase in volume is linear with time (Fig.3.9). The volume distribution curve was approximately normal indicating that the conidial population was homogeneous with respect to swelling. Although prolonged preincubation promoted the subsequent release of protoplasts, after 3 h the conidial count decreased due to

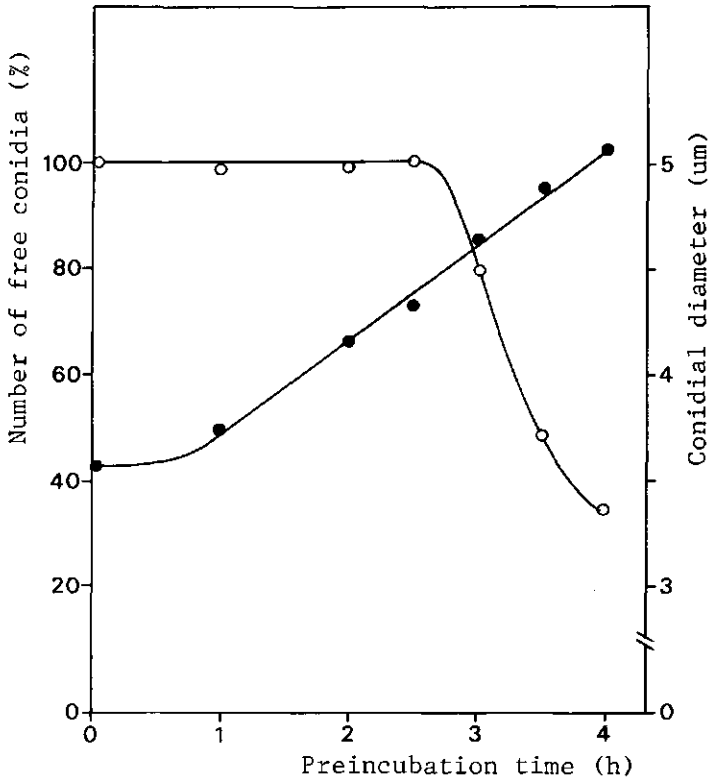


Figure 3.9 Swelling and aggregation of conidiospores during preincubation. Conidiospores of A.nidulans (strain WG179) were incubated in CP medium at 37°C. The diameters (●) and numbers of free conidia (○) were calculated from Coulter counter measurements in samples of 0.5 ml taken at different times during incubation.

aggregation (Fig.3.9). Microscopic examination showed that at that moment only a few conidiospores had a small "nose" and that no germination tubes were present.

The effect of preincubation medium (cf 3.1.6) on the release of protoplasts from A. nidulans is shown in Fig.3.10. Although swelling of the conidia during preincubation was about equal in the three media used, the citrate-pyruvate MM gave better results than the normal MM, whereas the lowest yields were obtained when spore germination medium (SG) was used for preincubation.

In another experiment the production of protoplasts from three different strains of A. nidulans (WG093, WG132, WG179) was compared. The yield was found to be strain independent, and in general, after 3 h of incubation with lytic enzymes, at least 50% of the conidia were converted into protoplasts (data not shown).

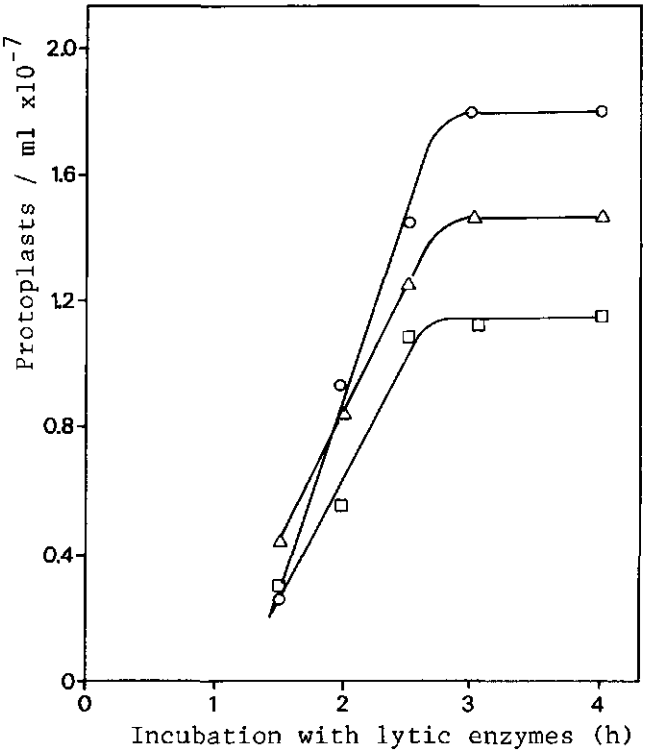


Figure 3.10 Effect of preincubation on protoplast yield.
Conidiospores of A.nidulans (strain WG179) were preincubated prior to treatment with lytic enzymes as described in 3.1.6. Three different media were compared:
○ CP △ MM □ SG (see 3.1.1).

The Oerskovia enzymes proved to be indispensable for protoplast formation, whereas the additional Aspergillus autolytic enzymes greatly promoted protoplast release (Fig.3.11). The latter could easily be replaced by a commercial glucanase from Penicillium, which gave even more reproducible and slightly higher yields of protoplasts. Jointly with Oerskovia enzymes, glucanase contributed 30% to the total yield, but when applied separately they produced almost no protoplasts. In this way often no less than 80% of the conidiospores could be converted into protoplasts.

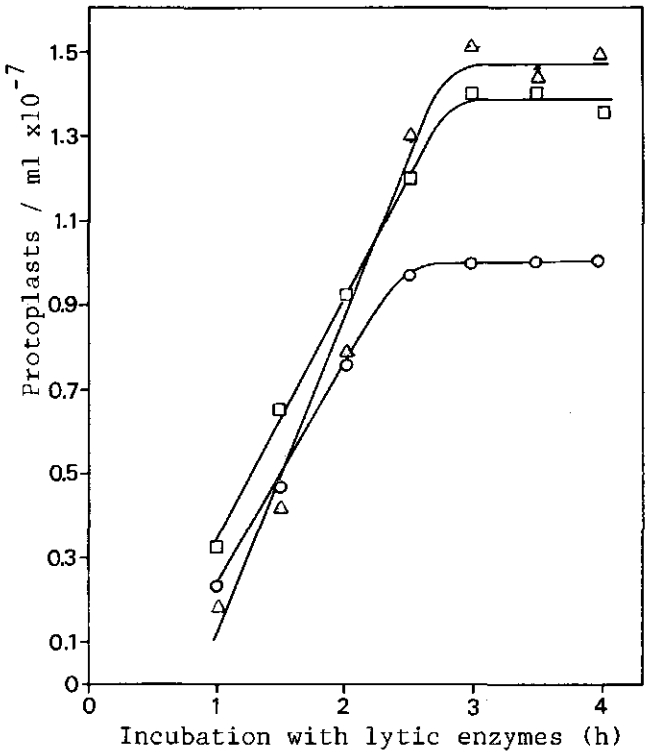


Figure 3.11 Effect of glucanases in combination with Oerskovia lytic enzymes on protoplast formation.
After preincubation conidiospores of A.nidulans (strain WG179) were treated with lytic enzymes from Oerskovia as described in 3.1.6, with or without glucanases.
○ without glucanases
□ with Aspergillus glucanases (0.1 ml/ml)
△ with Penicillium glucanases (0.1 mg/ml).

The residual conidia were removed by centrifugation of the protoplast suspension over a layer of 30% sucrose, which resulted in protoplast suspensions with less than 1%, and often less than 0.5% conidia. The effectiveness of this purification step was slightly strain dependent. After centrifugation almost all protoplasts were at the sucrose interface, whereas the conidia and cell wall fragments had penetrated the sucrose layer and formed a sediment. Removal of the interface and the top layer, followed by stepwise addition of 0.6 M KCl, resulted in a final recovery of protoplasts of at least 50%, often about 80% purified protoplasts. A representative example is given in Table 3.3.

Table 3.3 Summary of a typical isolation procedure for protoplasts from A.nidulans (strain WG132) conidia

Step in the procedure	Volume (ml)	Total count/ml	Protoplasts % of initial conidia	Protoplasts % of preced. step	Colonies *) formed/ml on complete medium plus 0.4 M KCl	Colonies formed/ml on complete medium
Inoculum (conidia, 2.5 um diameter)	1.14	4.4×10^7				
Preincubation for 2 h at 37°C	50.0	1.0×10^6				5×10^5(50)
Incubation for 4 h with lytic enzymes	2.0	1.3×10^7	52	52	5.5×10^6(42)	3×10^5(2.3)
Purification						
Amount used	1.6	1.3×10^7				
Purified protoplasts (5.1 um diameter)	1.3	1.5×10^7	49	87		
0.6 M KCl added stepwise	5.0	3.8×10^6	38	85	1.1×10^6(30)	3.3×10^3(0.1)
Washing by centrifugation; resuspension	1.0	1.9×10^7	38	100	6.7×10^6(35)	4.0×10^4(0.2)

*) Values in parentheses represent percentage of total count of protoplasts. Protoplasts were plated on CMK (CM + 0.4 M KCl; see 3.3.2).
Recovery of protoplasts e.g. after purification: input $1.6/2.0 \times 5 \times 10^7 = 4 \times 10^7$; output: 1.95×10^7 (→ 49%).

The complete procedure for the isolation of protoplasts required about 7 h. Microscopically it is seen that the protoplasts extrude through a pore in the cell wall and retract from it. The protoplasts were about twice as large as the conidiospores.

After release, the protoplasts develop a large vacuole and increase slightly in size with time. Vacuolization continues for about 30 min until the cytoplasm has wholly retracted against the cell wall. At this stage they resembled the protoplasts isolated from liquid-grown conidia of A. flavus after 12 h in lytic medium (Moore and Peberdy, 1976).

Microscopic examination after staining with orcein revealed that all initial conidia were uninucleate, but that the protoplasts isolated after 3 h of preincubation, followed by 4.5 h of incubation with lytic enzymes, mostly (>80%) had two nuclei. After less prolonged incubation (3 h of preincubation and 3 h of incubation with lytic enzymes), the protoplasts had one nucleus. Protoplasts of strain WG179 are shown in Fig.3.12. The strains WG093 and WG132 behaved identically.

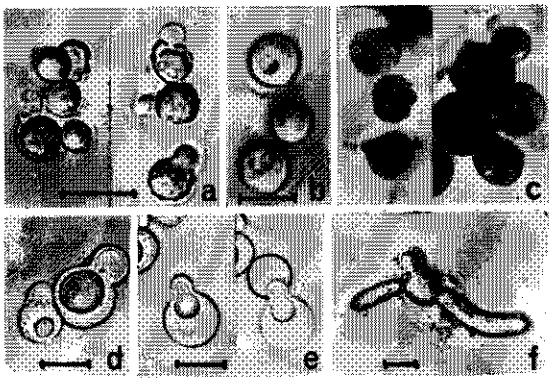


Figure 3.12 Microphotographs of protoplasts of A. nidulans WG179.
Bar equals 5 um in all Figures.
a) Protoplast release after 2 h of incubation with lytic enzymes at 37°C;
b) Protoplasts after purification, resuspended in 0.6 M KCl;
c) Protoplasts stained with lacto-acetic orcein: left, protoplasts obtained after 3 h of preincubation and 2.5 h of incubation with lytic enzymes mostly mononucleate; right, protoplasts obtained after 3 h of preincubation and 4 h of incubation with lytic enzymes, mostly with two nuclei;
d) and e) Protoplasts during regeneration in liquid CM + 0.4M KCl,
d) Protoplasts after 1 h incubation at 37 C,
e) A protoplast escaping from the newly synthesized cell envelope; right a drawing of the cell wall (difficult to see in the photograph);
(f) Regenerated cell with large germ tubes.

The procedure for isolation of protoplast from conidiospores was tested with some other fungi in a few pilot experiments. With dark coloured A.niger strains rather low yields were obtained, but from conidia of a light coloured mutant a high yield of protoplasts (64-92%) was obtained after preincubation in SG with 2-deoxy-D-glucose (Bos and Slakhorst, 1981). From A.carbonarius conididospores no protoplasts were obtained, but the procedure proved to be suitable for Cladosporium cumerinum, Colletotrichum lindemuthianum and Penicillium expansum.
Although this lytic procedure seems to have broad applicability to conidiospores of other fungi, modifications specific for each organism may be required for optimal results.

3.3.2 STABILIZATION AND REGENERATION OF A.NIDULANS PROTOPLASTS

Protoplasts were effectively stabilized in 0.6 M KCl. At lower stabilizer concentrations considerable losses occurred, but stepwise dilutions could also be made in liquid CM containing 0.4 M KCl. Protoplasts were plated either by spreading on agar, or by pouring them into a 3 ml top layer as usual in the case of high-density plating for the selection of fusion products or recombinants.

From the plating experiments summarized in Fig. 3.13 it was concluded that addition of 0.3 M KCl to the plating medium was sufficient; in general 0.4 M KCl was added.

Fig.3.14 shows that plating in a top layer had to be done quickly, since protoplasts did not survive a temperature of 45°C. Independent of plating conditions it was frequently found that only 20-50% of the plated protoplasts gave rise to colonies (cf Table 3.3), whereas upon plating conidia (with or without pre-incubation), in general 50-80% germination was found. Attempts to increase the plating efficiency by promoting the stability of the protoplasts were not successful.

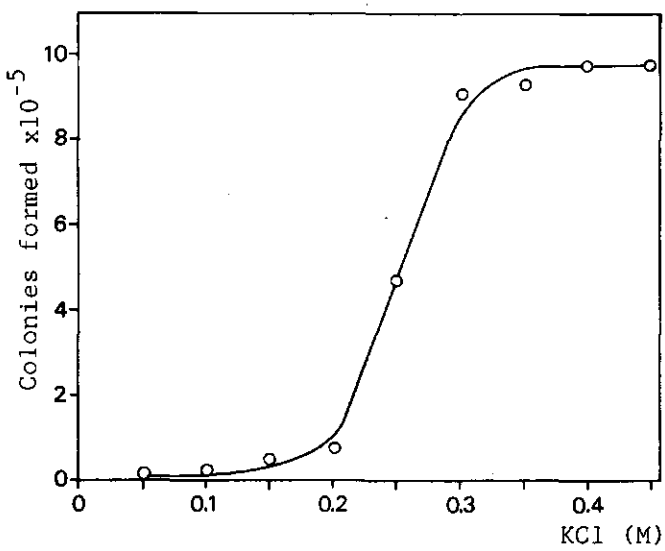


Figure 3.13 Stabilization of protoplasts on solid media with KCl.
Number of colonies upon plating of the same protoplast suspension (A.nidulans WG179) on complete media with different concentration of KCl (CMK).

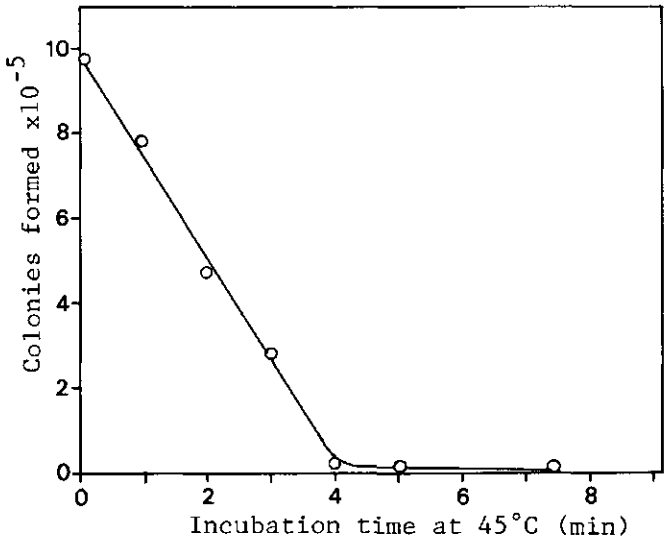


Figure 3.14 Effect of temperature when plating in a top layer.
Protoplasts of A.nidulans (strain WG179) were plated in a top layer of soft CMK (3.5 ml CMK with 0.8% agar at 45°C). After addition to the soft-agar the protoplasts were kept at 45°C for different times.

Storage in the cold (4-8°C) for 1 day prior to plating had no effect on the viability of the protoplasts and no aggregation occurred. The protoplasts were still sensitive to osmotic shock upon dilution in water, so it can be concluded that no regeneration had taken place. Freshly prepared and stored protoplasts showed no differences in fusion frequency (Section 3.4). In liquid CM containing 0.4 M KCl aggregation became first visible after incubation of protoplasts for 1 h at 37°C. After 1.5 h many aggregates were present, and some germ tubes could be seen.

Protoplasts prepared from A. nidulans conidiospores by the Oerskovia lytic enzymes and glucanases show a remarkable pattern of regeneration as shown in Fig. 3.12 d-f. Upon incubation in liquid CM + KCl at 30 to 37°C, regeneration starts immediately with the formation of a microfibrillar network that becomes denser with subsequent deposition of amorphous material (Peberdy, 1979). In a regenerating protoplast the vacuole vanishes, and subsequently the protoplast leaves the rigid network. Now the protoplast forms a new cell wall within 20 minutes, after completion of which two or three germ tubes are prodcued (see Fig.3.8).

3.3.3 DISCUSSION ON ISOLATION AND REGENERATION OF PROTOPLASTS

Methods for the isolation of protoplasts from fungal spores have been reviewed recently (Bos, 1985). Some procedures gave a low yield, whereas with others only protoplast-like structures could be obtained. The safest criterion for true protoplast formation is their release from the spore envelope through a pore in the cell wall and their osmotic lability. When lysing Fusarium culmorum protoplasts, prepared with Micromonospora lytic enzymes (Garcia Acha et al., 1966), the cellular content escaped from the spherical structure and a rather thick envelope was left. In this case no true protoplast were obtained. These structures rather are spheroplasts; the authors speak rightly about protoplast-like structures. In the review (Bos, 1985) it is suggested that in such situations addition of other enzymes might yield

true protoplasts. However, from our recent experiments with conidiospores of certain A.niger strains which produced also spheroplasts, carefully changing of the osmotic pressure of the stabilizing medium proved to be a way to obtain true protoplasts. This procedure may also be suitable for other fungi.

The procedure described in this Section is based on Oerskovia lytic enzymes with in addition commercial glucanases.

Fresh agar-grown conidia of A. nidulans appaer to be very insensitive to lytic enzymes of Oerskovia, but after a preincubation treatment, up to 99% (and rarely less than 80%) of the conidiospores can be converted into protoplasts.

It was surprising that commercial β-glucanases could replace the autolytic glucanases of A.nidulans since it was shown (Zonneveld, 1972; Van den Broek et al., 1979) that the α-(1,3)-glucanase activity was the most important component for degradation of cell wall material and release of protoplasts from hyphae of A. nidulans. This may reflect differences in cell wall constitution and structure, but it may also be due to heterogeneity of the glucanase preparation as indicated by Manners et al. (1976) for another fungal glucanase preparation. Moreover, the effect of glucanases in a crude extract may depend on the total complex system rather than on any single component (Clarke and Stone, 1965; Doi et al., 1976).

With protoplasts prepared from young mycelium, the conditions of mycelium cultivation have a great influence on the number of protoplasts released (Musilkova and Fencl, 1968; Zonneveld, 1972; Van den Broek et al., 1979). Our experiments showed that preincubation of conidia was an essential step for the release of protoplasts from Aspergillus conidiospores. The marked effect of 2-deoxy-D-glucose on the process was probably due to its interference with polysaccharide biosynthesis (Kratky et al., 1975). The viability of conidia was not affected by 2-deoxy- D-glucose, although germination was delayed.

The citrate-pyruvate medium turned out to be superior for the release of protoplasts in the subsequent incubation with lytic enzymes. Upon preincubation for more than 3 h the conidiospores begin to aggregate. For this reason vigorous shaking is necessary. Aggregation during preincubation was probably the

reason that the yield of protoplasts often varied from 50-80% under standard conditions. Their regeneration pattern was quite different from that observed for protoplasts obtained from young hyphae. Protoplasts from young hyphae form aberrant germination tubes sometimes resembling a chain of budding cells (Peberdy and Gibson, 1971; Gibson et al., 1976; Van den Broek et al., 1979). Yeastlike chains were also observed in regenerating protoplasts obtained from liquid-grown A. flavus conidia (Moore and Peberdy, 1976) which showed a striking resemblance to protoplasts from Saccharomyces calsbergensis isolated by Eddy and Williamson (1959).

One of the main advantages of preparing protoplasts from conidiospores is the resulting homogeneous suspension of protoplasts with either one or two nuclei, depending on the conditions during isolation. Sometimes this requirement of homogeneity is also fulfilled when protoplasts are prepared from hyphae, e.g. the protoplasts obtained from Pyricularia oryzae hyphae by Tanaka et al. (1981) were mostly mononucleate.

Although A.nidulans conidiospores are mostly mononucleate, during the isolation procedure mitosis obviously occurred after approximately 6 h of incubation. It was possible to obtain almost exclusively mononucleated protoplasts, however at the cost of yield. The observation of nuclear division during preincubation of conidia was in agreement with Fiddy and Trinci (1976) who found that in A.nidulans mitosis occurred when the cytoplasmic volume per nucleus attained a mean value of about 57 um^3. Bainbridge (1971) observed mitosis in germinating conidia of A.nidulans and found that after 6 h incubation (in which time 50% of the conidia produced germ tubes) approximately 70% of the conidia had two nuclei. Protoplasts from young mycelium may contain several nuclei (Aguirre and Villanueva, 1962), even up to a hundred or more (Emerson and Emerson, 1958). Often the total content between the septa of a hypha was extruded as a single protoplast. With more recently described methods the hyphal protoplasts were also highly variable in size and contained several nuclei (Anne and Peberdy, 1976; Peberdy, 1976; Ferenczy et al., 1976).

Secondly, protoplasts from conidiospores are homogeneous in physiological condition, whereas protoplasts derived from mycelium are heterogeneous as a result of age differences of the hyphal cells from which they have been extruded (Peberdy and Gibson, 1971; Anné et al., 1974). This is reflected by the regeneration process. Tanaka et al. (1981) observed for hyphal protoplasts three different patterns of regeneration: a chain of yeastlike buds; a chain where after some time a germ tube-like hypha protruded from the cell most distal to the original protoplast; and a type where the protoplast remained spherical for several hours before a germ tube-like hypha was formed directly from the spherical cell. These different regeneration types may be due to the origin of the protoplasts (older cells or hyphal tips).

In conclusion, the Oerskovia lytic system proved to be very suitable for the isolation of protoplasts from Aspergillus nidulans conidiospores. Starting with mononucleate conidiospores this isolation procedure gives suspensions of either mono- or binucleated protoplasts depending on the duration of incubation.

The protoplasts obtained from conidiospores proved to be very suitable for recombination experiments. They have been used for protoplast fusion (this study, Chapter 3.4) and for transformation (Wernars et al., 1985).

3.4 Protoplast fusion and karyogamy in A.nidulans

3.4.0 INTRODUCTION

Protoplast fusion and karyogamy too were studied in Aspergillus nidulans. In fusion experiments with Aspergilli and Penicillia large differences in fusion frequency have been found (Anné and Peberdy, 1976; Ferenczy et al., 1976). High fusion frequencies may be a consequence of extreme aggregation. With multinucleate protoplasts it is difficult to see whether the colonies on selective plates arise from aggregates of protoplasts or from protoplast fusion. Protoplast suspensions prepared from conidiospores of A.nidulans are either mono- or binucleate, depending on the duration of the incubation.
Therefore they are suitable to study protoplast fusion and karyogamy.

Protoplast fusion is mostly done in polyethylenglycol (PEG). It is reported to be stimulated by high pH and Ca^{2+}-ions (Anné and Peberdy, 1976; Anné, 1983; Ferenczy et al., 1975, 1976, 1977). These factors were varied in order to find optimal conditions for reproducible experiments. For studying how many protoplast participate in a fusion event three strains differing in colour of conidia were combined pairwise and in the combination of three.

As the fusion products proved to be ideal heterokaryons the next step was to study the frequency of karyogamy. In these experiments a yellow and a white strain were used so that green heterozygous diploid sectors or colonies could be identified.

3.4.1 CONDITIONS PROMOTING PROTOPLAST FUSION

Initially we followed the common procedures (e.g. Anné and Peberdy, 1976; Ferenczy et al., 1976) using a PEG solution buffered in 50 mM glycin. The pH was difficult to control as can be expected from the pK of glycin. Especially older solutions had a low pH (pH 4). In prelimary experiments 50 mM imidazole buffer gave better results than 50 mM glycin. Washing with 0.6M KCl gave a lower viable count than direct plating of the fusion mixture.

For studying the effect of different pH values on the fusion frequency a 50 mM imidazole buffer (pK = 7.0) was used for the higher pH range and a 25 mM malate buffer (pK = 5.0) for the lower pH range. Although the frequency of heterokaryons in consecutive experiments could differ with a factor 10, duplicates per experiment agreed well. The results of a representative experiment, in which also an unbuffered PEG solution was incorporated, are summarized in Table 3.4. When protoplasts of a single strain were incubated in the PEG solution and plated on CMK the viable count was mostly higher than 70%.

Table 3.4 Effect of pH on the fusion of non-purified protoplasts.

Input. : about 1.3×10^6 protoplasts per strain (WG132 and WG179).
Fusion : in 1 ml PEG (30% PEG, 50 mM $CaCl_2$, + or - buffer).

pH	Buffer	Recovery of colonies after PEG incubation		Heterokaryons	
		Viable count	% of input	Count on MMK	% in rel.to viable count
4	25 mM malate	535×10^3	41	409	0.08
5	,,	850	65	210	0.03
6	,,	1545	118	1473	0.05
7	50 mM imidazole	1085	83	558	0.05
8	,,	1060	81	420	0.03
5.9	without buffer	910	70	8000	0.88

These experiments show that a neutral pH is optimal for protoplasts fusion under these conditions. In similar experiments too, a freshly prepared PEG solution without buffer gave significantly the best results. We did not find differences between PEG4000 and PEG6000. There were however differencies between different batches of PEG. A 30% solution of PEG with lower molecular weight however is easier to handle.

In the experiments summarized in Table 3.4 the protoplasts were collected from the lytic mixture by centrifugation and resuspended in PEG buffer directly. Washing twice with 0.6 M KCl by centrifugation resulted in similar heterokaryon frequencies. Purification of protoplasts (see 3.1.7) often increased the heterokaryon frequency (Table 3.5).

In some other experiments (data not shown) the presence of Ca^{2+} -ions in the PEG solution proved to be essential and could not be replaced by K^+ or Al^{3+}. A concentration of $CaCl_2$ between 50 and 100mM gave the best results when using unbuffered PEG solutions. Furthermore a PEG concentration of 25-30 % resulted in maximum heterokaryon frequencies.

The effect of pH with imidazol buffer was analyzed again with purified protoplasts and compared with that in glycin buffer at high pH (Table 3.5). Also a treatment of unpurified protoplasts with imidazol buffer pH 6 was included. Purified protoplasts gave much higher heterokaryon frequencies than usually obtained with non-purified protoplasts. This experiment shows again that a neutral pH is optimal for protoplast fusion under our conditions. Often freshly prepared PEG solutions gave better results than in this experiment.

Table 3.5 Effect of pH on the fusion of purified protoplasts.

Input : about 10^6 protoplasts of each strain (WG132 + WG179)
fusion : in 1 ml PEG (30% PEG, 50 mM $CaCl_2$; + or - buffer)

pH	Buffer	Protoplasts	Viable count	Heterkaryons Count on MMK	% in rel. to viable count
5.9	without buffer,	purified	57 x10^3	15 x10^3	26
6	50 mM imidazole,	not purified	910	1.4	0.15
6	,,	purified	180	45	25
7	,,	,,	220	32	14
8	,,	,,	180	24	13
8	50 mM glycin	,,	190	20	10
9	,,	,,	100	16	16
10	,,	,,	160	20	12

3.4.2 NUMBER OF PROTOPLASTS IN A FUSION

In order to see how many protoplasts contributed to a fusion product an experiment with equal amounts of protoplasts per strain was performed (Table 3.6). The strains differed in colour and had two different auxotrophic markers each, so that the corresponding heterokaryons could be selected. In control experiments the strains were combined pairwise (Table 3.6). The three combinations of protoplasts showed about the same fusion ability. From two experiments where about $2x10^6$ protoplasts of each of the three strains were pooled in 1 ml 30% PEG, 50 mM $CaCl_2$, 50mM imidazole buffer pH6, the heterokaryon frequencies are summarized in Table 3.7. The average frequency of fusion products as % of the total viable count on CMK is 4.6. In these two experiments 6% respectively 10% of the fusion products consist of cells with three types of nuclei (Table 3.7).

Table 3.6 Fusion frequencies with pairs of strains.

Input : $2x10^6$ protoplasts per strain
Fusion : in 1 ml 30% PEG, 50mM $CaCl_2$, 50mM imidazole pH 6.0

Combination of strains	Heterokaryons (% of viable count) Exp. 1	Exp. 2
WG132 + WG179	3.8 %	3.8 %
WG076 + WG179	4.6 %	1.2 %
WG132 + WG076	10.0 %	6.2 %

Table 3.7 Relative frequencies of protoplast combinations participating in a fusion event.

Input :$2x10^6$ protoplasts of WG132 (white), WG179 (yellow), WG076 (green)
Fusion :in 1 ml 30% PEG, 50 mM $CaCl_2$,50 mM imidazole pH 6.0

		Exp.1	Exp.2
heterokaryons (% of viable count)		3.9	5.4
white - yellow heterokaryons	(WG132+WG179)	32 %	22 %
yellow - green heterokaryons	(WG179+WG076)	29 %	33 %
white - green heterokaryons	(WG132+WG076)	33 %	35 %
tricoloured heterokaryons	(WG132+WG179+WG076)	6 %	10 %

When combining two strains, 50% of the fusion products will be heterokaryotic, when equal amounts of protoplasts are mixed and assuming equal viability and fusability.

When three different types of protoplasts are combined, there are 27 permutations among triple fusions and 3x2x1 = 6 in 27 are tricoloured. So total triple fusions is calculated as 27/6 times the number of tricoloureds. For 6% and 10% tricoloured heterokaryons (see Table 3.7) the corresponding figures are 27 and 45 triple fusions. As 3 in 27 are homokaryons (go unnoticed) there remain 24% and 40% triple fusions among heterokaryotics. Consequently 76% and 60% heterokaryons result from pairs of protoplasts. Finally, with equal amounts of the three types of protoplasts, among pairwise fusions 1 in 3 are homokaryotic and go unnoticed, the corresponding figures being 38 and 30.

From this it can be calculated that among all fusions 27 / (76+38+27) = 19% resp 45 / (60+30+45) = 33% are between three protoplasts and consequently 81% resp. 67% between two protoplasts.

3.4.3 FREQUENCY OF KARYOGAMY

As the fusion of protoplasts isolated from conidiospores proceeded without appreciable interference of aggregation and predominantly only two or three protoplasts participated in fusion events, this system was suitable for quantitative studies on karyogamy. The yellow and white strain which were also used in the previous experiment were combined in a number of fusion experiments. The results are shown in Table 3.8. On the plates used for selection of heterokaryons (MMK), only few green diploid colonies were observed: 0.02% to 0.5% of the fusion products.

Table 3.8 Frequency of karyogamy upon protoplast fusion of WG132 and WG179.

Exp.	Protoplasts % survival	Heterokaryons % of viable protopl.	Green diploid colonies Number observed	Freq./heterok.
1	2	14	2	2.10^{-4}
2	6	4	3	3.10^{-3}
3	10	6	4	5.10^{-3}
4	30	3	2	10^{-3}
5	2	0.8	3	2.10^{-4}
6	1	1.1	3	3.10^{-4}

More frequently green sectors could be found. Sreening for green sectors was done with a dissecting microscope, although the sectors could be found by visual inspection of the plates. In three fusion experiments with strains WG132 (white) and WG179 (yellow) we found green sectors with frequencies of respectively 1/152, 3/1320 and 3/498 (= 0.35%). From the observations on regeneration in liquid minimal medium it was learned (cf Fig.3.12f) that regenerating protoplasts produce two, or more often three large hyphae. It was often seen that very young heterokaryons were composed of three sectors. So a green sector represents probably one third of the nuclei present in the heterokaryon, and from this the frequency of somatic karyogamy is estimated as 1/3 x 0.35 = 0.12%. In conclusion somatic karyogamy is at least a factor 100 higher then the frequency of heterozygous diploids found in heterokaryons (about 10^{-6}-10^{-5}).

3.4.4 DISCUSSION

Fusion of protoplasts isolated from A.nidulans conidiospores in 30% PEG and 50 mM $CaCl_2$ proceeded at frequencies of 2-20% (see Table 3.5 till 3.8). In contrast to observations of others (Anné, 1977; Ferenczy et al., 1976) fusion was better at neutral pH than at higher pH values. In fact a freshly prepared unbuffered PEG solution (pH 5.8-6.0) has proved very satisfactory. The high fusion frequencies at very high pH found by Ferenczy et al. (1976) are theoreticaly only possible if multiple fusions or aggregates are formed. With our system we could increase heterokaryon frequency, but at the same time the protoplasts viable count decreased, giving a smaller amount of heterokaryons. This is an indication for multiple associations. Under standard conditions (purified protoplasts in freshly prepared 30% PEG, 50 mM $CaCl_2$ solution at neutral pH) aggregation and fusions of larger numbers of protoplasts played a minor role as most fusion products were composed of two or three protoplasts.

Upon direct plating of the fusion mixture high survival of protoplasts was found, probably because the PEG protected the protoplasts from drying out on the plate. As has been mentioned in Section 3.3.2 plating on the surface of the plates gave equal-

ly good results as plating in a soft agar toplayer. With plating in a top layer the temperature was very critical and therefore we preferred surface plating.

Purification of protoplast did not result only in higher fusion frequencies, but also in more reproducible results. The lytic mixture contained 0.4 M $(NH_4)_2SO_4$, but the lytic enzymes were prepared by precipitation with ammonium sulphate and might contain varying amounts of sulphate. It is therefore possible that precipitation of $CaSO_4$ is one of the uncontrolled factors in fusions, which can be avoided by purification of protoplasts.

As only two or three protoplasts participated in a fusion event, this system is very suitable to distinguish between heterokaryosis and cross-feeding. Moreover, it is also a good system to study somatic karyogamy. As was concluded in section 3.2.4 the frequency of heterozygous diploid conidia could be a rather pronounced under-estimate of somatic karyogamy. With our protoplast fusion method reproducible data could be obtained which proved that somatic karyogamy occurred at a frequency of about 10^{-3}. Consequently protoplast fusion may also open a way for the isolation of hybrid diploids in cases where the frequency of heterozygous diploid conidia in a heterokaryon is low or in cases of heterokaryon incompatibility. It can also be useful when the isolation of heterozygous diploids by selective plating is not possible and visual identification is needed for distinguishing heterokaryons and hybrid diploids.

3.5 Protoplast isolation and protoplast fusion in Colletotrichum lindemuthianum

3.5.0 INTRODUCTION

The standard procedure for the isolation of protoplasts from Aspergillus nidulans conidiospores consists of a preincubation of the spores followed by incubation in a mixture of lytic enzymes and purification of the protoplasts formed (Chapter 3.3).

For A.nidulans strains with slow growth characteristics (e.g. strains which cannot grow on glucose) some adjustments in the procedure have to be introduced. Similary,with some modifications the procedure was also successful in pilot experiments with some phytopathogenic imperfect fungi. Protoplast were obtained from conidiospores of a phytopathogenic strain of Aspergillus niger, Penicillium expansum, Cladosporium cucumerinum and Colletotrichum lindemuthianum. Methods for isolation of protoplasts from conidiospores have been discussed elsewhere (Bos, 1985). Recently the procedures for lytic digestion of cell walls have been reviewed by Peberdy (1985)and Davis (1985).

In this Section the isolation procedures for C.lindemuthianum are reported in detail. Chu and Alexander (1972) were also able to digest Colletotrichum conidiospores in their studies on cell wall biosynthesis, but they took no precautions to stabilize the protoplasts and in addition the spores had been killed by ultraviolet radiation in advance.

In the studies on heterokaryosis in C.lindemuthianum (Chapter 3.2) no final evidence of heterokaryosis was obtained, since for the putative heterokaryotic colonies cross-feeding could not be excluded. However, when in protoplast fusion only few protoplast participate cross-feeding cannot play an important role, i.e. complementation must then be the result of heterokaryosis. In this section, apart from the isolation of protoplasts from conidospores of C.lindemuthianum, also fusion of mutants of different physiological races is described. From several physiological races auxotrophic mutants have been

isolated (see 3.1). For fusion experiments double auxotrophic strains were used in order to avoid complications caused by revertants and as extra precaution against cross-feeding.

3.5.1 ISOLATION OF PROTOPLASTS

From prelimary experiments it was obvious that the lytic enzymes from Oerskovia grown on Aspergillus cell walls in combination with Penicillium glucanases could effectively attack the Colletotrichum conidial cell walls. Under the standard conditions developed for A.nidulans the protoplasts collapsed and therefore conditions had to be modified. In the lytic mixture a higher $(NH_4)_2SO_4$ concentration proved to be necessary, and the best results were obtained with 0.8 M ammonium suphate. During lytic incubation small, probably anucleate, protoplast drops were pinched off. At 1.0 M $(NH_4)_2SO_4$ this effect is less, but the protoplasts yield was reduced by 50%. The protoplast procedures for A.nidulans and C.lindemuthianum are compared Table 3.9.

Table 3.9 Summary of conditions and results of protoplast procedures.

	A.nidulans	C.lindemuthianum
Conidiospores from	3 days old cultures on CM grown at 37°C	12 days old cultures on CM grown at 22°C
Conidial suspension	$5x10^7$ in about 1 ml saline +Tween; added to 50 ml CP	10^8 in 10 ml saline+Tween; collected on a membrane filter
Preincubation	in CP + 2-deoxy-D-glucose (2x25 ml in a 100 ml bottle) for 3 h at 37°C conidiospores collected by centrifugation or on a membrane filter	omitted
Lytic incubation	10 in 2 ml 0.4 M $(NH_4)_2SO_4$ = 0.2 ml PC-buffer + 0.4 ml Oerskovia enzymes + 0.4 ml glucanases (0.2mg) + 1 ml 0.8 M $(NH_4)_2SO_4$ for 3 h at 30°C	10 in 2 ml 1.0 M $(NH_4)_2SO_4$ = 0.2 ml PC-buffer + 0.4 ml Oerskovia enzymes + 0.4 ml glucanases (0.2mg) + 1 ml 2 M $(NH_4)_2SO_4$ for 5-6 h at 30°C
Purification of protoplasts	on 35% sucrose by centrifugation at low speed	washed with 0.6 M KCl by centrifugation.
Protoplast yield	60-80 %	95-100%

The formation of droplets was stimulated by shaking the suspension during lytic incubation, but ommission of shaking caused more aggregation of protoplasts. The preincubation step could be ommitted without decrease of yield, and prolonged lytic incubation up to 5 or 6 hours increased the yield. In general at least 90 % of the conidiospores were converted into protoplasts.

Since the mycelium of C.lindemuthianum hardly grows above 22°C and the spores are killed at higher temperatures (see Chapter 3.2.1) incubation with lytic enzymes at 25°C was compared with that at 30°C. The results are shown in Table 3.10. Incubation at 30°C during 5-6 hrs aparently was not harmfull and it even gave, like in some other experiments, a slightly higher yield of protoplasts than at 25°C. For the preparation of protoplasts from 10^8 conidiospores (in 2 ml) 0.5 ml Oerskovia enzymes and 0.5 mg glucanases were satisfactory.

Table 3.10 Isolation of protoplasts from C.lindemuthianum conidiospores.

Strains	Initial amount of spores ($x10^{-6}$)	Amount of protoplasts ($x10^{-6}$) at 25°C	at 30°C	% protoplasts at 25°C	at 30°C
C409-41	38	21	21	96 %	98 %
C409-47	6	5.6	7.5	98 %	100 %

Lytic incubation with Oerskovia enzymes and glucanases in 0.8 M PAC buffer. Conidiospores collected from cultures grown at 22°C for 12 days.

The structures found when conidiospores of C.lindemuthianum were incubated with the lytic enzymes resembled those of Saccharomyces carlsbergensis (Eddy and Williamson, 1959), except that the protoplasts protrude at the top of the cell (Fig.3.15). After one hour of incubation with lytic enzymes, the escape of the protoplasts from the cell wall can be readily observed.

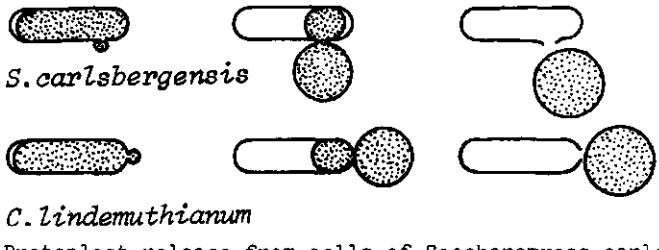


Figure 3.15 Protoplast release from cells of Saccharomyces carlsbergensis (after Eddy and Williamson, 1959) and from conidiospores of C.lindemuthianum.

3.5.2 PROTOPLAST FUSION IN C.LINDEMUTHIANUM

In prelimary experiments on fusion it was concluded that higher $CaCl_2$ concentrations should be used than with A.nidulans. In that case the protoplasts must be purified, because otherwise a precipitate of $CaSO_4$ will be formed. Protoplasts from the lytic mixture were diluted with two volumes 1.2 M KCl and brought in a centrifuge tube on a 30% sucrose cushion and centrifuged at 2000 rpm for 25 minutes. On the top some greasy cell material settled and above the sucrose a protoplast band was formed. The protoplasts were collected and washed twice with 1.2 M KCl by centrifugation. There was still no separation of protoplast and residual spores, but owing to the low percentage of spores this did not interfere in the fusion experiments. Protoplast could be plated on CMK or MMK, stabilized as usual with 0.4 M KCl. The fusion mixture was plated directly or diluted 10 times with 1.2 M KCl.

Table 3.11 Summary conditions and results of protoplast fusion.

	A.nidulans	C.lindemuthianum
Fusion	$2x10^6$ protoplasts in 2ml 30% PEG (pH 5.9); 50 mM$CaCl_2$ for 20 min at 30°C	10^7 protoplasts in 2 ml 30% PEG (50mM glycin-buffer pH8); 450 mM $CaCl_2$ for 20 min at 30°C.
Survival of protoplasts	non-purified: >40 % purified: 1-30 %	washed: 25-80 %
Heterokaryon frequency	non-purified: 0.03-1 % purified: 2-25 %	0.4-1 %

The protoplast fusion experiments (see Table 3.11) were performed with two double auxotrophic strains derived from the same wildtype strain. About equal amounts of protoplasts of both strains were collected by centrifugation and washed with 0.6M KCl. The protoplasts were resuspended in 30% PEG, 450 mM $CaCl_2$, 50 mM glycin buffer pH 8. After 15 - 20 minutes the protoplasts were plated on CMK and MMK and incubated at 22°C for respectively 5 and 10 days. Incubation in PEG for 60 min gave similar results. In Table 3.12 the results of two fusion experiments are presented.

Table 3.12 Protoplast fusion between two mutants of C.lindemuthianum (C409).

Exp.	Strain	Input total count $x10^{-4}$	Viable count on CMK $x10^{-4}$	Fusion treatment at different pH: pH 6 colonies CMK $x10^{-4}$	pH 6 colonies MMK	pH 7 colonies CMK $x10^{-4}$	pH 7 colonies MMK	pH 8 colonies CMK $x10^{-4}$	pH 8 colonies MMK
1	C409-41	90	4.5	5.2	225	3.9	220	2.2	220
	C409-47	60	1.8	(82%)	(0.4%)	(62%)	(0.6%)	(35%)	(1.0%)
2	C409-41	240	87	110	30	76	710	33	1200
	C409-47	330	44	(83%)	(0.04%)	(57%)	(0.09%)	(25%)	(0.38%)

Between brackets: CMK % protoplasts recovered; MMK % heterokaryons.

In Exp.1 800 mM $CaCl_2$ was used instead of 450 mM, but with 800 mM $CaCl_2$ the protoplast looked somewhat wrinkled and therefore normally 450 mM $CaCl_2$ was used, despite the higher heterokaryon frequencies in Exp.1.

As in this experiment the highest pH (pH 8) seemed to be the best choice, the pH range was extended in a next experiment. For pH 6 a 50 mM imidazole buffer was used and now for pH 7-10 a 50 mM glycin buffer (Table 3.13).

Table 3.13 Effect of pH on fusion frequency.

Strains Input total count	Number of colonies on CMK found after fusion: pH6	pH 8	pH 9	pH 10
C409-41: 12 $x10^5$ C409-47: 32 $x10^5$	1.8 $x10^5$	1.8$x10^5$	0.5$x10^5$	0.05$x10^5$
Heterokaryons on MMK	220	1010	350	130
Heterokaryon frequency	0.01%	0.56%	0.7%	2.6%

Incubation in 30% PEG, 450 mM $CaCl_2$.

Although at pH 10 the highest heterokaryon frequency was found this did not compensate for the loss of protoplasts. Optimal results in this experiment were obtained at pH 8.

Standard conditions for fusion of C.lindemuthianum protoplasts were: incubation in 30% PEG, 450 mM $CaCl_2$ at pH 8 and direct plating of the mixture. PEG 1000 could replace PEG 4000 without significant changes in yield. Incubation in 20% PEG at pH 9 was better than in 30% PEG at pH 9, but still less than in 30% PEG at pH 8.

In fusion experiments with double auxotrophic mutants from two different wildtype isolates (C409 and C420) a relatively high frequency of fusion products was found. The data from two of these experiments are given in Table 3.14. Also fusion between mutants derived from the same wildtype isolate is included in order to compare inter-strain with intra-strain fusion frequencies.

Table 3.14 Fusion of mutants of different physiolical races (C409 and C420).

Strain	Input protoplasts Total count	Count on CMK	Recovery count on CMK	Heterokaryon count on MMK	Fusion frequency
C420-12	3.4 x10^5	0.04 x10^5	0.4 x10^5	240	0.60 %
C409-41	12	0.2			
C420-12	3.4	0.04	1.8	150	0.05 %
C409-47	32	5.8			
C409-41	12	0.2	1.8	220	0.12 %
C409-47	32	5.8			

Incubation in 30% PEG, 450 mM $CaCl_2$.

The ratio between the strains was not optimal, since strain C420-12 sporulated poorly,so it was not possible to collect sufficient conidiospores. Protoplast fusion between mutants of different isolates proceeded equally well as between mutants of the same isolate. The variation between different experiments is such that the differences in this experiment can not be considered as significant.

3.5.3 DISCUSSION

As described in Chapter 3.3, with C.lindemuthianum putative heterokaryons (from hyphal anastomosis) could be selected on MM, but heterokaryosis was difficult to prove as cross-feeding could not be excluded. So, we resorted to protoplast fusion with two double auxotrophic strains. Here the problem of cross-feeding does not arise. This is in agreement with Genovesi and Magill (1976) who found no cross-feeding between double auxotrophic strains of Pyricularia oryzae. The same was found by others with

a number of fungi (Anné and Peberdy, 1976; Anné, 1977; Ferenczy et al., 1976). Also in our experiments with conditions not allowing fusion (e.g. no PEG or no Ca^{2+}) no cross-feeding was observed.

With the Oerskovia lytic system, developed for Aspergillus nidulans, protoplasts could readily be prepared from conidiospores of C.lindemuthianum. Already after one hour of lytic incubation most conidia showed protoplast formation. All protoplasts escaped from the top of the conidiospore. This is remarkable because conidiospores mostly form a germ tube at the side. The protoplasts leave the cell envelope through an aperture and one may suspect that the location at which the germ tube should be formed will be digested first. At least 90% and often up to 99% of the conidiospores could be converted into protoplast after 5 to 6 hours of lytic incubatión with 0.8 M $(NH_4)_2SO_4$ as stabilizer. As germination of conidia of C.lindemuthianum requires more than 15 h of incubation one may assume that the conidial protoplasts still have one nucleus. When shaking during lytic incubation is too vigorous, small probably anucleate protoplasts are pinched off.

For fusion experiments the protoplasts had to be washed prior to use for fusion to remove the sulphate, which could precipitate the calcium of the fusion buffer. If this was omitted lower fusion frequencies were found and a precipitate was observed. Fusion proceeded optimally at pH 8. At higher pH the heterokaryon frequencies were higher, but the number of heterokaryons showed a pronounced decrease. The high frequencies at high pH were strictly dependant on the presence of high calcium concentrations. This is in agreement with the high fusion frequencies at high pH found with Penicillium sp. (Anné, 1977) and Aspergillus nidulans (Ferenczy et al. , 1976). Probably multiple fusions occur under these conditions.

Usualy the fusion mixtures (in 30% PEG) were plated directly (without washing), giving the best results. Since incubation in PEG buffer for 20 minutes gave the same results as incubation for 60 minutes, it is probable that fusion is completed on the plates. Anyway the PEG buffer protects the protoplasts against

drying on the plate. It was not necessary to plate in a toplayer.

In our experience with C.lindemuthianum we observed that the plating efficiency of conidia was often not more than 10% (incidentally 20%) on CM and on MM even lower (see 3.2.0). So plating fused protoplasts on MMK may lead to underestimation of the heterokaryon frequency, since protoplast counts are done on CMK. Nevertheless, the heterokaryon frequencies (often 0.1-1 %) were not much lower than the frequencies obtained with A.nidulans (Chapter 3.4) and even higher than those obtained with Penicilium (Anné, 1977).

The heterokaryons of C.lindemuthianum obtained after protoplast fusion indicate that the presumed heterokaryons obtained by hyphal anastomosis can be real heterokaryons.

3.6 Conclusions

The phytopathogenic fungus Collectotrichum lindemuthianum which has low spore viability and is difficult to maintain can be preserved by lyophilization.

C.lindemuthianum probably is able to form intra- and inter-strain heterokaryons:

- Hyphal anastomoses occur, also between different races.
- Presumed heterokaryons of complementing strains could be established on MM, although this may be mimicked by cross-feeding.
- Heterokaryons were also obtained by protoplast fusion, where cross-feeding is excluded.
- Karyogamy could not be proven. Although presumed diploids could be isolated, no recombinants were found.

An effective procedure for the isolation of protoplasts from conidiospores was developed. This resulted in homogeneous suspensions of mono- or binucleate protoplasts. The procedure, developed for A.nidulans, was also suitable for other imperfect fungi. With certain strains (of A.niger) initially sferoplasts are formed, but by carefully lowering the osmotic pressure of the stabilizing medium protoplasts can be obtained.

The protoplasts prepared from conidiospores are very suitable for fusion experiments (heterokaryon frequency about 1 %). Under the fusion conditions used mostly only two or three protoplasts participate in a fusion event.

Studies on somatic karyogamy in A.nidulans with the help of protoplast fusion revealed that the frequency of somatic karyogamy may be some orders of magnitude higher (100x) as can be deduced from the frequency of heterozygous diploid conidiospores in a heterokaryon.

In balanced heterokaryons sorting out of nuclei results in homokaryotic hyphae and the heterokaryon is maintained by new anastomoses. Examinations on heterokaryons showed that hyphal tips from the border of a heterokaryon in general are homokaryotic. This agrees with the observations on karyogamy frequency.

4. Perspectives for genetic analysis of Aspergillus niger

4.0 Introduction

Although Pontecorvo et al. (1953) demonstrated that parasexual mechanisms occur in Aspergillus niger, the genetics of this fungus is still ill-explored despite its biotechnological importance. Pontecorvo, Roper and Forbes (1953) carried out a number of genetic experiments, but concluded that still much research had to be done before satisfactory genetic analyses could be achieved. Lhoas (1967) started a genetic analysis of A.niger by somatic recombination, but his work was only continued occasionally: Van Tuyl (1977) used among other fungi A.niger to study the genetic basis of resistance against fungicides, Azevedo and co-workers (Avezedo and Bonatelli, 1983) did genetic experiments with different strains of A.niger, including diploid strains, Pásková and Munk (1963) forced heterokaryons of gluconic acid producing strains; Ilczuk (1971) studied citric acid production by means of heterozygous diploids, and Fiedurek and Ilczuk (1983) the production of pectolytic enzymes.

Applications of somatic recombination to production strains are scarce, at least little has been published on the topic. In these experiments several obstacles will be met which are discussed in the present chapter.

In recent years attention is also paid to the isolation of protoplasts of A.niger. Some pilot experiments were reported by Anné (1977), Moore and Peberdy (1977), Bos and Slakhorst (1981), Lasure and Weber (1983). Recently, recombinant DNA techniques were applied to A.niger. Kelly and Hynes (1985) carried out experiments on the transformation of wildtype A.niger with plasmids containing the amdS gene of A.nidulans. The A.niger trpC gene was cloned and used in transformation of A.nidulans protoplasts (Kos et al., 1985)).

In our research group we started genetic research on A.niger in 1981. It was not possible to acquire the A.niger master strains constrcuted by Lhoas (1967). So we started with new wild

type strains obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn). One of these strains was chosen as reference strain. From this strain an extensive mutant collection will be made and test-strains for genetic analysis will be constructed. So genetic analysis can be performed in an isogenic background. Wildtype isolates may be polymorphic (i.e. contain different alleles) at several loci. Moreover chromosomal rearrangments may have occurred. Yet genetic analysis of strains of different origin is possible with the help of the reference strain. In this way one can use a single collection of genetic marker strains. Prerequesite for this approach is that somatic recombination between strains of different origin is possible. In the case of strains descending from different wildtype isolates, protoplast fusion can be used to overcome heterokaryon incompatibility.

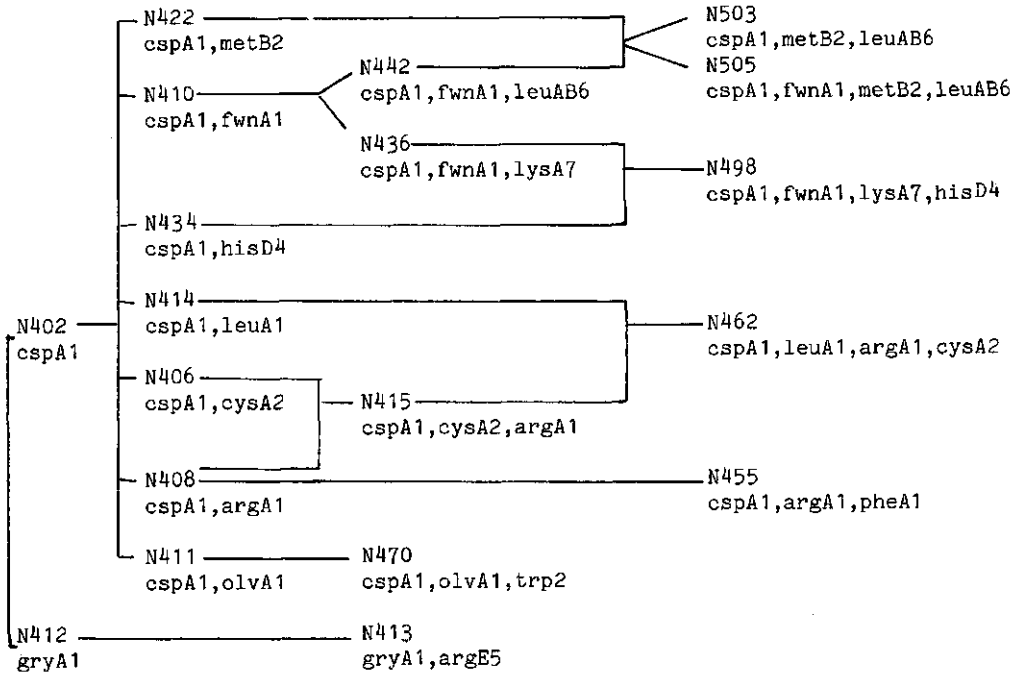
The building up of a collection of strains with suitable genetic markers and the construction of test-strains is time consuming. In this Chapter some of our collective results will be reported. First the strategy and result with the isolation of mutants will be given (4.2) and then a number of genetic analyses based on mitotic recombination will be presented (4.3).

4.1 Materials and methods

4.1.0 STRAINS

An Aspergillus niger wildtype strain was obtained from CBS (Baarn, The Netherlands): N400 = CBS120-49. In this wildtype strain a morphological mutant with low conidiophores (cspA1) was induced and from this mutant strain (N402) we derived auxotrophic and colour mutants (see 4.2). Combinations of different markers were in general obtained from recombination experiments (see 4.3). For convenience a list of genotypes showing the origin of the strains used in this Chapter is given:

Survey of strains descending from A.niger N400:



4.1.1 MEDIA

The A.niger strains were grown on complete medium (CM), minimal medium (MM), supplemented MM (SM) (according to Pontecorvo et al, 1953), and on malt extract (ME). These methods are described in Chapter 2.1. On CM and ME sporulating cultures were obtained after 3-4 days incubation at 30° C.

4.1.2 ISOLATION OF MUTANTS

Mutants were induced by UV-irradiation and occasionally by gamma-radiation, at survival levels of 70-80% according to the analyses presented in Chapter 2. Suspensions of $2x10^6$ conidia per ml were used since in denser suspensions the shelter effect was too large. For UV-irradiation 10-12 ml suspension was irradiated in an open Petri dish (see 2.1.3). Gamma-irradiation of 2 ml conidial suspensions ($5x10^6$/ml) in small polypropyleen tubes was carried out with a Co-source (ITAL, Wageningen) at a dose of 100 Gy (= 10 krad). The condial suspensions were made the day before and kept at 4 °C overnight.

Before and after irradiation, dilutions of the suspension were plated on CMT and spore counts were done with the Coulter counter to correctly determine the percentage survival.

The filtration enrichment procedure applied has been described in Chapter 2.1.3. After mutagenic treatment the conidia (about 10^7) were collected and added to 40 ml liquid MM, supplemented for the growth factors required by the parental strain. This suspension was incubated in a reciprocal shaker at 30 °C, in general for 18-24 hours. After 10-12 h the suspension was filtered through a cotton wool plug (50 mg in a 7 cm funnel) and the medium was refreshed. At the end of incubation the suspension was filtered again and the conidia that passed the cotton wool plug were collected by centrifugation and resuspended in 2 ml saline.

From this suspension samples were plated on CMT for survival count and the suspension was stored overnight at 4 °C. Then survival could be counted and an appropriate dilution was plated to obtain 50 to 80 colonies per plate. Sometimes the suspension

was plated on CMT enriched with arginine, tryptophane and uridine when specifically mutants of these types were required.

Subsequently, after 2-3 days the colonies rescued on CMT were transfered to MM. The colonies on the CMT plates which did not grow on MM were collected and tested again. In other experiments a replica plating technique was used. The CMT plates were covered with a piece of sterile filter paper and the suspension was plated on the filter paper (0.2 ml per plate). After two days incubation at 30°C the filter paper was transferred to the top of a wood block (Ø 8.5 cm) and replicas were made on MM by pressing the MM plate upside down on the filter paper. After one day at 30°C the MM plates could be scored. Auxothrophic mutants give only a dark spore print on the MM plate whereas prototrophs form a colony.

Several students and colleagues participated in the isolation of A.niger mutants. Especially the contributions of Miss Anja Huibers and Dr.K.Swart are gratefully acknowledged.

4.1.3 CHARACTERIZATION OF MUTANTS

The auxotrophic mutants isolated were identified by growth tests on media with different amino acids (20 mg/ml), vitamins (0.2 mg/ml) or nucleosides (20 mg/ml). Morphologic mutants were isolated directly from the CMT plates.

Phenotypically similar mutants were tested for complementation in heterokaryons. For some groups of mutants growth tests on metabolic intermediates and analyses of enzyme activities are currently carried out by Swart, Bos and others (unpublished).

4.1.4 HETEROKARYONS

Aspergillus niger heterokaryons were obtained according to Pontecorvo et al. (1953). Conidia of two strains with different auxotrophic markers were mixed in 2 ml liquid complete medium and incubated at 30 °C for 2 days. Pieces of the resulting mycelial material were transferred to MM plates and incubated at 30 °C for 4 to 5 days. The usually poorly growing mycelium produced vigorously growing sectors. By far not all pieces of mycelium give rise to heterokaryons, but from all combinations

of strains heterokaryons could be obtained. Uniformly growing balanced heterokaryons are secured by transfer of sector pieces to MM plates.

4.1.5 ISOLATION OF DIPLOIDS

For the isolation of heterozygous diploids from heterokaryons a spore suspension was made by collecting conidia from a heterokaryon in saline-Tween as described earlier. Viability counts were done by plating a diluted suspension on CMT. Diploids were isolated by plating a concentrated suspension (about 10^6/ml; filtered through a cotton wool plug) in MM with a thick MM overlay as described by Pontecorvo et al. (1953). The plates were incubated for 5-7 days at 30°C. Since in some experiments with A.niger we found a high frequency of heterokaryotic colonies from plated conidia, occasionally the suspensions were plated in a MM-toplayer poured onto a MM bottom-layer.

In view of the relatively high frequency of mitotic crossing-over the number of transfers of the diploids was limited as much as possible. The selection plates were preserved and the diploids were transferred once to MM master plates.

4.1.6 GENETIC ANALYSIS

Master strains for allocation of genes to linkage groups were not yet available, but several individual genes could be tested for linkage. To determine whether genes were on the same chromosome a masterplate with heterozygous diploid colonies was replicated on CM+benomyl (1/200 or 1/250 of a stock solution of 0.4 mg/ml in aceton) and/or on CM+fpa (2 mg p-fluorophenylalanine/ml). These plates were incubated for a week at 30°C and segregants were isolated on CM. Benomyl was preferred as haploidizing agent whenever possible, especially when using colour markers. Only in a few experiments CM+fpa proved to be more suitable. The segregants were purified, (usualy) twice on CM and tested for genetic markers. In case of doubt about ploidy the size of the conidia was determined by means of the Coulter counter.

For the determination of mitotic recombination diploid segregants, homozygous for a specific marker, were isolated and these were tested for homozygosity of linked markers descending from the same parent. Only markers in cis-position can be mapped in this way (see 1.2.3). For A.niger we used an enrichment method analoguous to that used for the isolation of mutants. This procedure had proven to be very suitable for the isolation of partially homozygous diploids in A.nidulans for a broad range of different characters, e.g. for pdhC mutants (Bos et al., 1981 and unpublished results). The auxotrophic segregants were identified by replica plating.

We verified the ploidy of the colonies of interest by measuring a sample of conidiospores with the Coulter counter. When suitable colour markers can be used for selection, a sample of diploid spores can be plated on CMT and inspected visualy.

4.2 Establishment of a mutant collection

4.2.0 INTRODUCTION

A wild type strain was chosen for the establishment of a collection of mutants to be used as genetic markers for the construction of master-strains. In this wildtype strain mutants are induced by UV or other mutagens at low doses (cf. Chapter 2). In order to avoid accumulation of background mutations or chromosomal rearrangements the number of consecutive treatments of a strain has to be kept to a minimum.

First a mutant was induced which had low conidiophores and from this mutant auxotrophic and colour mutants were isolated (see 4.1.0). In principle strains with more than two auxotrophic markers have to be obtained by recombination. Since we also worked with other wildtype strains we decided to have a fixed auxotrophic marker to label the strain used for the isolation of new mutants.

4.2.1 RESULTS

In a series of mutation experiments a collection of some 90 auxotrophic mutants were derived from the reference strain (N402) (See Table 4.1). Detailed characterization of the mutants is in progress. It was known from work with A.nidulans that some arginine, tryptophane and pyrimidine deficient mutants (mutants which we looked for with priority) need supplementation of CM. Therefore, except for the first experiments, these amino acids and uridine were always added to CM and CMT in the course of the mutant isolation procedure. Induction, isolation and prelimary characterization of mutants was carried out as described in Section 4.1. In general only mutants with full expression were retained, for leaky mutants are not always easy to handle in genetic analyses.

Table 4.1 List of auxotrophic mutants descending from N402

Gene symbol	Phenotype	Number of mutants	Number of loci on the basis of complementation
ade	adenine deficient	10	>2
arg	arginine or arg/pro deficient	14	5
bio	biotin deficient	1	1
cys	cysteine or cys/met deficient	4	3
his	histidine deficient	5	5
leu	leucine deficient	8	>3
lys	lysine deficient	23	4
met	methionine deficient	12	>3
nic	nicotinamide deficient	1	1
pab	p-aminobenzoic acid deficient	2	1
phe	phenylalanine deficient	1	1
pro	proline deficient	3	1
trp	tryptophan deficient	4	>2
	Total	88	>30

4.2.3 DISCUSSION

The procedure chosen for the isolation of auxotrophic mutants proved te be suitable for this A.niger strain (N402). It is obvious that the enrichment techniques favour the isolation of certain mutant phenotypes. By adjusting the conditions for enrichment and rescue, other mutants may be found too. The trp-mutants were only found when filter sterilized tryptophane was added to the CM. As was shown in Section 2.3, for the isolation of vitamine-less mutants repeated refreshing of the enrichment medium is recommended. In this way, variation in experimental conditions can supply sufficient different mutant fenotypes. It should be kept in mind that a great variety of markers is necessary for genetic analyses. The master strains should have phenotypically different markers because, for example, an argA and an argB marker in the same experiment will complicate the analysis. Apart from the auxotrophic mutants also some resistance markers and colour markers are used in recombination experiments.

4.3 Genetic analyses

4.3.0 INTRODUCTION

To assign genes to chromosomes a set of master strains is needed, each with marker genes on the different chromosomes. The construction of such master strains requires firstly the establishment of linkage relationships between the available genetic markers.

From several combinations of mono-auxotrophic strains double auxotrophic recombinants were obtained by mitotic recombination (Cf 4.1.0). Diploid analyses at this stage are not presented here, because little information about linkage was obtained. Rather the double auxotrophic recombinant strains were subsequently used for diploid analyses to identify linked genes (see below).

Non-disjunction in heterozygous diploids is induced by benomyl (Hastie, 1970). It is known that this agent interferes with spindle formation (Davidse and Flach, 1978; Sheir-Neiss et al, 1978), and consequently disturbs the disjunction of sister chromatids. Although Kappas et al. (1974) mentioned a mutagenic effect of benomyl on A.nidulans, Wood (1982) showed that it has little or no effect on mutation or mitotic crossing over in yeast.

It was already known that mitotic crossing over in A.niger is more frequent than in A.nidulans. Six linkage groups could be demonstrated (Lhoas, 1967). Also the genes studied by Van Tuyl (1977) assorted according to the known linkage groups.

On the basis of the compiled linkage data obtained in our experiments the interference of mitotic crossing over with the allocation of genes to chromosomes and the consequences for the construction of master strains are discussed.

4.3.1 SELECTION OF DIPLOIDS

Several combinations of mutant strains decending from strain N402 were used to make heterokaryons and heterozygous diploids. Upon the transfer of pieces of heterokaryon to fresh MM plates mostly well balanced heterokaryons were formed.

Usualy it was easy to collect 10^7 conidia from heterokaryons on a single MM plate. Although the suspension was filtered through a cotton wool plug, often many heterokaryons were found on the selection plates. In general one in 30 to 50 colonies was a diploid. The diploid frequency among conidiospores was about 10^{-6}. When the frequency of heterokaryons was high, plating in a toplayer was preferred over plating in a bottomlayer with a MM overlay as is usually done with A.nidulans.

Several presumptive diploid colonies were tested for growth on MM and the conidia were measured. One diploid colony was chosen for genetic analysis. This colony is maintained on MM with as few transfers as possible.

Often it is difficult to see whether a colony is a diploid or a heterokaryon. Also when colour markers are used, heterokaryons may show (sectors with) conidia with a dark phenotype like the heterozygous diploid. When e.g. olive-green and fawn colonies grew on the same CM plate, dark coloured conidia were formed at the border where the colonies adjoined.

4.3.2 ANALYSES BY HAPLOIDIZATION

A number of diploid analyses were carried out in order to identify linked genes. Haploid segregants were obtained from a heterozygous diploid on CM+benomyl, purified by repeated transfer to CM and tested for genetic markers.

The results of one of the experiments are presented in the Tables 4.2 and 4.3. The genotypes of the segregants are listed in Table 4.2. Segregants which differ in colour from the heterozygous diploid were easier to identify than segregants with wild type colour. It is seen that a number of segregations is disturbed (i.e. the segregation of the two alleles of a given gene deviates from the 1:1 ratio). Moreover, in this experiment prototrophic fawn segregants were very frequent. Probable these were mostly diploid homozygous for the fawn colour marker as a result of an earlier mitotic crossing-over event. In this experiment (one of the first analyses) these colonies were not screened for ploidy by measuring the conidia. For the analysis of linkage only arg-less segregants were used (* in Table 4.2) to circumvent the inclusion of diploid fawn segregants.

Table 4.2 Genotypes of segregants from diploid N413//N442

N413	argE5	gryA1	+	+		
N442	+	+	fwnA1	leuAB6		
	+	+	+	+	8	
	argE5	+	+	+	0	*
	+	gryA1	+	+	19	
	+	+	fwnA1	+	252	
	+	+	+	leuAB6	5	
	argE5	gryA1	+	+	8	*
	argE5	+	fwnA1	+	45	*
	argE5	+	+	leuAB6	3	*
	+	gryA1	fwnA1	+	29	
	+	gryA1	+	leuAB6	16	
	+	+	fwnA1	leuAB6	72	
	argE5	gryA1	fwnA1	+	38	*
	argE5	gryA1	+	leuAB6	6	*
	argE5	+	fwnA1	leuAB6	45	*
	+	gryA1	fwnA1	leuAB6	51	
	argE5	gryA1	fwnA1	leuAB6	31	*
				Total:	628	

*) arg5 segregants: 176

The frequencies of pairwise gene combinations are shown in the lower left half of Table 4.3. In the upper right half the recombination frequencies are given.

Table 4.3 Diploid analysis (N413//N442)

	N413 N442	gryA1 +	argE5 +	+ fwnA1	+ leuAB6
markers	gryA1	+	argE5 *)	+ fwnA1	+ leuAB6
gryA1 +			(53 %)	41 %	47 %
argE5 *)	83	93		(10%)	(48 %)
+	1	3	17		52 %
fwnA1	69	90	159		
+	46	45	91	8 83	
leuAB6	37	48	85	9 76	

* Only arg5 segregants were used for the analysis since there was a large excess of prototrophic fwnA1 colonies, probably descending from a mitotic crossing-over event.
Frequencies between brackets refer to single-class (within argE5) segregations. Here the assumption of non-disturbed overall segragation is made.

From this experiment it was learned that it is important to maintain the heterozygous diploid with as few transfers as possible to avoid mitotic crosssing-over. In the following experiments also special attention was payed to phenotypes which could represent diploids. In the case of suspect segregants ploidy was decided on spore size and diploid segregants were excluded. The results are summarized in the Tables 4.4 to 4.7. The parents of the diploids used for genetic analysis were chosen from the recombinants obtained in foregoing experiments (see 4.1.0). In the diploid analysis of N498//N503 (Table 4.4.) with five loci no linkage was found, and in the diploid analysis of N462//N498 (Table 4.5) only one pair of markers was linked (21% recombination). In the diploid analyses N455//N498 (Table 4.6) and N455//N505 (Table 4.7) three markers (pheA, argA, metB) showed close linkage. It should be noted that the pheA1 mutation was induced in an argA1 strain, so these two mutant alleles are in coupling phase. The linkage data are summarized in Table 4.8.

Table 4.4 Diploid analysis N498//N503

	N498 N503	fwnA1 +	lysA7 +	hisD4 +	+ metB2		+ leuAB6			
markers	fwnA1	+	lysA7	+	hisD4	+	+	metB2	+	leuAB6
fwnA1 +			54 %		53 %		55 %		51 %	
lysA7 +	66 89	63 64			47 %		50 %		51 %	
hisD4 +	18 137	12 115	13 116	17 136			50 %		55 %	
+ metB2	73 82	72 55	66 63	79 74	17 13	128 124			48 %	
+ leuAB6	87 68	77 50	74 55	90 63	19 11	145 107	87 58	77 60		

282 segregants were analyzed.

Table 4.5 Diploid analysis N462//N498

	N462 N498		leuA1 +		cysA2 +		argA1 +		+ fwnA1		+ lysA1		+ hisD4	
markers	leuA1	+	cysA2	+	argA1	+	+	fwnA1	+	lysA7	+	hisD4		
leuA1 +			44 %		42 %			51 %		47 %		57 %		
cysA2 +	37 81	53 136			41 %			52 %		21 %		70 %		
argA1 +	40 78	52 137	28 62	64 153				53 %		50 %		63 %		
+ fwnA1	58 60	96 93	42 48	112 105	42 50	112 103				47 %		48 %		
+ lysA7	55 63	80 109	80 10	55 162	36 55	98 117	72 82	63 90				58 %		
+ hisD4	105 13	161 28	70 20	196 21	83 9	183 32	136 18	130 23	111 24	155 17				

307 segregants were analyzed.
Note: When both single gene segregations are disturbed (deviate significantly from 1:1) the percentages cannot be considered as unbiased estimates of the recombination percentages.

Table 4.6 Diploid analysis N455//N498

	N455 N498		argA1 +		pheA1 +		+ fwnA1		+ lysA7		+ hisD4	
markers	argA1	+	pheA1	+	+	fwnA1	+	lysA7	+	hisD4		
argA1 +			1 %		50 %			44 %		59 %		
pheA2 +	90 3	0 225			51 %			44 %		59 %		
+ fwnA1	44 49	110 115	41 49	113 115				50 %		54 %		
+ lysA7	49 44	97 128	48 42	98 130	70 84	76 88				51 %		
+ hisD4	86 7	179 46	83 7	182 46	124 30	141 23	124 22	141 31				

318 segregants were analyzed.
See footnote to Table 4.5

Table 4.7 Diploid analysis N455//N505

	N455 N505	argA1 +	pheA1 +	+ fwnA1	+ metB2	+ leuAB6				
markers	argA1	+	pheA1	+	+	fwnA1	+	metB2	+	leuAB6
argA1 +			1 %		42 %		1 %		46 %	
pheA1 +	98 2	1 212			43 %		1 %		46 %	
+ fwnA1	64 36	97 116	62 37	99 115			42 %		52 %	
+ metB2	100 0	2 211	99 0	3 211	65 96	37 115			45 %	
+ leuAB6	66 34	109 104	65 34	110 104	87 74	88 64	68 34	107 104		

313 segregants were analyzed. See footnote to Table 4.5

Table 4.8 Compilation of recombination data from all diploid analyses.

	fwnA	gryA	argA	argE	cysA	hisD	leuA	leuAB	lysA	metB
gryA	41									
argA	53 50 42									
argE	(10)	(53)								
cysA	52		41							
hisD	53 48 54		63 59		70					
leuA	51		42		44	57				
leuAB	52 51 52	47	46	(48)		55				
lysA	54 47 50		50 44		21	47 58 51	47	51		
metB	55 42		1			50		48 45	50	
pheA	51 43		1 1			59		46	44	1

For percentages between brackets see Table 4.3.

4.3.3 ANALYSES BY MITOTIC CROSSING-OVER

The diploid N455//N505, which has three closely linked markers and two non-linked markers is suitable to study mitotic crossing-over:

N505	metB2	+	+	fwnA1	leuA1
N455	+	argA1	pheA1	+	+

(sequence arbitrary)

There are two drawbacks for an analysis of mitotic crossing-over. Firstly, as mentioned in Section 1.2.3, pairs of recessive markers must be in cis-position, i.e. on the same homologue, for recombinants which have become homozygous for the wildtype allele will go unnoticed as they have the same phenotype as the heterozygous diploid. Of course, the homozygous diploid recombinants can be analyzed by subsequent haploidization, but this is very laborious. Secondly, markers suitable for positive selection are not available. This is overcome by using enrichment procedures.

With the diploid strain N455//N505 three separate enrichment procedures (as described in 2.4) have been carried out: in SM without methionine (Exp.1), in SM without phenylalanine (Exp.2) and in SM without arginine (Exp.3). Each enrichment procedure started with conidia of the heterozygous diploid grown on CM (although MM would have been better, see Discussion). About $4x10^7$-10^8 conidia were incubated in 20 ml SM (e.g. SM −met) for two days. The medium was refreshed after one day. The remaining conidia were plated on filter paper on CMT supplemented with arginine and phenylalanine. The resulting colonies were replicated on MM. Colonies not growing on MM were collected by transfer to CM+arg+phe and tested for the other markers. Conidia were measured for determining the ploidy level. In this way haploid and partially homozygous diploid recombinants are selected. The results are summarized in Table 4.9.

Since the numbers of segregants obtained by enrichment procedures provide no information on the frequency of spontaneous haploidization, spontaneous fawn-segregants were isolated after plating of conidiospores from a heterozygous diploid colony (Exp.4). Starting from a monospore colony the diploid was

Table 4.9 Enrichment for recessive recombinants from an A.niger diploid N455//N505.

Exp.	Enrichment for		Number of colonies rescued and tested	Segregant colonies: total	haploid	diploid
1.	met-requirement		3600	66	64	2
2.	phe-requirement		331	27	25	2
3.	arg-requirement		2096	90	87	3
4.	no enrichment	from CM	4020	10	8	2
	fwnA1 colonies	MM	5260	6	5	1

Conidiospores were incubated for enrichment (see 4.1 and 2.4). After enrichment the conidospores were plated on 20 plates CMT (40 in Exp.3) and tested for deficiencies. Segregants were measured for determining ploidy.
In Exp.4 no enrichment was applied; fwnA1 segregants were isolated visually after plating of conidia directly on CMT.

grown on CM and on MM at 30°C for 4 days. Spore suspensions (about 10^7 spores) were made from both cultures and plated on CMT+arg+phe so that about 200 colonies per plate could be expected. Spores harvested from CM and from MM gave rise to fawn colonies in frequencies of 0.2% and 0.1%, respectively. The fawn segregants were isolated by transfer to CM+arg+phe, grown for 3 days at 30°C and the spore sizes were determined to assess the ploidy level. The results were: spores from fawn colonies obtained from the culture on CM: 8 haploids and 2 diploids; from MM: 5 and 1, respectively (see Table 4.9).

The diploid recombinants from Exp.1-3 were each transferred to a master plate (CM+arg+phe) and replicated on plates CM+arg+phe+benomyl. Segregants were isolated and tested for markers. In the diploids from Exp.2 and Exp.3 only the metB2-marker had to be analyzed in this way; argA1 respectively pheA1 could be scored in the diploids, since they would be present either as heterozygotes (wildtype phenotype) or as homozygous recessives (mutant phenotype). The results are presented in Table 4.10.

To assess the linear order of the three linked genes and the centromere (cen), we first turn to the assumed genotypes of the homozygous diploids (see Table 4.10).

Table 4.10 Analysis of homozygous diploid recombinants

Exp.	Diploids Number 1)	Assumed genotype 2)	Segregants obtained by haploidization Genotype	From diploid number a	b	c
1.	a,b	metB2 + +	metB2 + + :	127	148	
		metB2 argA1 pheA1	metB2 argA1 pheA1 :	51	98	
2.	a,b	metB2 argA1 pheA1	metB2 argA1 pheA1 :	28	16	
		+ argA1 pheA1	+ argA1 pheA1 :	51	48	
3.	a,b	metB2 argA1 +	+ argA1 + :	4	1	
		+ argA1 pheA1	+ argA1 pheA1 :	40	11	
			metB2 argA1 + :	22	22	
			metB2 argA1 pheA :	8	0	
	c	metB2 argA1 pheA1	metB2 argA1 pheA1 :			0
		+ argA1 pheA1	+ argA1 pheA1 :			20

1) *For numbers of diploids, see Table 4.9.*
2) Most probable genotype as deduced from the phenotype of the diploid; linear order still arbitrary.

In Exp.2 the pheA1//pheA1 diploids (2 a,b) are also homozygous for argA1 and it can be concluded that pheA and argA are on the same side of the centromere. Since in Exp.3 two out of three argA1//argA1 diploids (3 a,b) were heterozygous for pheA1 and one (3 c) was homozygous for pheA1, the simplest explanation is the linear order cen-pheA-argA. These diploids represent two cases of exchange, i.e. one between pheA and argA and one between pheA and the centromere. Haploidization confirmed the assumed genotypes of the diploids 2 a,b and 3 a,b,c (see Table 4.10).

From the metB2//metB2 diploids (1 a,b) both $argA^+$ $pheA^+$ and argA1pheA1 haploid recombinants were obtained. This can be expected from the assumed genotype, if metB is distal on the pheA-argA arm or is on the other side of the centromere.

However, haploidization of the pheA1//pheA1 diploids (2 a,b) revealed that these diploids were heterozygous for metB and consequently metB cannot be distal to pheA, in other words metB must be on the other chromosome arm.

The same conclusion follows from the two argA1//argA1 diploids (3 a,b) which were also heterozygous for metB2. The most probable linear order then is: metB-cen-pheA-argA.

From the argAlpheA1//argAlpheA1 diploid (3 c) only metB$^+$ haploids were obtained. So the diploid is probably homozygous for metB$^+$ and the presumed genotype of this diploid (see Table 4.10, 3 c) is not correct. At first sight the homozygosity for metB$^+$ may suggest that metB is distal of argA, but this possibility has been rejected. Probably this diploid results from an additional crossing-over on the other chromosome arm between metB and the centromere.

From diploid 3 a two types of haploids were obtained (viz. metB2 argA1 pheA1 and metB$^+$argA1 pheA$^+$ with frequencies 8 and 4 respectively) which do not seem to fit in the scheme. Also from diploid 3 b one haploid of the latter type was found. These rare recombinant genotypes might have resulted from a later crossing-over (see Table 4.11).

Table 4.11 Origin of rare recombinant genotypes by mitotic crossing-over.

	Genotype of diploids			Numbers of haploids
original diploid:	metB2	+	+	
	+	argA1	pheA1	
first crossing-over:	metB2	argA1	+	22
	+	argA1	pheA1	40
second crossing-over:	+	argA1	+	4
	+	argA1	pheA1	
	metB2	argA1	+	
	metB2	argA1	pheA1	8

Although a joint interpretation of all data is not easy, the linear order of the three linked markers is well established. It is obvious that frequently more than one crossing-over between two homologues can occur, although the overall frequency of mitotic crossing-over is low.

4.3.4 DISCUSSION

The frequencies of heterozygous diploids found by us showed much variation (around 10^{-6}) and is much lower than reported by Lhoas (10^{-4}). Also we often found many times (e.g. 20 times) more heterokaryons than diploids on the diploid selection plates, a phenomenon not mentioned by Lhoas (1967). Our results agree with the observations of Chang et al. (1974). The heterokaryons on the selection plates probably originate from heterokaryotic conidia which can be present at a low frequency.

With mitotic division one expects that markers located on different chromosomes segregate independently, whereas markers on homologous chromosomes show no recombination. However, due to mitotic crossing-over, recombinants can occur at low frequencies. So one expects two classes of recombination frequencies, e.g. <10%, and about 50%.

When estimating recombination frequencies a number of points should be kept in mind. Firstly, the individual recombinants are not wholly independent in origin. In the diploid small recombinant clones can occur (e.g. conidia from one chain), and occasionally even larger recombinant sectors can be present. This was the case in the first haploidization experiment described here (see Table 4.2). Secondly, with some digenic combinations both segregations are disturbed, which leads to biassed estimates of the recombination frequencies. However, from their order of magnitude one can safely make conclusions about linkage groups.

Among 11 loci studied in these experiments (Table 4.8) only three showed close linkage: argA, pheA and metB. The low percentage of recombination (1%) observed between the three linked loci can be explained by mitotic crossing over.

The genes fwnA, hisD, leuAB, and lysA are not linked to this linkage group and are mutually unlinked. So at least 5 linkage groups are present. CysA showed 21 % recombination with lysA. Further experiments should be done to see if these loci are linked or not. The remaining two genes, gryA and argE, are mutually unlinked, but otherwise insufficient information on the two genes is available.

The low percentage of recombination (by mitotic crossing-over) between the three linked loci is in agreement with the observations of Lhoas (1967). He found among haploid segregants induced on CM+p-fluorophenylalanine between the loci pab, arg and a of linkage group I 0.3 % respectively 6.3 % recombinants resulting from mitotic crossing over. In two diploids, however, he found up to 15 % recombinants between arg and a. This can be due to the small number of segregants analyzed (66 and 67, respectively). By ommitting the diploids with a high fraction of recombinants Lhoas obtained homogeneous data. He concluded that in two diploids mitotic crossing-over took place several cell generations before haploidization. This illustrates that complications can be expected in gene mapping by mitotic crossing-over when scoring is done with haploid segregants.

As mentioned above, we found 21 % recombinants between cysA and lysA (Table 4.5). In one of his analyses Lhoas found 17 % recombinants (7 out of 42) between two markers (met and his), whereas in other diploids independent segregation between these two markers was observed. He concluded that the diploid might be heterozygous for a translocation. In our strains translocations are unlikely, because we used only few rounds of mutagenic treatment and applied a low dose. Therefore, the interpretation might be that the loci are located on different chromosomes or are on the same chromosome, either far from each other or mitotic crossing-over must have occurred some cell generations before haploidization. The markers cysA and lysA will be included in further analyses.

When mitotic crossing over is studied on the basis of haploid segregants the two processes of recombination may interact. It may well be that benomyl does not have any effect on mitotic crossing-over (Wood, 1982), but it may still influence the selection relationships. Lhoas (1967) found for the loci arg and a (chromosome I) in 20 separate diploid analyses mitotic crossing over frequencies to vary from 3 - 15 %. At any rate cloning of mitotic recombinants and selection may disturb the analysis.

Upon plating of conidia from different diploid single spore colonies Lhoas (1967) found 0.4-1.4 % haploid acr-resistant

segregants. He estimated the incidence of mitotic crossing-over in a population of diploid nuclei to be at least 20%. This was based on a crossing-over frequency of 6.27% between arg and a (on chromosome I) and on the assumption that A.niger has six chromosomes. Although we too found low frequencies (1%) of mitotic crossing-over among haploid segregants, haploid spontaneous recombinants were found to be 20-30 times more frequent than homozygous diploid recombinants. For our marker fwnA1 (probably on the same chromosome as the acr marker of Lhoas) we found a somewhat lower frequency of haploids (0.1 - 0.2%). So, even if we assume a haploidization frequency of 1%, our conclusion is that the frequency of mitotic crossing-over per nucleus will be much lower than found by Lhoas. It can then be estimated to be at least about 0.3% (6 x 1/20 x 1%). Further experiments will be done to obtain a more exact estimate. As said earlier when mitotic crossing-over is studied on the basis of haploid segregants the two processes of recombination may interact and so Lhoas (1967) arrived at too high an estimate of the frequency of mitotic crossing-over. However, it should be mentioned that Lhoas used strains derived from a different wildtype isolate.

The analysis of homozygous diploids by haploidization revealed that a second crossing-over occurs more frequently than expected. This suggests that a special physiologic condition in the cells promotes mitotic crossing-over. This differential capability of cells for mitotic recombination is an interesting point for further studies for which A.niger would be a suitable object.

4.4 Conclusions

A start has been made with the establishment of a mutant collection in an isogenic background as a source of genetic markers. Up to now about 90 independent auxotrophic mutants have been isolated after induction by UV at a low dose followed by enrichment procedures. Complementation tests showed that the mutants are at least at 30 different loci.

Somatic heterozygous diploids can be obtained in the usual way (frequency about 10^{-6}), but often far more heterokaryons are obtained. Probably this is caused by a certain number of binucleate conidia.

Genetic analysis of 11 loci by haploidization showed that at least 5 different linkage groups could be distinguished. So it is quite possible that A.niger has more than the 6 chromosomes as proposed by Lhoas (1967).

The filtration enrichment procedure was used successfully to isolate recessive recombinants so we can do without specific selective markers terminal on the chromosome arms, since any auxotrophic marker can now be used for selection.

The frequency of spontaneous haploid recombinants was somewhat lower than found by Lhoas (1967), but not very much so. However, the estimated frequency of mitotic crossing-over per nucleus (0.3%) is much lower than the 20% assumed by Lhoas. The frequency of haploid segregants was 20-30 times higher than that of homozygous diploid segregants of similar phenotype.

By relating the frequency of mitotic crossing-over to the frequency of spontaneous haploid recombinants, quantitative data on the frequency of spontaneous mitotic crossing-over can be obtained and the results of different expriments become comparable. These frequencies are not dependent on the position of the selection marker which happens to be used in the experiment, so gene-maps can be made in a consistent way.

From these studies it can be concluded that these procedures for the isolation of mutants and for genetic analysis offer good perspectives to further development of the genetics of A.niger, as well as for application in breeding programs.

5. Summary

Many fungi which are important in Agriculture as plant pathogens or in Biotechnology as producers of organic acids, antibiotics or enzymes, are imperfect fungi. These fungi do not have a sexual stage, which implies that they lack a meiotic recombination mechanism.

However, many imperfect fungi have effective recombination mechanisms operating during mitotic divisions. The first step in somatic recombination is the fusion of somatic cells. In nature this occurs by hyphal fusion followed by exchange of nuclei, which results in a heterokaryotic mycelium, if the partners are genetically different. Owing to fusion of somatic nuclei (karyogamy), which occurs at a very low frequency, heterozygous diploid nuclei may then arise. Heterozygous diploid strains can be isolated and maintained by transfer of conidia. During division of somatic nuclei (mitosis) recombination of genes can occur by mitotic crossing-over and by loss of chromosomes leading to haploid recombinants.

In the laboratory heterozygous diploid strains can also be obtained via protoplast fusion.

This study concerns the biotechnologically important fungus Aspergillus niger and the phytopathogenic fungus Colletotrichum lindemuthianum (the causal organism of bean anthracnose), whereas studies on fundamental aspects or on the development of procedures were carried out with the genetically well known fungus Aspergillus nidulans. The latter has both a sexual stage and well studied processes of somatic recombination. We used it as a model for studies on mutation induction, heterokaryosis and protoplast fusion.

The chapter on induction and isolation of mutants (Chapter 2) presents studies on UV-survival curves for conidiospores (2.2), the frequency of mutants (2.3) and mutant enrichment procedures (2.4).

The interpretation of the shape of survival curves was discussed. Processes generating an initial shoulder in log-survival curves have quite different effects: A multi-hit

process leads to a much more pronounced initial shoulder than a multi-target process does. In the experimental part is was argued that the initial shoulders in logS curves of haploid uninucleate A.nidulans conidiospores (single target cells) probably are the result of an inherent repair capacity which becomes saturated at a certain UV dose. An increase in target number (number of genomes in the spore) results in an increase of the logS-intercept due to a larger shoulder. At the same time, however, the repair capacity may have increased and complementation of lethal lesions may occur. In general such increases of the logS-intercept lead to overestimation of the target number.

As in practice often high mutagen doses are applied so that mutants are isolated at low survival, the relationship between mutant frequency and survival was analyzed to see whether such high mutagen doses are necessary at all. High doses produce chromosome rearrangements and unnoticed mutations which disturb the genetic background. For several types of mutation it was shown that the highest yield of mutants is found at low mutagen dose (i.e. at about 20-50 % survival). The frequency of mutants among the survivors increases with the dose of mutagen, but levels off and even decreases at higher doses. It was also found, contrary to what is suggested in litterature, that often no simple linear relationship exists between frequency of mutants and the logarithm of the dose or of the surviving fraction. In conclusion our experiments show that mutants can be well isolated at low doses of mutagen. So taking into account the risk of disturbance of the genetic background by unnoticed mutations and chromosomal rearrangments, mutation induction should be done at a survival level of at least 70%.

To compensate for the relatively low frequency of mutants among the survivors, we require procedures for the selection of mutants or for the elimination of the non-mutant cells. The effectiveness of filtration enrichment procedures was demonstrated for different types of mutations. Next, the procedures have been applied to establish a collection of A.niger strains providing genetic markers for genetic analysis and breeding. The first results are reported in Chapter 4.

In Chapter 3 studies on different aspects of somatic recombination are presented: heterokaryosis in C.lindemuthianum, isolation of protoplasts from condidiospores, protoplast fusion and karyogamy in A.nidulans and protoplast fusion in C.lindemuthianum.

To see whether somatic recombination plays a role in the evolution of physiological races of Colletotrichum lindemuthianum the possibilities for heterokaryosis and somatic karyogamy in this fungus were studied (3.2). Although this fungus fulfils some essential requirements for such experiments, the phenomenon of cross-feeding hampered these studies. Between different strains hyphal fusion was observed, but no definite prove for heterokaryosis could be given. Somatic karyogamy could not be demonstrated.

As protoplast fusion seemed a promising alternative for inducing heterokaryosis, methods for isolation (3.3) and fusion (3.4) of conidial protoplasts were first developed with A.nidulans. It was demonstrated that by these methods also in C.lindemuthianum heterokaryons can be formed and maintained. Fusion of protoplasts from conidiospores of strains with different auxotrophic markers resulted in well growing heterokaryons. So at least heterokaryosis may play a role in the development of genetic variation of this phytopathogenic fungus.

In the fusion experiments with A.nidulans protoplasts (3.4) we found that only few protoplasts fused. The system was used to estimate the frequency of somatic karyogamy, which we found to be much higher than the frequency of heterozygous diploid conidia on a heterokaryon. These results confirm that only a small portion of a balanced heterokaryon consists of heterokaryotic hyphae. This was already indicated by an experiment in which hyphal tips were analyzed.

The conclusion is that a heterokaryon is a dynamic system based on hyphal fusions and segregation of homokaryotic hyphae of the parental types.

In Chapter 4 the start of a program for genetic analysis in A.niger is described. The first results in establishing a collection of mutants providing genetic markers are presented (4.2). Nearly a hundred different mutants are now available, located on at least 30 different genes.

Genetic analysis by haploidization revealed that the 11 loci analyzed belong to at least five linkage groups. In general haploidization was induced by benomyl, but for estimating the frequency of spontaneous haploidization we screened for spontaneous light coloured (fawn) segregants.

The isolation of (homozygous diploid or haploid) recessive recombinants was succesfully done by the enrichment procedure as used for the isolation of mutants. So, for genetic analysis and gene mapping there is no need for special terminal selection markers. In this way genetic analysis and mapping of genes on the chromosomes is possible. The process of mitotic crossing-over proved to be 20-30 less frequent than spontaneous haploidization.

Starting from a reference strain, mutant strains should be induced by as few rounds of mutagenic treatment as possible. Instead, combinations of markers have to be made by recombination. In this way well characterized master strains can be obtained in an isogenic background. These can be used for genetic analysis of other A.niger strains.

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7. Samenvatting

7.1 INLEIDING

Veel schimmels die belangrijk zijn als veroorzakers van planteziekten of in de biotechnologie als producenten van organische zuren, antibiotica of enzymen zijn imperfecte schimmels. Het zijn schimmels die geen geslachtelijk stadium hebben en juist bij de generatieve reproductie spelen zich de processen voor recombinatie van erfelijke eigenschappen af. Het lijkt er op alsof de evolutie van natuurlijke populaties van imperfecte schimmels alleen gebaseerd is op het optreden van mutaties (toevoegen of verloren gaan van bepaalde kenmerken) zonder dat er uitwisseling of recombinatie van bijvoorbeeld genen voor virulentie mogelijk is. Een dergelijk systeem is veel minder flexibel dan wanneer er wel recombinatie mogelijk is. Bij de voor de industrie belangrijke schimmels zou het betekenen dat veredeling alleen mogelijk is door herhaalde mutatie inductie en het selecteren van betere stammen. Kruising van stammen met goede kenmerken is dan niet mogelijk.

Veel imperfecte schimmels hebben echter toch effectieve recombinatie mechanismen die zich afspelen in gewone somatische cellen. De eerste stap daarin is de fusie van somatische cellen. Dat kan gebeuren door de vorming van verbindingsbruggetjes tussen schimmeldraden (hyphe-fusies of anastomoses). Dit proces kan onder laboratorium condities geforceerd worden indien beide stammen verschillende voedingsdeficienties hebben. Het resultaat is dan een mycelium met twee verschillende typen kernen (heterokaryon).

Een heterokaryon kan ook verkregen worden door fusie van protoplasten. Deze weg is interessant wanneer zich tussen schimmelstammen geen bruggetjes kunnen vormen. Een heterokaryon verkregen door protoplastenfusie heeft nòg iets voor op een gewoon heterokaryon: Een klein heterokaryon, net ontstaan door fusie van twee protoplasten, heeft van beide typen kernen evenveel; een gebalanceerd gewoon heterokaryon kan voor een groot deel bestaan uit hyfen met maar eéń type kern. Om deze twee redenen maakten wij gebruik van protoplasten fusie.

Er komen in dit onderzoek drie vragen aan de orde:

- Op welke wijze kunnen we het beste mutanten isoleren?

Dit is van belang, omdat voor alle genetisch onderzoek mutanten nodig zijn als genetische kenmerken. Het probleem is ook van belang voor de veredeling van productiestammen (Hoofdstuk 2).

- In hoeverre speelt somatische recombinatie een rol bij de imperfecte schimmel Colletotrichum lindemuthianum? (Hoofdstuk 3). Hier wordt ook beschreven hoe protoplasten geisoleerd kunnen worden uit conidiosporen en hoe deze gebruikt worden voor het aantonen van somatische karyogamie.

- Tenslotte: kunnen we een systeem opzetten voor de genetische analyse van de biotechnologisch belangrijke schimmel Aspergillus niger? (Hoofdstuk 4).

7.2 INDUCTIE EN ISOLATIE VAN MUTANTEN

De experimenten over mutatie inductie zijn grotendeels uitgevoerd met UV en met A.nidulans als object. Een van de aspecten die kenmerkend zijn voor de effectiviteit van een mutagene behandeling is de inductie van letale mutaties. Zo verschaffen overlevingscurves belangrijke informatie over de wijze waarop mutanten kunnen worden geisoleerd. Mutaties zijn in het algemeen recessief, dat wil zeggen dat ze niet tot uiting komen als het oorspronkelijke gen (wildtype gen) ook aanwezig is. Wanneer nu een conidiospore meer dan één kern heeft of ook wanneer de chromosomen in de kernen van deze cellen al verdubbeld zijn (uit twee chromatiden bestaan) zal door mutatie een heterokaryon ontstaan en selectie van recessieve mutanten zal nagenoeg niets opleveren.

Verschillende schimmels blijken een initiële schouder te vertonen in de logS UV-overlevingscurve (logarithme van de fractie overlevenden uitgezet tegen de bestralingstijd). In hoofdstuk 2.2 worden enkele theoretische achtergronden van het optreden van initiële schouders in de logS-curves besproken. Er is aangetoond dat dit bij haploide A.nidulans stammen in de regel een gevolg is van een mechanisme dat zorgt voor herstel

van UV-schade. Dit bijzondere 'repair'-mechanisme is bij een bepaalde dosis verzadigd en pas daarna is het effect van UV op de overleving te zien. Sommige stammen hebben diploide of meerkernige conidiosporen. Dat veroorzaakt een extra grote schouder in de logS-curve. De toename is echter sterker dan op grond van het grotere 'target-number' (bv aantal kernen per cel) verwacht mag worden. Dat komt omdat ook de'repair'-capaciteit toeneemt.

In de mutatieveredeling worden bij mutagene behandelingen vaak hoge doses toegepast. Een voordeel is dat veel van de overlevende sporen wel een of andere mutatie hebben opgelopen. Het nadeel is echter dat er dan nogal wat additionele ongewenste mutaties onopgemerkt blijven en bovendien treden er chromosoombreuken en uitwisselingen op. Hoewel de gewenste mutanten er door selectie wel uitgezocht kunnen worden, wordt op deze wijze de genetische achtergrond een vergaarbak van allerlei afwijkingen. In een serie experimenten (2.3) waarin naar verschillende typen mutaties werd gekeken, is aangetoond dat al bij relatief lage doses het hoogste aantal mutanten wordt verkregen, al is de frewkentie onder de overlevenden dan niet het meest gunstig. Bij hoge doses neemt trouwens de frekwentie van de mutanten onder de overlevenden af.

Om mutanten te isoleren uit een monster met betrekkelijk veel niet gemuteerde sporen van het oorspronkelijke type kunnen verrijkingstechnieken worden toegepast (2.4). Een effectieve methode is: het laten kiemen van de sporen in een medium waarin alleen de oorspronkelijke sporen kunnen kiemen en de gewenste mutanten niet. De gekiemde sporen worden na enige tijd afgefiltreerd door een wattenprop en daarna worden de dan nog aanwezige niet gekiemde sporen uitgezaaid op een compleet medium. Onder de dan nog groeiende kolonies wordt vervolgens gezocht naar mutanten. Enkele condities voor een dergelijke verrijkingsprocedure zijn bestudeerd, waarbij duidelijk is geworden dat deze methode heel effectief is indien ze aangepast wordt aan het gewenste type mutanten.

7.3 SOMATISCHE RECOMBINATIE

In eerste instantie (3.2) is onderzocht of heterokaryose en somatische karyogamie een rol spelen bij de ontwikkeling van de genetische variatie in de fytopathogene schimmel Colletotrichum lindemuthianum (veroorzaker van bruine vlekken op slabonen). Hoewel daarvoor onder meer door de waarneming van bruggetjes tussen verschillende schimmeldraden wel aanwijzingen werden verkregen, was er geen sluitend bewijs. Waarschijnlijk speelde het optreden van 'cross-feeding' (het elkaar voorzien van noodzakelijke voedingsstoffen via het medium) hierbij parten. Daarom zijn er later (3.5) met behulp van protoplastenfusie experimenten gedaan die met zekerheid aangetoond hebben dat bij deze schimmel heterokaryons kunnen ontstaan.

Er werden procedures voor de isolatie van protoplasten uit condidiosporen ontwikkeld (3.3). Er was reeds bekend dat protoplasten uit mycelium verkregen konden worden. Deze protoplasten zijn echter zeer heterogeen. Protoplasten uit conidiosporen zijn fysiologisch homogeen en ze bevatten maar een of twee kernen. Ze bleken dan ook zeer geschikt voor kwantitatieve genetische experimenten. Met behulp van deze protoplasten is aangetoond dat somatische karyogamie bij A.nidulans met veel hogere frekwentie optreedt (minstens 100x) dan gesuggereerd wordt door de frekwentie van heterozygoot diploide sporen op een heterokaryon (3.4).

Deze protoplasten zijn ook geschikt voor transformatie (opname van DNA) en zijn in elders beschreven experimenten gebruikt voor de ontwikkeling van een gastheer-vector systeem ten behoeve van genetische manipulatie met recombinant DNA. Binnen onze sectie worden de protoplasten gebruikt voor de hybridisatie van A.niger stammen die van nature geen heterokaryons vormen.

7.4 PERSPECTIEVEN VOOR GENETISCHE ANALYSE VAN ASPERGILLUS NIGER

Sinds enkele jaren concentreert het schimmel genetisch onderzoek zich op A.niger, een schimmel die van belang is in de biotechnologie. De bedoeling daarvan is om uitgaande van een bepaalde wildtype stam een collectie op te bouwen van stammen met geschikte kenmerken voor genetische analyses. Deze wildtype

stam dient daarbij tevens als referentie voor de analyse van stammen met een andere herkomst. Ook worden test-stammen geconstrueerd waarmee genetische analyses uitgevoerd kunnen worden. Daarnaast wordt in het kader van toegepaste projecten veredelingsonderzoek uitgevoerd.

In dit hoofdstuk (4.2) wordt duidelijk dat de isolatie van mutanten op basis van de in hoofdstuk 2 beschreven methoden goede resultaten oplevert. Er zijn nu een kleine honderd mutanten van in totaal tenminste dertig verschillende genen. In dit hoofdstuk (4.3) is ook beschreven de aanzet tot genetische analyse op basis van somatische recombinatie. Een dergelijk onderzoek was reeds eerder (1952, 1967) in Engeland begonnen maar niet voortgezet. Er zijn echter goede perspectieven, mede doordat verrijkingstechnieken voor de isolatie van mutanten ook toepasbaar bleken voor de isolatie van homozygoot diploide recombinanten. Hierdoor zijn we niet afhankelijk van zeer specifieke voor selectie geschikte genen. De lokalisatie van de genen op de chromosomen is uitgewerkt aan de hand van enkele beschikbare gekoppelde genen. Dit type onderzoek zal in de toekomst veel aandacht krijgen.

Aan het in dit proefschrift beschreven onderzoek hebben veel studenten en stagiaires bijgedragen. Vaak waren het experimenten met een duidelijk explorerend karakter, waardoor zij zelf geen grote oogst aan resultaten konden binnenhalen, omdat voor hen het 'leerdoel' voorop stond. Het bood ons vaak wel de mogelijkheid om met enige aanvullende experimenten een aantal onderdelen af te ronden.

Curriculum vitae

De auteur van dit proefschrift werd op 21 juni 1936 te Westeremdem geboren. Na het behalen van het diploma MULO B (1953), een jaar praktijk op een landbouwbedrijf, het behalen van het diploma van de Rijks Middelbare Landbouwschool (nu HLS) te Groningen (1957) en een jaar militaire dienst werd in 1958 de studie aan de Landbouwhogeschool begonnen.

In 1964 studeerde hij met lof af aan de Landbouwhogeschool, studierichting Plantenziektenkunde, met als verzwaard hoofdvak de Fytopathologie en als bijvakken de Biochemie en de Virologie. De praktijkstage werd doorgebracht in het Institut für Spezielle Botanik van de Eidgenossische Technische Hochschule in Zürich, terwijl een deel van het hoofdvak werd gedaan op het Genetisch Instituut van de Rijksuniversiteit van Utrecht.

Sinds 1964 is hij werkzaam bij de vakgroep Erfelijkheidsleer van de Landbouwhogeschool als wetenschappelijkmedewerker, sinds 1972 als wetenschappelijk hoofdmedewerker, voor het onderwijs en het onderzoek in de Microbiele Genetica. Het onderzoek betreft in het bijzonder mutagenese, somatische recombinatie en genetische manipiulatie bij Aspergillus. Het richt zich vooral op uiteindelijke toepassingen bij de veredeling van industriëel belangrijke stammen.

Naast het werk binnen de vakgroep zijn er andere activiteiten zoals voor het HBO, met name voor het laboratorium onderwijs: eerst als docent Genetica, later als cursusleider van de botanische avondopleiding in Wageningen en nu nog als lid van een adviescommissie voor het laboratorium onderwijs (CALPA). Verder is hij o.m. actief betrokken geweest bij de studierichting biologie bijvoorbeeld als studiecoördinator, bij de herprogrammering en momenteel als secretaris van de richtingsonderwijscommissie Biologie. Hij is lid van de Vaste Commissie Studiebegeleiding en tevens voorzitter van de Coördinatiecommissie Studievoorlichting.