Chromosomal Studies in Higher Fungi: Challenges for Cytological Investigation.

Dr. Dipan Adhikari

ASSISTANT PROFESSOR

PG DEPT OF BOTANY, PLANT CELL AND MOLECULAR BIOLOGY LABORATORY, HOOGHLY MOHSIN COLLEGE, CHINSURAH, HOOGHLY-712101, WEST BENGAL, INDIA.

Introduction

The cytological investigations and analysis of fungi remains as an unexplored area in mycological research. Owing to its minuscule morphology and very short period of division, in addition to partial penetration of present stains as a result of retention of nuclear envelope during division, fungal chromosome remained as very unpopular subject of scientific interest. Over the years numerous attempts have been made to elucidate the finer details of the nuclear as well as its divisional stages during vegetative filaments mainly from the meiospores. Over the years, we can come across few well known techniques like mapping the gene of interest with linkage groups by recombinational analysis, light microscopic observations of meiotic chromosomes, electron microscopic observations of synaptonemal complexes of homologus chromosomes, and separation of individual chromosomes as bands in pulse field gel electrophoresis. But presently, a good number of high throughput investigational devices are also available which can be easily accomplished to get the inner findings done. The rapidly developing, large-scale use of electron microscopy today has made the study of these "critical minutes" lucid. Owing to which the whole structural cytology has entered a new era, so impressively, that the intricate characters of a cell are primarily interpreted as an electron image. From the early 1980's the wide use of pulse field gel electrophoresis (PFGE) has yielded fascination results about the occurrence of intraspecific chromosome polymorphisms, which is a very common phenomenon in fungal chromosomes. These intraspecific polymorphs are somewhat interpreted as a cytological marker of chromosomal variation in size and number between strains. Except these methods, PFGE coupled with restriction fragment length polymorphism genetic linkage analyses are widely employed to elucidate the inconsistencies occurred through light microscopy. Analysis of the ultrastructure of chromosome and chromatin materials has not only been fascinating because of its novelty but also can highlight on the chromatin compaction into condensed chromosomes during nuclear division and migration in vegetative filaments. Presently, all these highthroughput devices are being exploited to expose the finer nuances of fungal chromosomes.

Key words: Higher Fungi, cytology, propionic orcein staining, PFGE, SEM, EM.

Introduction:

Fungal chromosomes are too small and very difficult to observe under compound microscope; hence there are few reports available of successful examination of them compared with reports on plant and animal chromosomes. Furthermore, chromosomes usually differ little in size, so it is difficult to distinguish between them to prepare for karyotyping. In filamentous fungi also, mitotic chromosomes have rarely been studied. Such studies have been hampered by several obstacles such as the small size of the mitotic chromosomes in comparison with meiotic chromosomes, the asynchronous movement of chromatids during nuclear division and the lack of proper techniques for the preparation of chromosome spreads. Consequently, information concerning the morphological nature of chromosomes in filamentous fungi is heavily biased toward meiotic chromosomes, in marked contrast to the situation in higher plants and animals. (Taga and Murata 1994). Although mitotic chromosomes of filamentous fungi are more difficult to observer than the meiotic chromosomes, mitotic observations are necessary in some cases. For example, sexual stages are when unknown or difficult to form in laboratory in a considerable number of species including many important plant pathogenic fungi.

Not many fungal species have been examined cytogenetically to date. They are mainly Ascomycotina. There is shortage of data concerning Basidiomycotina (especially wild agaricales) and Zygomycotina. The results of most chromosome visualizations by different authors are summed in **Table 1**. Some of the authors have stated that it has been generally easier to observe meiotic chromosomes than those from mitosis. Mitotic chromosomes are usually smaller in comparison to the meiotic ones. Furthermore, usually a well-defined "metaphase plate" remains absent in addition to the disjunction of sister chromatids, which is asynchronous (Aist and Morris, 1999). Here mitotic observations are necessary in some cases, for example, when a given fungus does not undergo sexual processes or does not perform them in laboratory conditions. The use of two different methods examining the same species:

conventional light microscopy and GTBM (germ tube burst method) reveals that results might not coincide. In some cases counts of chromosomes in asci, by conventional light microscopy, leads to underestimation of chromosome number comparing with data of PFGE (Pulse field Gel Electrophoresis) or GTBM; for example in *N. haematococca* (Taga et al., 1998) or *Cochliobolus* (Tsuchiya and Taga, 2001). This suggests that re-examination of data from past cytological studies is needed.

Cytogenetic studies of chromosomes during mitosis or meiosis leads to the clarification of the number of chromosomes and their behavior during cell division. They also reveal peculiarities of chromosome structure. Knowing these features, adding data from other molecular tools, gives a full and complex overview of any fungus being studied, at three conceptual levels viz: genome, chromosome and DNA.

The estimated number of chromosomes in fungal species by microscopy visualization

Table 1

Species	Number of chromosomes (n)	Reference
Ascomycotina:		
Aspergillus nidulans	8	Robinow and Caten (1969)
Cochliobolus heterostrophus	8	Guzman *et al*. (1982)
Cochliobolusheterostrophus	15 to 16	Tsuchiya and Taga (2001)
Cochliobolus carbonum	13 to 15	Tsuchiya and Taga (2001)
Cochliobolus sativus	6 to 8	Huang and Tinline (1974)
Cochliobolus sativus	15	Tsuchiya and Taga (2001)
Erysiphe graminis f.sp. *hordei*	7 to 8	Borbye *et al*. (1992)
Erysiphe pisi	6	Singh *et al*. (1984)
Fusarium graminearum	4	Gale *et al*. (2005)
Nectria haematococca	12 in homothallic	Taga *et al*. (1998)
	15–17 in heterothallic	Taga *et al*. (1998)
Neurospora crassa	7	Singleton (1953)
Penicillium chrysogenum	5 or 6	Crackower and Bauer (1970)
Penicillium notatum	5 or 6	Crackower and Bauer (1970)
Tuber aestivum	5 to 6	Poma *et al*. (1998)
Basidiomycotina		
Agaricus bisporus	9	Lemke et al. (1975)
Gomphidius rutilus	8	Duncan and Galbraith (1973)
Deuteromycotina:		
Alternaria alternate	10	Taga and Murata (1994)
Botrytis alli	16	Shirane *et al*. (1989)
Botrytis byssoidea	16	Shirane *et al*. (1989)
Botrytis cinerea	16	Shirane *et al*. (1989)
Botrytis squamosa	16	Shirane *et al*. (1989)
Botrytis tulipae	16	Shirane *et al*. (1989)
Cladosporiumherbarum	4	Crackower (1972)

After Wioletta Wieloch, 2006.

Various nuclear stains are available, but only a limited number of them are useful in elucidation of fungal nuclear staining. (Burpee *et al*, 1978; Herr 1979; Mogford 1979; Tu and Kmibrough 1973). The available stains and techniques often do not give the necessary results for nuclear studies in fungi and also are useful only in particular isolates, species, or groups of fungi (Kangatharalingam and Ferguson 1984). This may be due to the fact that fungal nuclei have a very poor affinity for most ordinary stains (Mogford 1979). The wall materials also may have varying degree of affinity to these stains, thus interfering with the clarity of the stained nuclei.

In nuclear studies of fungi, the location of nuclei in cells, number of nuclei per cell, size of nuclei, and their behavior during different phases of growth and development are important. To study these details it is necessary to develop techniques to stain nuclei with maximum clarity, rapidity and simplicity.

Various methods have already been established to study fungal chromosomes by light microscopy based on typical dyes like Giemsa or aceto-orceine, as well as by fluorescent dyes (DAPI).

In the Ascomycetous fungus *Tuber aestivum* chromosome number was determined in spores and hyphae (Poma *et al*, 1998). The chromosomes were observed and measured in metaphases of haploid ascospores and hyphal dikaryotic or diploid metaphases, by acetoorcein staining. Treatment, including cell wall dissolving enzymes were used prior to squashing and staining and this was found to improve the results. Five chromosomes (or six in some cases) were observed in cells at metaphase during mitoses of haploid nuclei in spores; medium chromosome size had been found to be approximately 0.95 μm. All chromosomes were round-to-oval in shape with no obvious demarcations.

Another ascomycetean fungus *Erysiphe graminis* f. sp. *hordei* had been examined by Borbye *et al*. (1992). In this case a protocol incorporated simple acetoorcein staining which was found not sufficient to make the chromosomes of this fungus visible. Treatments including the use of colchicine 0.05% w/v, dimethylsulphoxide, hydrolamine hydrochloride and iron alum, prior to orcein staining, were found to improve the results. Seven to eight chromosomes could be well detected in cells at somatic metaphase establishing a lower number of n=7. In contrast to *T. aestivum*, chromosomes of *E. graminis* differed in size and shape. It was noticed that all metaphase cells were having a prominent nucleolus; thus the persistence of the nucleolus prevented a more exact assessment of the size of the nucleolus-forming chromosome. The other chromosomes could be assigned to at least three groups according to their size. The three largest chromosomes were triangular in size of 1.2×0.8 μm (approx). Two medium-sized chromosomes, approximately 0.8×0.3 μm, were rod-shaped. The two smallest chromosomes (0.4 μm) were circular. It was also observed that two of the largest chromosomes were often connected forming a butterfly-shaped body; they may be components of one very large chromosome. The results obtained from field inversion gel electrophoresis seemed to confirm these findings.

The other Erysiphe species that has been analysed cytogenetically was *E. pisi*, has n=6. The chromosomes were easily stained with aceto-orceine without any prior treatment (Singh *et al*, 1984).

Study of mitosis in the ubiquitous mould *Cladosporium herbarum* by Crackower (1972) put forth interesting results. He stained the chromosomes with aceto-orcein which revealed small dot-like, but countable, chromosomes; eight at the stage of prometaphase.

Similar work examining mitosis in *Aspergillus nidulans* by Robinow and Caten (1969) had revealed 16 chromosomes in diploid cells and eight in haploid cells. They were more filamentous in shape.

Analysis of mitosis in *Penicillium chrysogeum* and *Penicillium notatum* (Crackower and Bauer, 1970) depicted approximately five or six chromosomes in both the species. Fluorescent dyes to stain fungal nuclei and chromosomes were tested by Lemke *et al* (1975). Auramin-O proved to be of general use.

Nine pairs of chromosomes at pachytene stage had been observed in *Agaricus bisporus*. Good chromosome visualization had been obtained also with the use of embedded and sectioned material (Duncan and Galbraith, 1973) prior to Giemsa staining. Analysis, by this method, of the pachytene stage of meiosis in *Gomphidius rutilus*, revealed the presence of eight bivalents.

Shirane *et al*, (1989) had established a method to spread mitotic chromosomes of *Botrytis* called the germ tube burst method (GTBM). In this, growing tips of either conidial germ tubes or young hyphae adhering to a glass slide were made to burst by treating them with methanol–acetic acid solution. Growing tips of hyphae usually contain several dividing nuclei and, upon bursting, condensed chromosomes are usually discharged from the hyphal cells to spread on the surface of the slide. Materials prepared in this way were subjected to stain with Giemsa. Following this protocol, five species had been examined: *B. byssoidea*, *B. cinerea*, *B. squamosa*, *B. tulipae* and *B. alli*. The chromosome number was found to be 16 in the first four species. Out of seven strains of *B. alli*, three isolates had 32 chromosomes and the other four had 16. The measured volume of conidia of *B. alli* was correlated with the chromosome number. They suggested that the isolates with 16 chromosomes might have been the haploids and those with 32 chromosomes could have been the diploids. It was also observed that all *Botrytis* species examined had one of the chromosomes with a thread-like structure of unknown identity.

A few years later, using the germ tube burst method, mitotic chromosomes of *B. cinerea* and *Alternaria alternata* had been examined by Taga and Murata (1994). Chromosomes were stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI). The authors found that the ratio of methanol to acetic acid solution in the fixative had a crucial role to play for making good quality specimens. A higher methanol proportion tended to increase the frequency of burst germlings, but damaged chromosomes to a greater degree that with treatments with less methanol. The genome of *A. alternata* has been thought to be composed of ten chromosomes; two of them could be easily distinguishable owing to their curved shape and larger size, more than 2 μm in lengths versus 1 μm for the smallest chromosomes.

In the case of *B. cinerea*, counts of chromosome number per genome had been found to be inconsistent Although 16 chromosomes were often counted, numbers other than 16 were also observed in some preparations. In all cases variation in chromosomal size in a genome was obvious; for example, the largest chromosome was more than twice as long as the smallest one. Some of the chromosomes of both fungi had a centromeric-like constriction. The authors also, were the first to successfully apply ‘fluorescence in situ hybridisation’ (FISH) to the chromosomes of filamentous fungi (Taga and Murata, 1994; Taga et al., 2003). This revealed

one ribosomal RNA gene cluster in the genome of each fungus. By this method, in *B. cinerea*, a long attenuated chromatid thread (the "threadlike structure") was reported by Shirane *et al*, (1988), which extended from a condensed metaphase chromosome, was proved to correspond exactly to the location of nucleolus organizer region (NOR). Thread like structures were also observed in other species examined. The procedure of GTBM was applied to the ascomycetous fungus *Cochliobolus*. This revealed the strain differences in chromosome number per nucleus—15 or 16 in *C. heterostrophus*, 13 to 15 in *C. carbonum* and 15 in *C. sativus* (Tsuchiya and Taga, 2001).

Studies employed in fungal cytology

1. *Simple staining by Propionic Orcein*: Simple propionic orcein staining (2% orcein in 45% propionic acid has been employed by the present author in the laboratory to evaluate the vegetative mycelium in *Lentinus squarrulosus* (after fixing in Newcomer's Solution (Isopropyl alchol: Propionic acid:Petroleum ether: Acetone= 6:2:1:1) (Adhikari et al, 2018) . In this observation the author could establish the different stages of vegetative nuclei in different parts of the fruiting body. In the present study the karyokinesis in the developing basidium of straw mushroom *Volvoriella volvacea* (Bull ex. Fr.) Sing., has been tried and clear nuclear division could be well elucidated. The basic development of young basidium and basidiospore could be established nicely with propionic orcein staining.

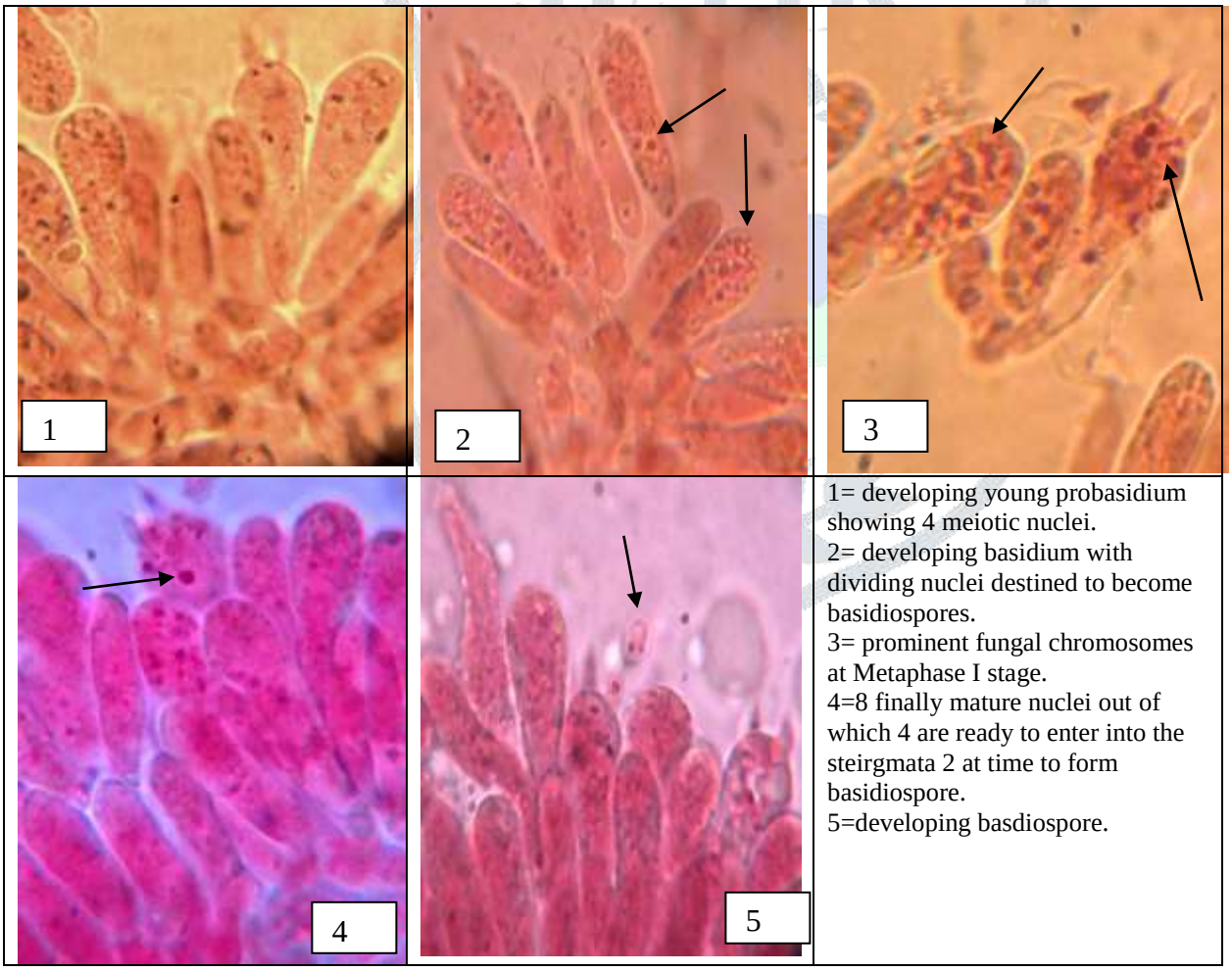

Fig 1: elucidation of Basidial Cytology in *Volvoriella volvacea* (Bull ex. Fr.) with propionic orcein taing

2. *Scanning electron microscopy (SEM)*

One example of an attempt to examine fungal chromosomes by SEM is the recent work of Tsuchiya *et al*, (2004). The three-dimensional ultrastructure of nuclei and mitotic chromosomes of *Cochliobolus heterostrophus* and *Neurospora crassa* had been visualized showing the irregular 'knobbly' surface of chromosomes, similar to those of mammals or higher plants.

3. *Pulse field gel electrophoresis (PFGE)*

The application of pulse field gel electrophoresis (PFGE) to study fungal chromosomes (Mills and McCluskey, 1990) has greatly contributed to the knowledge of chromosome number in fungi. The advantage is applicability to various fungi irrespective of sexual stage, and the ability to visualise minute chromosomes and differences in size between chromosomes.

PFGE in fungi revealed that not only different isolates of the same species frequently have chromosomes that differ in size, but they may differ in number. These additional chromosomes are called supernumerary, conditionally dispensable, B or minichromosomes. The term "supernumerary chromosome" seems to be the most descriptive and is recommended for general use in fungal genetics (Covert, 1998). The experimental criterion for their identification has been to visualize their size on the gel, in addition to the absence of sequences, characteristic of such chromosomes, in at least one other, normally growing in the isolates of the very species. This problem seems to be checked by DNA–DNA hybridization technique. It might happen that sequences on supernumerary chromosome present in one isolate got attached or had translocated to another chromosome in the genome. The supernumerary chromosomes are usually inherited less frequently otherwise predicted by Mendelian genetics (Orbach et al., 1996), at least in species that undergo sexual reproduction. However, many filamentous fungi do not reproduce sexually, so it is impossible to determine if their extra chromosomes are inherited in a Mendelian or non-Mendelian fashion. Some of the supernumerary chromosomes carry functional genes, like antibiotic resistance (Miao *et al*, 1991) or ability to cause disease on host plant in *Nectria haematococca* (Wasmann and VanEtten, 1996).

A supernumerary chromosome had been visualized cytogenetically by in situ hybridisation in *N. haematococca* (Taga *et al*, 1999). The morphology of this additional chromosome was similar in appearance to the other chromosomes at metaphase and no peculiarities were evident when stained with DAPI.

There are some limitations of this technique however. Only fragments up to 10 Mbp can be resolved on the gel, and there is still uncertainty if the fragments on the gel represent intact chromosomes, unless other methods are involved. It might also happen that one band is a cluster of chromosomes of the same size and the efforts to separate them are unsuccessful. PFGE does not reveal any morphological characteristic of chromosomes other than their size expressed in Mbp.

4. Fluorescence Staining coupled with Fluorescence in situ hybridization (FISH):

The mitotic chromosomes of the platn pathogenic filamentous fungi *Botrytis cinerea* and *Alternaria alternate* had been examined. The chromosomes were prepared by the germ tube burst method and were stained with the fluorescent dye DAPI which yielded figures with good resolutions. (Taga and Murata 1994). The results successfully depicted the chromosome number and proper visualization of chromosomes. The reporters also could employ fluorescence in situ hybridization (FISH) to reveal one ribosomal RNA gene cluster or nucleolus organizer region (NOR). The pictures revealed a long attenuated chromatid thread expanding from a condensed metaphase chromosome which had been named "a thread-like structure" in B. cinerea and had been proved to be the NOR. This was the very first one of its kind to employ FISH successfully in revealing the chromosomes of filamentous fungi.
Recently Taga *et al*, (1999) had also visualized a conditionally dispensable chromosome in the filamentous Ascomycetean fungi *Nectria haematococca* by fluorescence in situ hybridization.

In 2003 Taga *et al*, had reported the dynamic changes of rDNA condensation in state during mitosis in filamentous fungi revealed by fluorescence in situ hybridisation.

Agents that arrest nuclear division in metaphase

In many organisms arresting nuclear division at metaphase is usually employed to increase the frequency of metaphase nuclei. The most widely used agents include colchicine and colcemid. These two chemicals have been reported as relatively ineffective in fungi (Heath, 1978), although colchicine in the concentration 0.01% and 0.05% had been in practice in some of the previously mentioned studies (Borbye *et al*, 1992; Poma *et al*, 1998). Colchicine was found to be ineffective in mitotic segregation assays with *Aspergillus nidulans* (Crebelli *et al*, 1991) as well as in yeast cells (Albertini, 1990). Cooling on ice to arrest mitotic events at metaphase had no effect on germling cells of *B. cinerea* (Taga and Murata, 1994). Thiabendazole treatment was effective in arresting nuclear division at metaphase in *N. crassa* (Tsuchiya *et al*, 2004), but not in *Cochliobolus* (Tsuchiya and Taga, 2001). Another way to increase the metaphase frequency is by using hydroxyurea. This agent arrests the cell cycle of germ tube cells at the entry of S phase (DNA synthesis inhibitor) and, when washed out, allows synchronous nuclear division in the germ tube (Tsuchiya and Taga, 2001; Tsuchiya *et al*, 2004).

So from the above exhaustive literature search we have come to know that although different staining protocols have been attempted for the elucidation of the karyological characteristics of different fungal species, till date a very few species have been subjected to proper experimentation. Different hardships have been encountered while these studies employed in staining fungal cell. The ideal solution would be to combine cytological studies (with simple staining, DAPI and Giemsa staining) and PFGE in karyotyping fungi, as in the cases of *N. crassa* (Orbach *et al*, 1998), *E. graminis* (Borbye *et al*, 1992), *N. haemotococca* (Taga *et al*, 1998), and *C. heterostrophus* (Tsuchiya and Taga, 2001). Sometimes PFGE is not useful when chromosomes are large and similar in size, as in *Fusarium graminearum*. In this case cytological observation of four chromosomes is confirmed by a molecular method like genetic mapping (Gale *et al*, 2005). The advantage of molecular tools, widely used nowadays, like amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) or rDNA sequencing (Soll, 2000) is that they can be conducted on DNA without any (or with little) knowledge about the structure of fungal genomes.

Conclusion:

Cytogenetic studies of chromosomes during mitosis or meiosis leads to the clarification of the number of chromosomes and their behavior during cell division. They also reveal peculiarities of chromosome structure. Knowing these features together with data from other molecular tools, can give a clear picture of a full and complex overview of any fungus being studied, at three conceptual levels viz: genome, chromosome and DNA.

Funding Support:
This work was supported by the University Grants Commission (*UGC*) in the form of Minor Research Project [F.PSW-70/12-13(ERO)] to Dr. Dipan Adhikari.

References:

Adhikari, D. and Ghosh R. Inter. Jr. High. Edn. Res. 2018. 8(2): 168-179.

Aist, J.R., Morris, N.R., 1999. Fungal Genet. Biol. 27, 1–25.

Albertini, S., 1990.M. Mutagenesis 5, 453–459.

Borbye, L., Linde-Laursen, I., Christiansen, S.K., Giese, H., 1992. Mycol. Res. 96 (2), 97–102.

Burpee, L. L., Sander, P. L., Cole Jr. H., Kim, S. H. 1978. Mycologia 70:1281-1283.

Crackower, S.H.B., 1972. Mitosis in Cladosporium herbarum. Can. J. Microbiol. 18, 692–694.

Crackower, S., Bauer, H., 1970. Can. J. Microbiol. 17, 605–608.

Crebelli, R., Conti, G., Conti, L., Carere, A., 1991. Mutagenesis 6, 131–136.

Covert, S.F., 1998. Curr. Genet. 33, 311–319.

Duncan, E.G., Galbraith, M., 1973. Stain Technol. 48, 107–110.

Gale, L.R., Bryant, J.D., Calvo, S., Giese, H., Katan, T., O'Donnell, K., Suga, H., Taga, M., Usgaard, T.R., Ward, T.J., Kistler, H.C.,2005. Genetics 171, 985–1001.

Guzman, D., Garber, N.C., Yoder, O.C., 1982. Can. J. Bot. 60, 1138–1141.

Heath, I.B., 1978. Int. Rev. Cyt. 64, 1–80.

Herr, L. J. 1979. Phytopathology 69:958-961.

Huang, H.C., Tinline, R.D., 1974. Can. J. Bot. 52, 1561–1568.

Kangatharalingam N and Ferguson M. W. 1984, Current Microbiology, 10: 99-104.

Lemke, P.A., Ellison, J.R., Marino, R., Morimoto, B., Arons, E., Kohman, P., 1975. Exp. Cell Res. 96, 367–373.

Miao, V.P., Covert, S.F., VanEtten, H.D., 1991. Science 254, 1773–1776.

Mills, D., McCluskey, K., 1990. Mol. Plant-Microb. Interact. 3, 351–357.

Mogford, D. J. 1979. Journal of South African Botany. 45:263-265.

Orbach, M.J., Chumley, F.G., Valent, B., 1996. Mol. Plant-Microb. Interact. 9, 261–271.

Orbach, M.J., Vollrath, D., Davis, R.W., Yanofsky, C., 1998. Mol. Cell. Biol. 8, 1469–1473.
Plamman M, 1996. *J.Genet.* 75(3), 351-360.

Poma, A., Pacioni, G., Ranalli, R., Miranda, M., 1998. FEMS Microbiol. Lett. 167, 101–105.

Robinow, C.F., Caten, C.E., 1969. J. Cell Sci. 5, 403–431.

Shirane, N., Masuko, M., Hayashi, Y., 1988. Phytopathology 78, 1627–1630.

Shirane, N., Masuko, M., Hayashi, Y., 1989. Phytopathology 79, 728–730.

Singh, U.P., Singh, H.B., Sakai, A., 1984. Trans. Br. Mycol. Soc. 83, 481–485.

Singleton, J.R., 1953. Am. J. Bot. 40, 124–144.

Soll, D.R., 2000. Clin. Microbiol. Rev. 13, 332–370.

Taga, M., Murata, M., 1994. Chromosoma 103, 408–413.

Taga, M., Murata, M., Saito, H., 1998. Mycol. Res. 102, 1355–1364.

Taga, M., Murata, M., VanEtten, H.D., 1999. Fungal Genet. Biol. 26, 169–177.

Taga, M., Tsuchiya, D., Murata, M., 2003. Mycol. Res. 107, 1012–1020.

Tsuchiya, D., Taga, M., 2001. Phytopathology 91,354–360.

Tsuchiya, D., Koga, H., Taga, M., 2004.Mycologia 96, 208–210.

Tu, C. C., Kimbrough, J. W. 1973. Mycologia 65:941-944.

Wasmann, C.C., VanEtten, H.D., 1996. . Mol. Plant-Microb. Interact. 9, 793–803.

Wioletta Wieloch, 2006. http://dx.doi.org/10.1016/j.mimet.2006.05.022.