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Fungal Biology Reviews

Review

# Six decades of *Neurospora* ascus biology at Stanford☆

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*Keywords:*
Ascospore development
Ascus biology
*Cochliobolus heterostrophus*
*Coniochaeta tetraspora*
Meiosis
Meiotic drive
Meiotic silencing
*Neurospora crassa*
*Neurospora tetrasperma*
Spore killers

ABSTRACT

Ascus is the largest cell in the entire life cycle of *Neurospora*; it is where the transient diploid nucleus undergoes meiosis and a postmeiotic mitosis. The eight haploid nuclei are then sequestered into eight linearly ordered ascospores. Dodge's pioneering work on *Neurospora* and its simple nutritional requirements inspired Beadle and Tatum of Stanford University to use *N. crassa* for their landmark demonstration that individual genes specify enzymes. McClintock visited Stanford in 1944, and showed that meiosis and chromosome behaviour in *Neurospora* are similar to those of higher eukaryotes. Most of the subsequent *Neurospora* ascus biology work was carried out in David Perkins' laboratory at Stanford from 1960–2007. Since 1974, I have extensively used an iron-haematoxylin staining procedure, the DNA-specific fluorochrome acriflavine, and GFP-tagged genes for visualizing meiotic chromosome behaviour and gene silencing during ascus and ascospore development. Our recent discovery of meiotic silencing, and the availability of genome sequence and GFP-tagged genes will no doubt pave the way for molecular analysis of complex processes during ascus development.



## 1. Historical background

B.O. Dodge discovered the sexual cycle and mating types in a *Monilia* fungus, and named the genus *Neurospora* because of the characteristic ascospore ornamentation (Shear & Dodge 1927). He showed that the linearly ordered ascospore pairs in the elongated asci reflect the underlying genetic events during meiosis, and enthusiastically advocated *Neurospora* for genetic research. It was Dodge's work on *Neurospora* and its simple nutritional requirements that inspired George Beadle and Edward Tatum of Stanford University to use *N. crassa* for their landmark demonstration that individual genes specify enzymes that carry out biochemical reactions in the cell (later known as the 'one gene-one enzyme' or 'one gene-one polypeptide' hypothesis). At Beadle's invitation, Barbara McClintock visited Stanford in 1944 and applied Belling's aceto-orcein squash method for meiotic chromosome studies in *Neurospora* (McClintock 1945). Singleton (1953) extended McClintock's studies, and showed that meiosis and chromosome behaviour in *Neurospora* are very similar to that of higher plants and animals. E.G. Barry has subsequently used the aceto-orcein method for analysing numerous chromosome rearrangements (see Perkins 1992, 1997 for references), and Lu (1993) has successfully spread synaptonemal complexes of *Neurospora*. Most of Lu's and Barry's pachytene chromosome observations, and all of my *Neurospora* ascus studies have been carried out in David Perkins' laboratory at Stanford. Since 1974, I have extensively used an iron-haematoxylin staining procedure, which stains chromosomes, nucleoli, spindles, spindle pole bodies (SPBs), and ascus apical pores very well (Raju 1980; Raju & Newmeyer 1977). The DNA-specific fluorochrome acriflavine has also been used for detailed meiotic chromosome analysis (Raju 1986a; Perkins *et al.* 1995). More recent work has employed GFP-tagged genes for visualizing meiotic

☆ This article is dedicated to the memory of David D. Perkins (1919–2007).

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1749-4613/$ – see front matter 
doi:10.1016/j.fbr.2008.03.003

chromosomes, and meiotic gene silencing and its suppression (Freitag *et al.* 2004; Jacobson *et al.* 2008; Raju *et al.* 2007; Shiu *et al.* 2001, 2006; Zickler 2006). Several well-documented reviews on *Neurospora* sexual biology are available (Raju 1980, 1992b, 1994, 2002b). Here, I give an overview of more than three decades of my cytogenetic work on *Neurospora* ascus development, chromosome rearrangements, meiotic drive-inducing Spore killers, meiotic silencing and its suppression, programmed ascospore death in *Coniochaeta tetraspora*, and meiosis and ascospore development in *Cochliobolus heterostrophus*.

## 2. Normal ascus development in *Neurospora* species

Dodge (1927) described nuclear phenomena associated with heterothallism in *N. crassa* and *N. sitophila*, and (pseudo)homothallism in *N. tetrasperma* (Fig 1). Ascus is the largest cell ($20 \times 200$ μm) in the entire life cycle of *Neurospora*. Nuclei, chromosomes, spindles and the associated organelles are clearly seen in the light microscope (Fig 2). After fertilization, the two haploid nuclei of opposite mating type (*mat A* and *mat a*) proliferate in the ascogenous tissue, which give rise to croziers and ascus initials inside the developing perithecium (ascocarp) (Fig 2A). The two compatible nuclei fuse in the young ascus to give rise to a diploid nucleus, which immediately undergoes meiosis (two divisions) and a postmeiotic mitosis. All three nuclear divisions occur in the common cytoplasm of the ascus prior to ascospore delimitation (Fig 2A-H). Premeiotic DNA replication is shown to occur prior to karyogamy in the penultimate binucleate cell of the crozier or in the ascus initial in a related ascomycete *Neottiella rutulans*. In *Neurospora*, repeat-induced point mutation (RIP), which serves as a major genome defence mechanism, also occurs premeiotically between the stages of fertilization and karyogamy (Selker 2002). Chromosomes are short at karyogamy as they soon begin to pair in the young asci, but they elongate throughout zygotene and pachytene stages (7–18 μm long). This is in contrast to the chromosome behaviour in the mushroom fungus *Coprinus* (*Coprinopsis*), where the chromosomes are fully extended at karyogamy (Lu & Raju 1970; Raju & Lu 1970; Raju 1980). The paired and extended pachytene chromosomes in *Neurospora* appear as railroad tracks (Fig 2B, C). Following a diffuse diplotene stage, chromosomes condense and segregate in a manner resembling the higher eukaryotes (Raju 1980, 1986a). The spindles at the two meiotic divisions are oriented longitudinally and in tandem (at the second division), parallel to the ascus wall (Figs 1, 2E). A second round of DNA replication occurs during a prolonged interphase II, following meiosis, in preparation for the postmeiotic mitosis. It is during this interphase, the ascospore-delimiting double membranes are formed around the ascus cytoplasm, and spindle pole bodies (SPBs) duplicate and form greatly enlarged outer plaques (Fig 2F). The SPB plaques separate and migrate to opposite sides of the ascus to form transverse spindles during the postmeiotic mitosis (Fig 2G). The four pairs of sister nuclei, which are initially on opposite sides of the ascus realign in single file, with the sister nuclei located adjacent to one another, and all eight SPB plaques facing the same side of the ascus (Fig 2H). Preformed ascospore wall membranes invaginate around individual nuclei to cut out eight uninucleate ascospores. A SPB plaque is always seen at the lower end (relative to ascus base) of each incipient ascospore (see Raju 1980; Read & Beckett 1996). Actin microfilaments and microtubules emanating from SPB plaques are shown to play a major role in the realignment of nuclei and ascospore delimitation in *Sordaria macrospora*, *Podospora anserina*, *N. crassa*, and *N. tetrasperma* (Thompson-Coffe & Zickler 1992, 1993, 1994).

A second postmeiotic mitosis occurs in the young ascospores soon after they are delimited (Fig 2 I). Four or five additional mitoses occur in the mature black ascospores, even before they are ejected from the perithecia. The multinucleate condition was first observed in freeze-etch and thin-section studies of mature ascospores of *N. crassa* (Byrne 1975), and subsequently shown in the hyaline ascospores of the perithecial colour mutant *per-1*, and with GFP-tagged histone H1 (Fig 3A) (Freitag *et al.* 2004; Raju 1980). In several homothallic species of *Neurospora*, where there are no mating type distinctions, ascus development and nuclear divisions follow exactly the same stages as in the heterothallic *N. crassa*, including the fusion of two haploid nuclei and the formation of linearly ordered ascospores, except in *N. pannonica* (Raju 1978, 2002a). In *N. pannonica*, the immature asci are broad and the young ascospores are not linearly ordered, although mature asci show linearly arranged ascospores. A similar behaviour is found in several large-spored species of *Gelasinospora* (Glass *et al.* 1990). Each *N. crassa* perithecium produces 200–400 asci, and most of the asci mature normally in crosses between

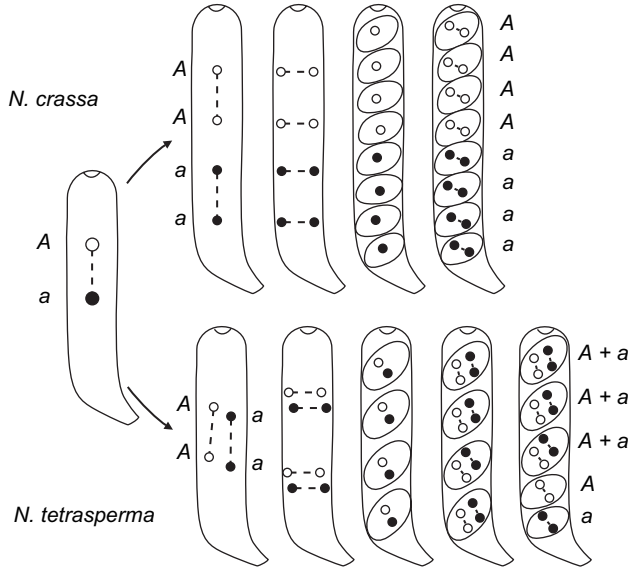


**Fig. 1 – A schematic diagram of ascus development in the heterothallic species *N. crassa*, and in the pseudohomothallic species *N. tetrasperma*. Mating types (*A*/*a*) segregate at the first division of meiosis in both species. In *N. crassa*, the second division spindles are aligned in tandem, and the four spindles at the third division (mitosis) are aligned equidistant and across the ascus. Subsequently, all eight nuclei line up in single file and cut out eight uninucleate ascospores, four *mat A* and four *mat a*. In *N. tetrasperma*, the second and third division spindles overlap, and each of the four ascospores encloses two nuclei of opposite mating type. (From Jacobson *et al.* 2008.)**

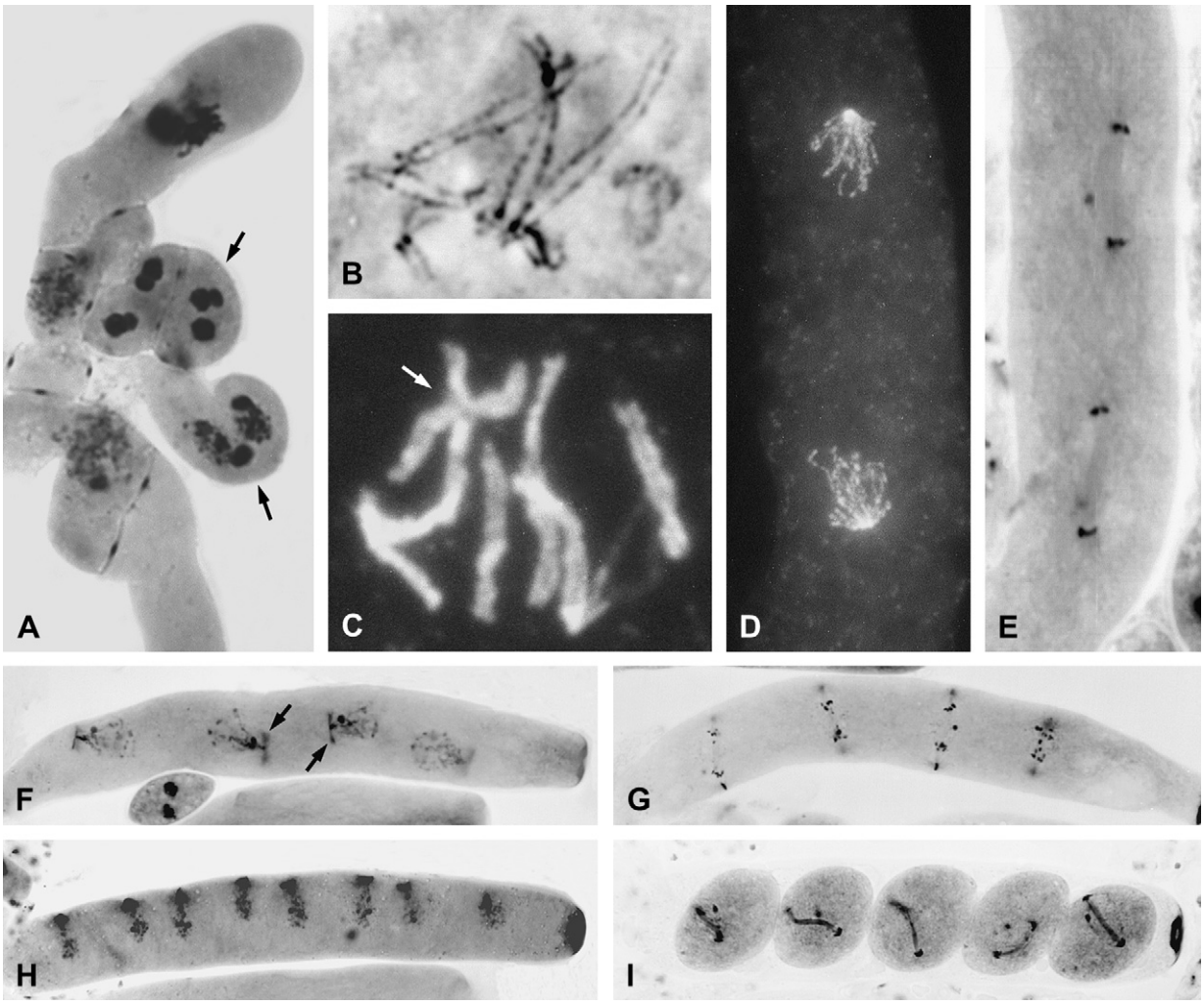


**Fig. 2 – Meiosis and ascospore genesis in *N. crassa* (and *N. discreta*). Haematoxylin staining except where noted otherwise. A. Croziers (arrows), and a young ascus. B. Pachytene chromosomes stained with aceto-orcein; paired chromosomes appear as railroad tracks (7–18 μm long). C. Pachytene chromosomes from Normal × Reciprocal translocation (*T [IR;IIR] 4637*), showing the cross-shaped configuration of rearranged chromosomes (arrow). Acriflavine staining. D. Interphase I following meiosis I (acriflavine; from Raju 1986a). E. Telophase II (from Raju & Perkins 1994). F. Interphase II following meiosis II. Note the enlarged spindle pole body plaques (arrows). G. Anaphase III. The four spindles are aligned across the ascus. H. Interphase III prior to ascospore delimitation. I. Telophase IV that results in young binucleate ascospores (see one such ascospore in F) (*N. discreta*).**

unrelated parents (Fig 3B). However, crosses between highly inbred laboratory strains result in a high proportion of ascus abortion, following ascospore delimitation (Raju *et al.* 1987). Fig 3C shows segregation of *cys*-3, which has a pleiotropic effect on ascospore maturation and viability. A new eight-spored heterothallic species, *N. discreta*, was described based on infertility of certain wild isolates with the previously established species testers (Perkins & Raju 1986).

*N. tetrasperma* is a four-spored pseudohomothallic (also called secondarily homothallic) species. Ascus development is reprogrammed so that each of the four ascospores encloses two nuclei of opposite mating type; single-ascospore cultures are thus self-fertile (Figs 1, 4). This is accomplished by the complete linkage of the mating-type locus to the centromere (no crossing over), overlapping spindles at the second and third divisions, and precise alignment of nonsister pairs of nuclei for sequestration into four ascospores (Fig 4A-D) (Dodge 1927; Raju 1992a). A large genetically determined recombination block is positively correlated with a cytologically detectable, long unpaired region in linkage group I (Gellegos *et al.* 2000; Jacobson *et al.* 2008). Of the six pseudohomothallic species examined, only *N. tetrasperma* evolved a recombination block in the mating-type chromosome and overlapping spindles at the second division of meiosis. Five other species (*Apiosordaria verruculosa, Coniochaetidium savoryi, Gelasinospora tetrasperma Podospora anserina, and P. tetraspora*) have apparently evolved an obligate crossing over proximal to mating type, and tandem spindles at the second division to accomplish the same end result – self-fertile ascospore progeny. The ascus programming may have evolved independently because it shows much variation among different species (Raju & Perkins 1994, 2000). In all six pseudohomothallic

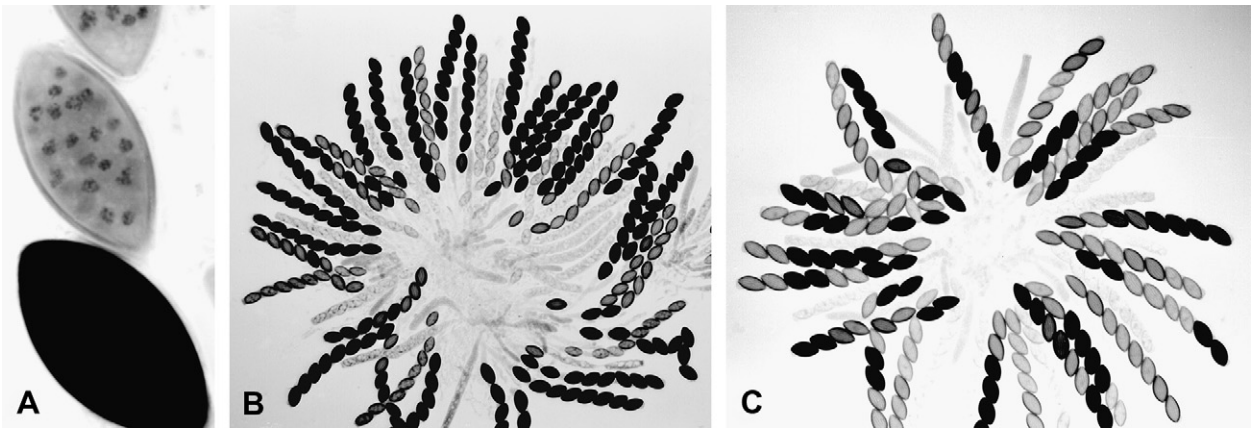


**Fig. 3 – Maturing ascospores in *per*-1 (perithecial colour mutant), *cys*-3, and wild type. A. Wild type × *per*-1. Mature ascospores of per-1 are hyaline and clearly show their highly multinucleate condition (32–64 nuclei; from Raju 1980). B. A rosette of maturing eight-spored asci in *N. sitophila* (*Sk*-1 × *Sk*-1; from Raju 1979). C. Maturing asci from wild type × *cys*-3. Ascospores that received the mutant *cys*-3 allele fail to pigment or mature. The linearly ordered ascospores show either first-division segregation (4 black: 4 white) or second-division segregation (2:2:2:2 or 2:4:2).**

species, pairs of nonsister nuclei are sequestered into four heterokaryotic ascospores. In some asci, pairs of small, homokaryotic, single-mating-type ascospores replace one or more heterokaryotic ascospores. Such 5–8 spored asci are more common (up to 10 %) in highly inbred laboratory strains than in wild-collected strains (1–2 %). Several mutant strains produce mostly 5 to 8-spored asci (e.g., *E*; Fig 4*G*), and certain wild strains produce all 8-spored asci in outcrosses (Jacobson

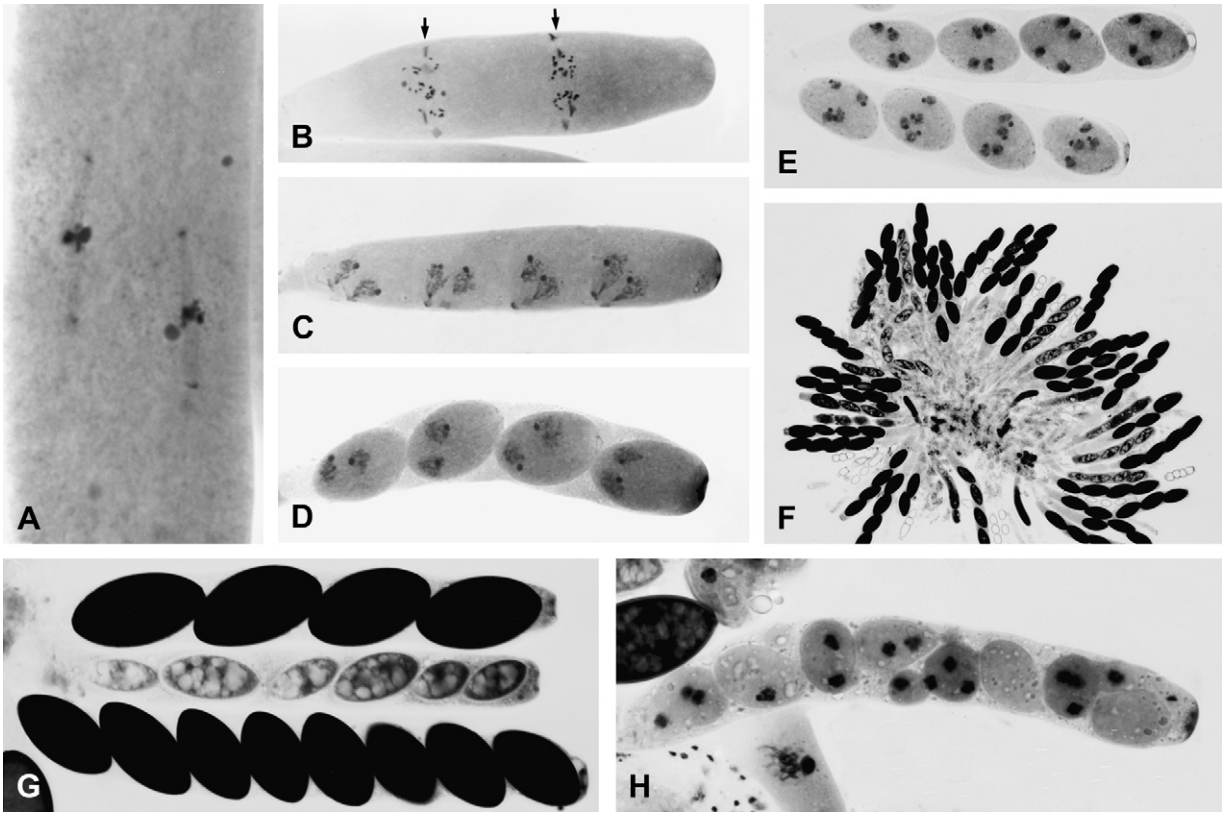


**Fig. 4 – Ascus development in *N. tetrasperma*. A. Metaphase II, showing the overlapping spindles. B. Anaphase III. The four nuclei are dividing across the ascus as two pairs of spindles at each arrow. C. Interphase III at spore delimitation. D. Binucleate young ascospores. E. Following a mitosis in young ascospores, each spore now contains four nuclei (2 *mat A*, 2 *mat a*). F. A rosette of four-spored asci. G. Four to eight-spored asci from wild type × *E*. Note the ascospore size difference. H. Abnormal ascospores in the *bud* mutant (*bud* × *bud* ). Irregular distribution of nuclei and incomplete spore cutting result in budded ascospores and some buds without nuclei. (B-F, from Raju & Burk 2004.)**

1995) The self-sterile small-ascospore progeny, as well as the cultures from the homokaryotic conidia, can outcross. Apparently, *N. tetrasperma* and other pseudohomothallic species have the best of both worlds: self-fertile cultures routinely undergo sexual cycle without needing a compatible mate, and the self-sterile cultures can outbreed bringing in a new gene pool (Raju 1992a).

## 3. *Neurospora* mutants that affect ascus development

Dodge initiated the studies on ascus mutants in *N. tetrasperma*, and later Srb and associates at Cornell University described several mutants of *N. crassa* and *N. tetrasperma* that affect meiosis or ascospore development (Srb *et al.* 1973; Raju & Burk 2004). Numerous sexual phase mutants have also been isolated and studied in *N. crassa* (Raju 1992b). The first ascus mutant of *N. crassa* I have studied is *Banana* (*Ban*), which produces giant ascospores in >95 % of asci. It is dominant, female sterile, shows abnormal vegetative morphology, and highly multinucleate crozier and precrozier cells. In wild type × *Ban*, all three nuclear divisions occur as in normal asci. However, the four pairs of sister nuclei fail to realign in single file, and cut out a single giant ascospore enclosing all eight nuclei, four *mat A* and four *mat a* (Fig 5A). The giant ascospores mature, germinate, and give rise to mixed hyphae that can be separated into individual components (Raju & Newmeyer 1977). I have used *Banana* for studying synchronous mitosis in the abnormal multinucleate crozier cells (Raju 1984), for the analysis of Spore killers (Raju 1979), and for visualizing the expression and transport of a GFP-tagged protein through the cytoplasm of the giant ascospores (Freitag *et al.* 2004). Another giant-spore mutant, *Perforated* (*Prf*), differentiates multiple apical pores at the ascus apex, instead of a single pore, resembling a saltshaker. The giant ascospores of *Prf* are nevertheless ejected forcefully by rupturing the perforated apex. *Prf* also shows abnormal multinucleate croziers, and carries a vegetative lethal (Raju 1987). A swollen ascus mutant (*peak*) causes non-cylindrical asci in which the eight ascospores are not ordered linearly (Fig 5B). The balloon-shaped swollen asci fail to differentiate apical pores, or eject their ascospores (Raju 1988). A dominant round-spore mutant (*R*) produces all round ascospores in crosses of wild type × *R*. The round ascospore shape is apparently determined by the genotype of the ascus rather than the genotype of individual ascospores (Fig 5C; Raju 1992b). Two temperature-sensitive four-spore mutants, *Fsp-1* and *Fsp-2*, delimit ascospores at the four-nucleate stage, without undergoing a postmeiotic mitosis. *Fsp-1* produces almost all eight-spored asci at 16–20 °C, and mostly two to four-spored asci above 25 °C. The reverse is true with *Fsp-2*, i.e., four-spored asci at 16–20 °C and mostly eight-spored asci above 25 °C. *Fsp-1, Fsp-2* double mutant is temperature-independent and produces mostly four-spored asci from 16–30 °C. The intercrosses of *Fsp-1* × *Fsp-2* are also temperature independent and produce mostly four-spored asci from 16–30 °C (Raju 1986b). I have also examined other mutants that affect meiosis (*mei-2*, *mei-3*), postmeiotic mitosis (*mus-8*), and ascospore development (Raju 1986c; Schroeder & Raju 1991). We have tested ~100 wild isolates and found that on average, each isolate harbours one recessive sexual-phase mutation that is expressed only when made homozygous (Leslie & Raju 1985; Raju & Leslie 1992). Raju (1992b) reviewed the literature on many sexual-phase mutants.

## 4. Chromosome rearrangements

David Perkins championed the analysis of over 350 chromosome rearrangements in *N. crassa*, mostly by scoring the

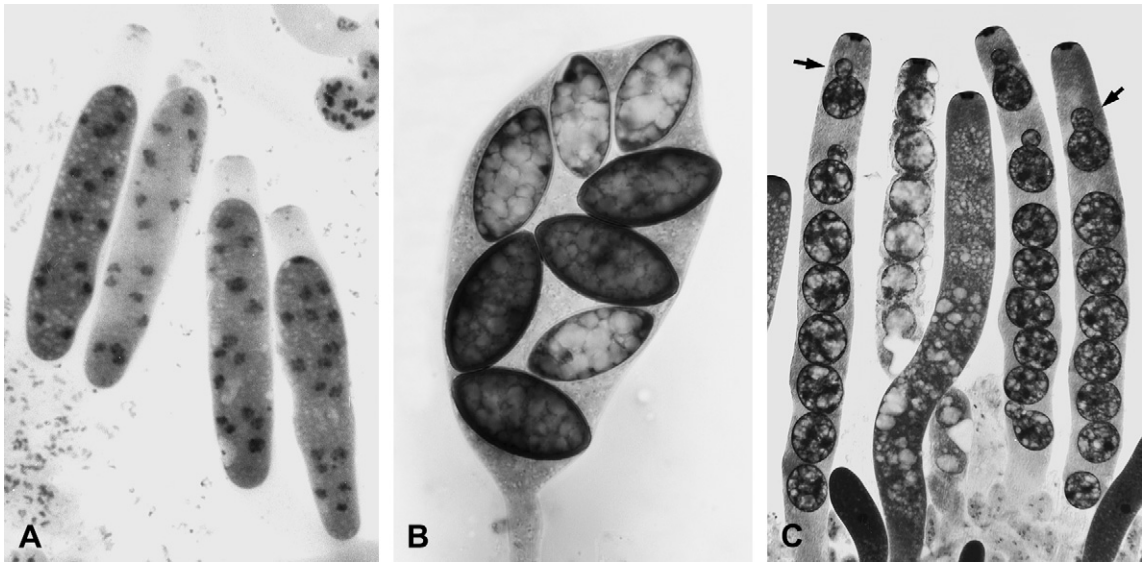


**Fig. 5 – Abnormal asci or ascospores in three mutants of *N. crassa*. A. In the giant ascospore mutant *Banana* (wild type × *Ban*), each ascus cuts out a single giant ascospore that encloses all eight nuclei (from Raju & Newmeyer 1977). B. A swollen ascus from a dominant allele of the mutant *peak*. The ascospores are not linearly ordered in the swollen asci, and the asci fail to differentiate an apical pore; consequently the ascospores are not ejected out of perithecia (from Raju 1992b). C. In crosses of wild type × R, all eight ascospores of each ascus are round, even the ones that are genotypically $R^+$. The round ascospores developed bud-like structures on one or more ascospores in some but not all asci (arrows).**

ejected groups of eight ascospores from individual asci. His analysis is based on the knowledge that only the euploid ascospores blacken and mature, whereas the ascospores carrying duplication-deficiencies fail to pigment or mature. Perkins (1974) showed that a cross of Reciprocal translocation × Normal produces 8 Black:0 White, 4B:4W (2:2:2:2 or 2:4:2), and 0B:8W asci in characteristic frequencies, and that Insertional translocations × Normal produce 8B:0W, 6B:2W, and 4B:4W asci in characteristic frequencies (see Perkins 1997).

Unordered tetrads are generally very useful for quick detection and analysis of various translocation strains. However, a class of rearrangements, where one or both translocation break points are close to the centromere, produced mature intact asci containing 4B:4W ascospores, rather than the expected 2B:2W:2B:2W (or 2:4:2) patterns for crossover asci. The 4B:4W first-division-segregation asci were subsequently shown to have resulted from 3:1 segregation of the translocation quadrivalent. This study required a direct microscopic examination of intact mature asci for distinguishing whether the 4:4 asci are the result of crossing over (2:2:2:2 or 2:4:2) or of nondisjunction (4B:4W) (Perkins & Raju 1995). One-third of the random viable progeny from Insertional translocation × Normal sequence are duplicated for the inserted chromosome segment, and these are barren both in heterozygous and homozygous crosses (Raju & Perkins 1978). The barrenness was thought to be due to extensive repeat-induced point mutations (RIP) or meiotic silencing by unpaired DNA. However, the crosses remain barren even in RIP deficient background, and when homozygous. In other studies, I have used an iron-haematoxylin or acriflavine staining for the analysis of ~15 rearrangement strains that involved the nucleolus organizer region, which served as a cytological marker during ascus and ascospore development (Perkins *et al.* 1980, 1984, 1986a, 1995).

## 5. Meiotic drive causing Spore killers

Spore killers (*Sk*), first discovered in *Neurospora*, are chromosomal elements that distort genetic ratios of *Sk*-linked genes. They are meiotic drive elements that closely resemble the segregation distorters in *Drosophila*, *t* complexes in mouse, pollen killer in wheat, and female gamete eliminator in tomato (Raju 1979, 2002b; Turner & Perkins 1979, 1991). In crosses of killer × sensitive (normal), four of the eight ascospores of each ascus that contain the sensitive allele fail to mature and are inviable (Fig 6). Meiosis, postmeiotic mitosis, and ascospore genesis are completely normal. A second mitosis occurs in all eight ascospores before there is any sign of death of four of the eight ascospores in each ascus. All survivors are killers. Three different Spore killers have been found among natural populations of *N. sitophila* (*Sk-1*) and *N. intermedia* (*Sk-2* and *Sk-3*). *Sk-2* and *Sk-3* have been found in only four *N. intermedia* isolates from Borneo (Brunei, Sabah), Java, and Papua New Guinea among ~2500 isolates of this species from around the world, whereas *Sk-1* killer is found in up to 30 % of *N. sitophila* isolates from many parts of the world (Turner 2001).

*Sk-2* and *Sk-3* have been introgressed into *N. crassa*, and *N. tetrasperma* for detailed genetic analysis. When either one is heterozygous in a cross (*Sk-2* or *Sk-3* × wild type), crossing over is blocked in a 30 map-unit region that includes the centromere of linkage group III (Campbell & Turner 1987). There is neither killing nor recombination block when either killer is homozygous. When *Sk-2* is crossed with *Sk-3*, all eight ascospores are killed because of mutual killing, i.e., *Sk-2* is sensitive to killing by *Sk-3*, and *Sk-3* is sensitive to killing by *Sk-2*. Sensitive nuclei are sheltered from killing when a killer nucleus is also enclosed in the same ascospore. This was first shown in *N. crassa* by using *Ban*, where all eight nuclei of each ascus (4K + 4S) are enclosed in a single giant ascospore, and subsequently, in the naturally heterokaryotic four-spored asci of *N. tetrasperma*. Progeny cultures from the heterokaryotic ascospores contain both killer and sensitive nuclei, which remain unchanged through subsequent generations (Raju 1979, 1994; Raju & Perkins 1991). The sheltering of sensitive nuclei in the heterokaryotic ascospores prompted Raju and Perkins (1991) to suggest that pseudohomothallism in *N. tetrasperma* may have evolved to circumvent the deleterious effects of Spore killers in heterothallic species. The exact chromosomal location of *Neurospora* Spore killers is not known because of the recombination block in linkage group III (Campbell & Turner 1987). Thus none of the Spore killers have yet been cloned for molecular analysis (see reviews by Raju 1994, 2002b; Raju & Perkins 1991; Turner & Perkins 1991). *Sk-2* and *Sk-3* have since been used for determining centromere distances of marker genes by simple scoring of ejected unordered half-tetrads (Perkins *et al.* 1986b), and in studies of meiotic silencing and its suppression (Raju *et al.* 2007; see below).

Spore killers have also been found in *P. anserina*, *Gibberella fujikuroi*, and *Cochliobolus heterostrophus* (Raju 1994).

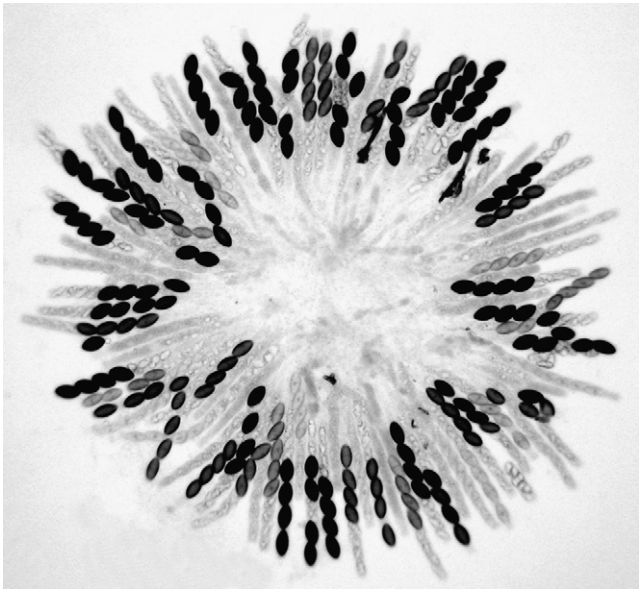

**Fig. 6 – A rosette of maturing asci of *N. sitophila* from *Spore killer-1* × wild type (*Sk*-sensitive), showing the death of four (*Sk*-sensitive) ascospores in each ascus. All survivors contain the *Sk*-killer haplotype. Ascospore development is completely normal in homozygous killer × killer and sensitive × sensitive crosses.**

In *G. fujikuroi* and *C. heterostrophus*, crosses between killer and sensitive strains result in the death of four of the eight ascospores that do not contain the killer allele, just as in *N. crassa*. In *P. anserina*, killing of two of the four ascospores occurs when the killer and sensitive alleles segregate at the first division of meiosis. Sensitive nuclei are sheltered in the heterokaryotic ascospores following second-division segregation resulting from crossing over in the centromere proximal region (see Raju 2002b).

## 6. Meiotic silencing in *N. crassa*

Meiotic silencing by unpaired DNA (MSUD), first discovered in *N. crassa*, is a posttranscriptional gene silencing process related to quelling in *Neurospora* and cosuppression in plants, but its effect is expressed only during meiosis and postmeiotic mitosis. Any gene without a homolog in the same chromosomal position during meiotic prophase generates a sequence-specific signal that prevents expression of all copies of that gene (Aramayo & Metzenberg 1996; Shiu *et al.* 2001). Meiotic silencing is epigenetic, and mutations in several genes of RNA silencing machinery (*Sad-1*$^{\Delta}$, *Sad-2*$^{RIP}$, *sms-2*) suppress MSUD. Wild-type *sad-1* gene, which codes for RNA-directed RNA polymerase (RdRP), is an essential component of the silencing mechanism. We have recently shown that *sad-2*$^{+}$ is required for the perinuclear co-localization of SAD-1 and SAD-2 proteins for meiotic silencing (Shiu *et al.* 2006). In addition, a dicer-like ribonuclease, DCL-1, also colocalizes in the perinuclear region and is involved in meiotic silencing (Alexander *et al.* 2008). Early observations on MSUD utilized *asm-1*, *actin*, *β-tubulin*, *mei-3* etc, and their effects on ascus development, when silenced, could be readily monitored cytologically. For example, in crosses of wild type × *actin*$^{+}$ ectopic insert, all copies of the actin gene are silenced during meiosis and the asci are swollen like balloons. There is no silencing when the ectopic gene inserts are paired in homozygous crosses (*actin*$^{+}$ × *actin*$^{+}$), where asci elongate and develop normally. Similarly, actin is not silenced in *Sad-1*$^{\Delta}$ × *actin*$^{+}$, because *sad-1*$^{+}$ itself is unpaired and self-silenced. GFP-tagged histone H1 (*hH1*) and β-tubulin genes have since been used for visualizing the expression of meiotic silencing, and their suppression by *Sad-1*$^{\Delta}$ and *Sad-2*$^{RIP}$, using fluorescence microscopy (Fig 7A-C) (Freitag *et al.* 2004; Shiu *et al.* 2001, 2006). The meiotic drive-inducing *Neurospora* Spore killers *Sk-2* and *Sk-3* also suppress meiotic silencing of several ectopic gene inserts (e.g., *asm-1*, *actin*, *mei-3*, *β-tubulin*, *hH1-GFP*, *β-tubulin-GFP*), as effectively as *Sad-1*$^{\Delta}$ and *Sad-2*$^{RIP}$ (Fig 7D, E) (Raju *et al.* 2007).

We have recently used *N. tetrasperma* to evaluate both the generality of meiotic silencing within the genus and its

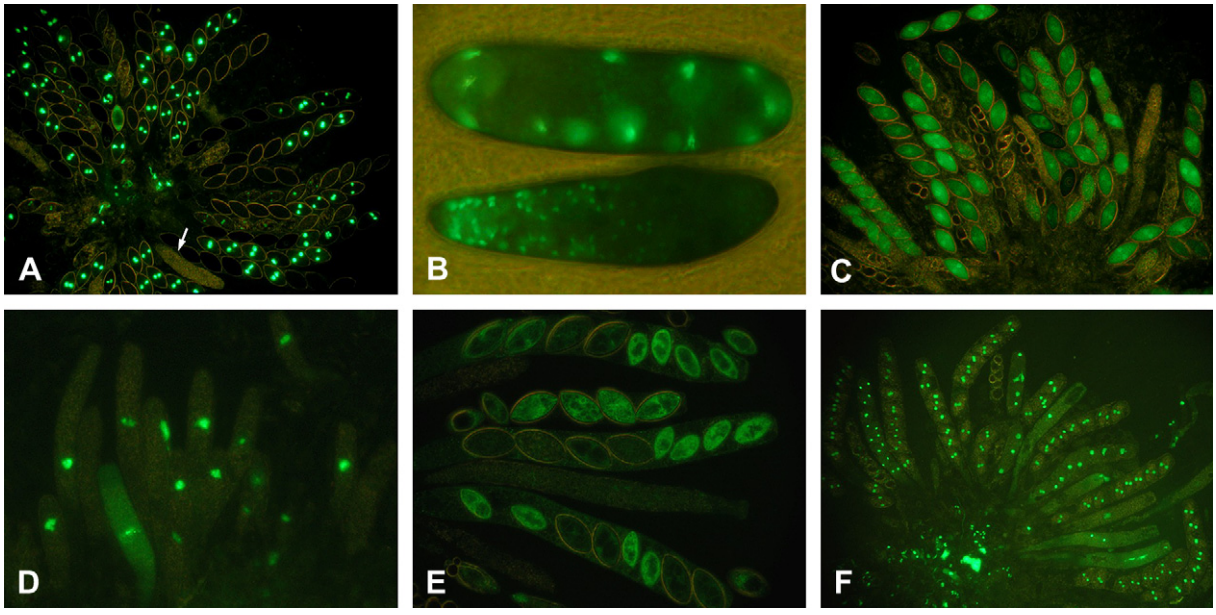


**Fig. 7 – Visualization of meiotic silencing and the suppression of silencing using GFP-tagged *histone H1* and *β-tubulin* genes. A. A rosette of maturing asci from wild type × *hH1-GFP*. Histone H1 is completely silenced during meiosis (see arrow) but it is re-expressed following ascospore delimitation. The *hH1-GFP* nuclei fluoresce in four of the eight ascospores. B. Two giant ascospores from *hH1-GFP* × *Ban*, each encloses all eight nuclei of the ascus (4 *hH1-GFP* + 4 non-*hH1-GFP*). The bottom spore is highly multinucleate following several mitoses. *Ban* is used here to study the transport of GFP-tagged histone H1 from one end of the giant spore to the other end, as it is gradually incorporated into all nuclei in the cytoplasm. C. Normal ascus development in *β-tubulin-GFP* × *β-tubulin-GFP*, where there is no silencing of *β-tubulin*. The expression of *β-tubulin* is completely silenced in heterozygous crosses, where young asci are arrested and aborted prior to metaphase I. D. *Sk-3* × *hH1-GFP*. The silencing of *hH1-GFP* is completely suppressed by *Sk-3*, and the nuclei fluoresced throughout meiosis. E. *Sk-2* × *β-tubulin-GFP*. The silencing of *β-tubulin-GFP* is suppressed by *Sk-2* and asci developed normally through spore delimitation. Shortly thereafter, both *β-tubulin-GFP* and *Sk*-induced spore death are expressed. F. Eight-spored asci from *E* × *hH1-GFP* showing the absence of meiotic silencing in *N. tetrasperma*. Meiotic silencing is also absent in wild type × *hH1-GFP* crosses of *N. tetrasperma*.**

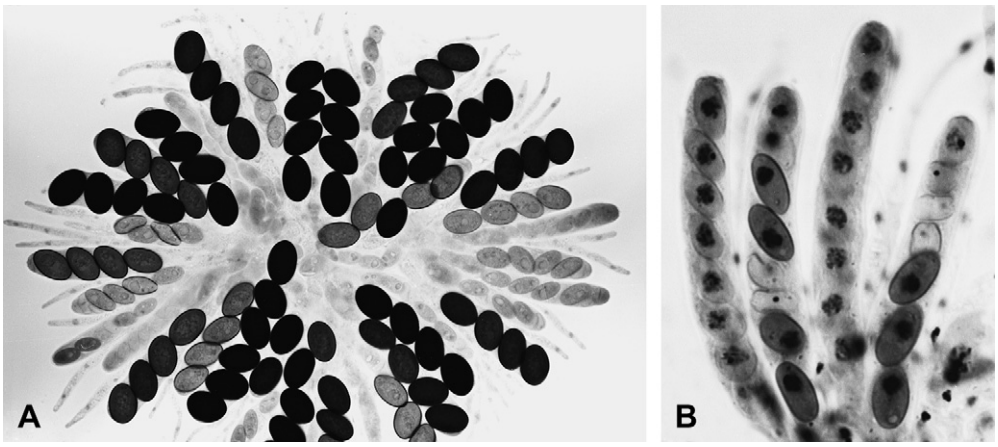


**Fig. 8 – Ascus development in the homothallic fungus *Coniochaeta tetraspora*. A. A rosette of maturing asci. All asci are eight-spored at inception, but the mature asci show only four black viable spored. B. Immature asci showing the nuclei, and death of four of the eight ascospores (from Raju & Perkins 2000).**

possible evolutionary significance. Several *hH1-GFP* constructs were introgressed from *N. crassa* into various chromosome locations in *N. tetrasperma*. Our results indicate that there is no meiotic silencing of *hH1-GFP* in this pseudohomothallic species, presumably because *sad-1* is naturally unpaired and self-silenced during meiosis by structural differences between *N. tetrasperma* mating-type chromosomes (Gallegos *et al.* 2000; Jacobson *et al.* 2008). There is also no meiotic silencing in a cross of $E \times hH1\text{-}GFP$, where almost all asci produced eight ascospores (Fig 7F).

## 7. Programmed ascospore death in *Coniochaeta tetraspora*

Cytological studies with *C. tetraspora* were initiated with the assumption that it is pseudohomothallic, similar to *N. tetrasperma* and *P. anserina*. However, each ascus initially contained eight ascospores, and the four-spored condition resulted only secondarily by disintegration of two pairs of sister ascospores. Meiosis and postmeiotic mitosis are similar to those in *N. crassa*, and that all eight ascospores are uninucleate at inception (Raju & Perkins 2000). However, four of the eight ascospores soon abort and disintegrate, leaving only four mature ascospores, which showed either the first (4 viable: 4 inviable) or the second-division-segregation patterns (2:2:2:2 or 2:4:2) for ascospore death (Fig 8A, B). Progeny analysis showed that single-ascospore cultures of each ascus are self-fertile and again produce four viable and four inviable ascospores generation after generation. Thus *C. tetraspora* is primarily an eight-spored homothallic species, and not a pseudohomothallic species like *N. tetrasperma*. The ascospore death in *C. tetraspora* superficially resembles that of *Neurospora* spore killers, but the death cannot be due to interaction of *killer* and *sensitive* haplotypes as in *Neurospora*, because *C. tetraspora* is homothallic and there are no such genotypic differences. Raju and Perkins (2000) discussed similar phenomena in several other fungi, and attributed them to epigenetic mutational changes in one of the two nuclei that go into meiosis.

## 8. Meiosis and ascospore development in *Cochliobolus heterostrophus*

*Cochliobolus heterostrophus* causes southern corn leaf blight. It produces eight filiform ascospores per ascus, following meiosis and a postmeiotic mitosis. Early ascus development and nuclear divisions in *C. heterostrophus* resemble those of

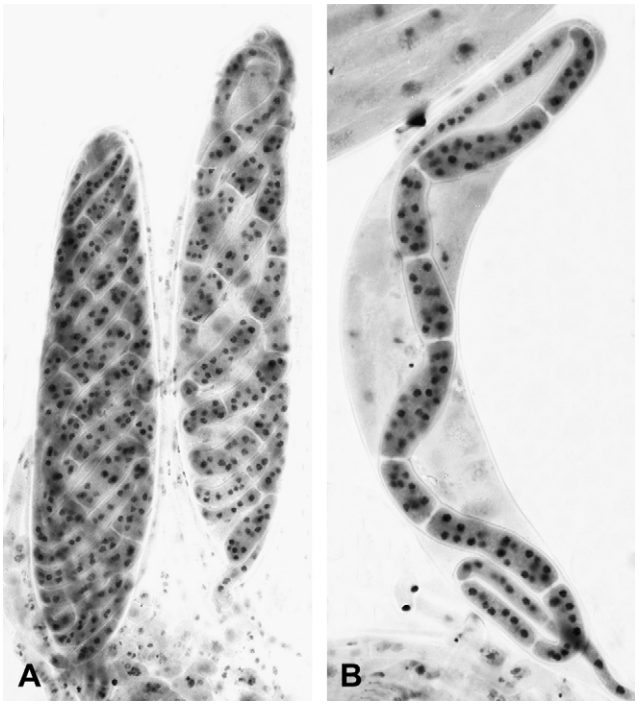


**Fig. 9 – Helically coiled filiform, multinucleate, multiseptate ascospores in the corn pathogen *Cochliobolus heterostrophus*. A. Two asci containing eight and four mature ascospores. B. An ascus showing a single mature ascospore; the remaining seven ascospores have aborted shortly after spore delimitation. (From Raju 2008).**

*N. crassa*. However, the two fungi differ in several important details owing to differences in ascus and ascospore shape, SPB behaviour during spore delimitation, and ascospore development. The two spindles at meiosis II, and the four spindles at the postmeiotic mitosis are aligned irregularly, unlike the tandem or ladder rung-like orientation of spindles in *N. crassa*. Prior to ascospore delimitation, all eight nuclei reorient themselves and their SPB plaques migrate toward the base of the ascus. The SPB plaques facilitate demarcation of the lower end of each incipient ascospore. The ascospores are uninucleate and unsegmented at inception but they become highly multinucleate, multisegmented, and helically coiled when mature (Fig 9A, B). An illustrated account of ascus and ascospore development is given in Raju (2008).

## 9. Epilogue

The Perkins' laboratory at Stanford (1949–2007) played a pivotal role in the development of *Neurospora* as a model for genetic, cytogenetic and cytological studies, and more recently for the molecular analysis of its sexual cycle. Since 1974, I have contributed to the elucidation of normal processes underlying the ascus and ascospore development, abnormal processes in numerous mutant strains, chromosome rearrangements, Spore killers, and meiotic silencing. Admittedly, much of my focus was on light microscopy studies of ascus and ascospore development relevant to our laboratory's genetic and cytogenetic interests in *Neurospora*. Now with the *Neurospora* genome sequenced, mutations in specific genes can be readily correlated with the observed cytological defects in the sexual stage. It is hoped that our recent discovery of meiotic silencing in *Neurospora*, and the use of immunofluorescent labelling and GFP-tagged genes for studying gene expression (or silencing) will pave the way for the molecular analysis of complex processes during ascus and ascospore development.

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