

## Life-history of *Neurospora intermedia* in a sugar cane field

ALKA PANDIT and RAMESH MAHESHWARI\*

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

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**Abstract.** The life-history of *Neurospora* in nature has remained largely unknown. The present study attempts to remedy this. The following conclusions are based on observation of *Neurospora* on fire-scorched sugar cane in agricultural fields, and reconstruction experiments using a colour mutant to inoculate sugar cane burned in the laboratory. The fungus persists in soil as heat-resistant dormant ascospores. These are activated by a chemical(s) released into soil from the burnt substrate. The chief diffusible activator of ascospores is furfural and the germinating ascospores infect the scorched substrate. An invasive mycelium grows progressively upwards inside the juicy sugar cane and produces copious macroconidia externally through fire-induced openings formed in the plant tissue, or by the mechanical rupturing of the plant epidermal tissue by the mass of mycelium. The loose conidia are dispersed by wind and/or foraged by microfauna. It is suggested that the constant production of macroconidia, and their ready dispersal, serve a physiological role: to drain the substrate of minerals and soluble sugars, thereby creating nutritional conditions which stimulate sexual reproduction by the fungus. Sexual reproduction in the sugar-depleted cellulosic substrate occurs after macroconidiation has ceased totally and is favoured by the humid conditions prevailing during the monsoon rains. Profuse microconidiophores and protoperithecia are produced simultaneously in the pockets below the loosened epidermal tissue. Presumably protoperithecia are fertilized by microconidia which are possibly transmitted by nematodes active in the dead plant tissue. Mature perithecia release ascospores *in situ* which are passively liberated in the soil by the disintegration of the plant material and are, apparently, distributed by rain or irrigation water.

**Keywords.** *Neurospora*; life-history; ascospores; macroconidia; microconidia; dispersal; dormancy; furfural; germination.

### 1. Introduction

The heterothallic species of *Neurospora* produce prodigious quantities of vivid orange-coloured macroconidia by which the fungus can be unambiguously recognized in nature. The fungus had been reported from several countries on various types of material, notably, on bread from bakeries (Perkins 1992), vegetation that had been scorched by fire (Kitazima 1925; Perkins 1992), corn cobs (Perkins *et al* 1976), and filter mud from the sugar mills (Shaw 1990). Shear and Dodge (1927) described the morphology and life-cycle of several *Neurospora* species from laboratory-grown cultures. Dodge also made genetic crosses and recognized the advantages for biological research of this haploid organism with its rapid life-cycle (Dodge 1939). These features led Beadle and Tatum (1941) to use *N. crassa* for the production and analysis of nutritional mutants. Since then a vast literature has accumulated on the use of *Neurospora* in genetics and biochemistry (Perkins 1992). *Neurospora* presented new possibilities in population biology for making comparisons with organisms whose life

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\*Corresponding author (Fax, 091-80-3341683; Email, fungi@biochem.iisc.ernet.in).

cycle is predominantly diploid (Perkins *et al* 1976; Perkins and Turner 1988). However, despite its proven and projected importance in general biological research, the biology of the fungus in nature has remained largely unknown. Thus, although the biologists who use *Neurospora* in the laboratory are impressed with its rapidly creeping mycelium, the tendency of the mycelium to grow towards openings, the rapid production of powdery masses of macroconidia on aerial mycelium, the stimulation of production of microconidia and sexual reproduction under low-nutrient conditions, the forcible discharge of ascospores, their constitutive dormancy and thermoresistance of ascospores—the significance of these features in the context of the growth of the fungus in nature is not understood. Moreover, virtually no information is available on the factors in nature which contribute to its selective colonization of burnt vegetation, its morphology in natural substrates, the significance of its production of different types of spores and the conditions and the timing of their production, its mode of dissemination and method of propagation.

To gain a deeper understanding of the growth and reproduction of *Neurospora*, we have made observations of the fungus in agricultural fields where burning of sugar cane is regularly practised and *Neurospora* occurs commonly. Material collected from these fields was brought to laboratory for microscopic examination and experimentation in the laboratory with burnt sugar cane. Although our observations were chiefly on *N. intermedia*,—the most abundant species on burnt substrates (Perkins and Turner 1988)—there is no reason to believe that other heterothallic species of the fungus have significantly different life-histories. Our observations show that some aspects of the life history of *Neurospora* in nature are different from those surmised or inferred from laboratory-grown cultures.

We demonstrate that *Neurospora* is a soil-borne fungus and that chemical(s) diffusing from burnt sugar cane into soil can trigger the germination of ascospores in soil. The chemical trigger is identified as furfural. The morphogenetic development of the fungus in relation to histology of its substrate was studied for the first time and the timing of sexual reproduction in relation to conidiation determined. The micro-environment and conditions of sexual reproduction were investigated. The spore content of air around fields where *Neurospora* occurs in nature was determined. A new hypothesis on the role of macroconidia in sexual reproduction by the fungus is presented.

## 2. Materials and methods

### 2.1 Collection of material

Observations of *Neurospora* were made in plantation fields of sugar cane at different times of the year (1991-1993). The study site is located in Maddur district in the state of Karnataka (India), approx. 80 km southwest of Bangalore. Soil and plant material from the sugar cane fields were brought to the laboratory for examination. Colonies of *Neurospora* on sugar cane were sampled as described by Perkins *et al* (1976).

### 2.2 Fungal isolates and culture conditions

Table 1 lists the strains of *Neurospora* used in this study. The techniques for growth and genetic crossing of *Neurospora* were as described by Davis and de Serres (1970). The

**Table 1.** *Neurospora* strains used in this study.

Strain	Species	Colour	Source
RM 114-1a (FGSC 7462)	<i>N. intermedia</i>	Albino	Laboratory collection
RM 114-2A (FGSC 7463)	<i>N. intermedia</i>	Albino	Laboratory collection
RM 125-1a (FGSC 7466)	<i>N. intermedia</i>	Orange	Laboratory collection
RM 125-2A (FGSC 7467)	<i>N. intermedia</i>	Orange	Laboratory collection
Maddur 1993-41	<i>N. tetrasperma</i>	Orange	Laboratory collection
RM 36-10A	<i>N. discreta</i>	Orange	Laboratory collection
RM 48-23a	<i>N. discreta</i>	Orange	Laboratory collection
74-OR23-IVA (FGSC 2489)	<i>N. crassa</i>	Orange	Fungal Genetics Stock Center
74-ORS-6a (FGSC 4200)	<i>N. crassa</i>	Orange	Fungal Genetics Stock Center
FGSC 4762A	<i>N. sitophila</i>	Orange	Fungal Genetics Stock Center
FGSC 4763a	<i>N. sitophila</i>	Orange	Fungal Genetics Stock Center

mating types of strains collected from nature were determined by spot crosses on lawns of fluffy (*fl*) tester strains (Perkins *et al* 1989). Identification of species was based on fertility in crosses with tester strains as recommended by Perkins and Turner (1988). Ascospores bearing colour marker genes were obtained from homozygous crosses using *al*<sup>+</sup> (orange) or albino (*al*) strains of opposite mating types.

### 2.3 Microscopic examination

Observations of the fungus in sugar cane stumps were made using a Wild Stereomicroscope. Material for scanning electron microscopy was fixed in 3% (v/v) glutaraldehyde in 50 mM phosphate buffer, pH 7, for 6 h, washed with distilled water and dehydrated through an ascending alcohol series. After critical-point-drying, samples were gold-coated and examined in a scanning electron microscope. The plant tissue bearing perithecia was dried in a vacuum desiccator over anhydrous calcium chloride, sputter-coated with gold and examined in a JEOL JSM 840 A or a Cambridge Instruments S-120 scanning electron microscope. For histological examination, material was dehydrated and embedded in paraffin wax. Sections were cut with a microtome, stained with cotton blue in lactophenol and observed under a Leitz microscope.

### 2.4 Sampling of air-borne spores

A volumetric, rotating arm impaction device (Model 82 Rotorod Sampler, Sampling Technologies Inc., USA) was used to sample air-borne spores in the environment of burnt sugar cane fields. Sampling was done 10 times during January-August covering

the entire phase of *Neurospora* growth cycle. Each sampling was for at least 10 min and 20 samples were taken each time. The coated rods were examined under a microscope for *Neurospora* ascospores, identifiable by grooved black wall. Alternatively, Petri dishes containing sorbose plating medium (Davis and de Serres 1970) supplemented with 0.02% (w/v) chloramphenicol were exposed for 5-10 min. These were brought to the laboratory and placed over water at 60°C for 30 min, a treatment known to kill *Neurospora* conidia but to activate ascospores. After incubation at 34°C for 5 days, plates were examined for development of *Neurospora* colonies. Control Petri dishes were not exposed to heat. Orange colonies appearing in control plates were considered to have originated from air-borne conidia.

### 2.5 Soil plating

For isolation of *Neurospora* from soil, 100-1000 mg soil was suspended in 5 ml of warm 0.6% agar and heated at 60°C for 30 min in a water bath. The suspension was poured over sorbose agar medium containing 0.02% (w/v) chloramphenicol. Petri dishes were incubated at 34°C.

### 2.6 Reconstruction experiments

In order that *Neurospora* development on sugar cane could be monitored closely, reconstruction experiments with burnt sugar cane were carried out in the laboratory. Sugar canes were purchased from a local market. These were cut into small portions and scorched by covering with dried rice straw which was burned. In some experiments the pieces were scorched by dipping in 70% alcohol and flaming. *Neurospora* ascospores for use in these experiments were obtained from culture tubes and freed of contamination of viable asexual conidia by treatment with 0.8% (w/v) Na<sub>2</sub>EDTA (30 min) or by 16-fold diluted aqueous solution of sodium hypochlorite that contained approximately 4-6% available chlorine (5 min). The ascospores were washed thrice with distilled water before use.

### 2.7 Isolation, assay, purification and identification of ascospore activator

Scorched sugar cane pieces were soaked for 1-24h in distilled water containing 0.04% (w/v) chloramphenicol to inhibit bacterial growth. After filter-sterilization, the biological activity of the aqueous diffusate was determined. Ascospores were incubated in this solution for 4 h with constant shaking, following which they were plated on 2% water agar. Germinating ascospores were counted after 3-5 h under a Wild binocular microscope.

Reverse phase HPLC of samples was done using a C-18 column in a Shimadzu LC-6A or a Varian 5000 high performance liquid Chromatograph (HPLC). The elution protocol employed a linear gradient of water with increasing methanol concentration. The absorbance was monitored at 276 nm. For mass spectral analysis a Hewlett Packard mass spectrometer was used in combination with HP-ultra 2 column (5% methyl silicone).

## 2.8 Sugar estimation

For estimation of total soluble sugar in sugar cane, approx. 0.5-3.0g (wet wt) tissue pieces were crushed in 10 ml distilled water in a mortar. The suspension was boiled for 10 min, following which the insoluble material was removed by centrifugation. Aliquots of the clarified extract were taken for estimation of sugar by the anthrone reagent (Morris 1948).

## 3. Results

### 3.1 Fields observations

**3.1a Production and liberation of macroconidia:** Five to seven days after the trash of cut leaves in a harvested sugar cane field (figure 1A) had been burned (figure 1B), pink-orange conidiating pustules of *Neurospora* were commonly visible on the scorched, juicy stumps. A sampling of over 150 pustules showed that 95 % of these belonged to *N. intermedia* and those remaining to *N. tetrasperma*. In a field which had been burned approximately two weeks before, in nearly 70% of the stumps the pustules were at the basal node (figure 1C), or at the internode above it. By contrast, only 30 % of the scorched stumps had pustules on the exposed cut surface. Conidiation was intense and distributed in stumps in a field that had been burned approximately three-four weeks before and had been irrigated.

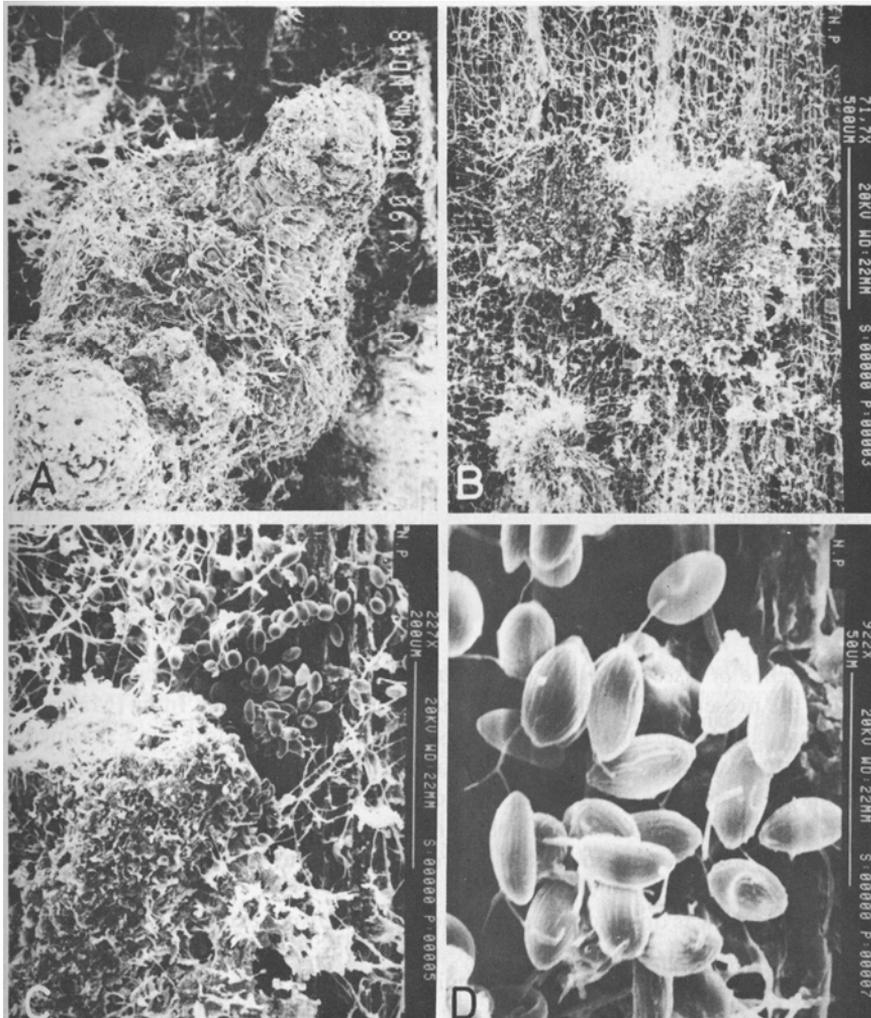
In the *Neurospora*-infected canes, in many places, the epidermal tissue had become separated from the ground tissue and could be removed easily. *Neurospora* mycelium was essentially submerged in the infected canes. On removing the epidermis a weft of pale yellow mycelium was exposed that rapidly turned orange. In places, the mycelium tended to form cushion like structures.

The scorched, juicy stumps supported rapid and profuse production of macroconidia which were constantly dispersed by wind. Macroconidia continued to be produced for 3-6 weeks after which the stumps had begun to shrivel. These stumps were now surrounded by a ratoon crop of sugar cane which had sprouted from the underground portions of the clump of stumps and had grown 1-1.5 metre tall (figure 1D). The pustules were no longer visible, except for some desiccated orange-whitish patches. The weft of submerged mycelium that had been seen during conidiation had mostly disappeared; presumably it was foraged by the microarthropods which were active in these stumps.

**3.1b Sexual reproduction:** Approximately 12 weeks after a field had been burnt, the stumps had become desiccated despite the regular irrigation of the field. The stumps were hidden among 1-1.5 metres tall ratoon sugar cane (figure 1D). Infected stumps bearing the remnants of macroconidia were searched, uprooted and brought to the laboratory. The epidermal tissue was carefully removed with forceps under a binocular microscope. A variety of fungal fructifications were observed. Perithecia of *Neurospora* were located on the exposed ground tissue in 20 out of some 200 stumps examined (figure 1E). In a few stumps, perithecia of *Neurospora* protruded through the cracked epidermal tissue (figure 1F). Usually the perithecia were disfigured but some of them had distinct beaks (figure 1G and 2A). Inside the perithecia were asci in different stages



Figure 1.



**Figure 2.** Scanning electron micrographs of perithecia of *Neurospora* on burnt sugar cane from field. Epidermal tissue was removed to expose perithecia. (A) A perithecium of *Neurospora* (scale bar 100  $\mu\text{m}$ ). (B) A perithecium of *Neurospora* and shot ascospores (arrow) (scale bar 500  $\mu\text{m}$ ). (C) A magnified view of ascospores (scale bar 200  $\mu\text{m}$ ). (D) Ascospores showing longitudinal grooves (scale bar 50  $\mu\text{m}$ ).

**Figure 1.** *Neurospora* on sugar cane in field. (A) A view of sugar cane field after harvest showing trash of cut leaves on ground, covering stumps. (B) View of a sugar cane field after trash had been burned. (C) Conidiating *Neurospora* pustules at root openings on a burnt stump (approx. two weeks after burning). (D) A cluster of stumps approx. after 12 weeks. Note shrivelled stumps with cracked epidermis and ratoon shoots. (E) A view of perithecia (arrow) exposed by removing epidermal tissue. (F) A cluster of *Neurospora* perithecia protruding through cracked plant epidermis (arrow). (G) A magnified view of perithecia. (H) A rosette of asci exposed by opening a perithecium.

of development (figure 1H). Some perithecia had discharged ascospores and some of these were sticking to the under surface of the epidermal tissue (figure 2B, C). Mature ascospores were black, grooved (figure 2D) and measured  $27 \times 15 \mu\text{m}$ . Ascospores were isolated on Vogel's N medium +1.5% glucose and heat-shocked. Nearly 40% of these germinated and produced the characteristic pink-orange cultures belonging to *N. intermedia*. Ten cultures, each of the opposite mating type, were crossed in all combinations and all were fertile as determined by the black ascospores which were shot on to the wall of the culture tubes.

3.1c *Dispersal*: To determine whether ascospores are air-borne and play the role in dispersal as postulated by Perkins and Turner (1988), the air-spores in sugar cane fields was sampled.

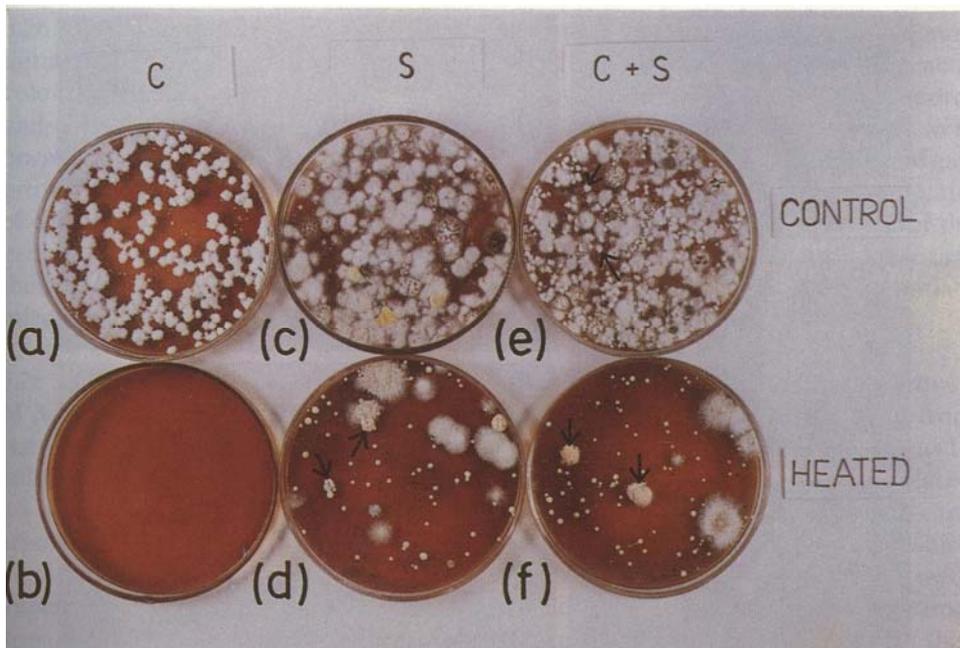
The sampling of air by means of a Rotorod Sampler did not reveal black, oval, grooved ascospores of *Neurospora* although dust particles, wings of insects, blackened tissue pieces from burnt sugar cane, plant trichomes and pollen grains, conidiophores and conidia of unidentified fungi were commonly found. In air-sampling by the exposed plate method, orange *Neurospora* colonies appeared in varying numbers in the control (unheated) plates but the heat-treated plates were consistently devoid of any type of fungal colony. The results indicate that macroconidia but not ascospores are air-borne.

3.1d *Presence of ascospores in soil*: Earlier, we had provided circumstantial evidence for the presence of ascospores of *Neurospora* spp. in soils in India (Maheshwari and Antony 1974; Palanivelu and Maheshwari 1979). Therefore we examined the possibility that ascospores are liberated upon the decomposition of the plant tissue and disseminated in soil by water from rain or irrigation. Species of *Neurospora* were isolated by plating a suspension of field soils after these had been heat-treated. In the present study, we were able to isolate *Neurospora* much more consistently from sugar cane field soil.

An experiment was done to ascertain whether *Neurospora* colonies which appeared in the soil plates in fact originate from heat-activated ascospores or from macroconidia. Approximately 500-1000 viable macroconidia of albino *N. intermedia* were mixed per 100 mg or 1 g of sugar cane field soil and plated without or after a heat-treatment (30 min at 60°C). As seen from figure 3, only the wild-type (orange) *Neurospora* colonies were recovered from soil after heating although albino macroconidia had been mixed in the soil. However, albino *Neurospora* colonies were recovered from the unheated soil. These results showed that heat-treatment selectively kills the conidial population of *Neurospora* in soil. The orange *Neurospora* colonies, which were repeatedly recovered from heat-treated soil, could have originated only from the wild-type ascospores present therein.

## 3.2 *Reconstruction experiments*

3.2a *Inoculum is present in soil*: The field observation that the visible *Neurospora* pustules were at the lower region of stumps, and subsequently were also produced apically, indicated that infection might come from soil and not from air. Had air-borne inoculum initiated infection of the stumps, the pustules would be more widely



**Figure 3.** Soil plating. Abbreviations: C, *al* conidia; S, soil; C + S, *al* conidia + soil. Upper panel was not given heat shock. Lower panel was given heat shock, (a) *Neurospora* colonies developing on sorbose medium from plating of a suspension of albino (*al*) conidia, (b) Absence of *Neurospora* colonies demonstrating that conidia are killed by the heat-treatment. (c) Fungal colonies from plating of unheated field soil. Note the absence of *Neurospora*. (d) As (c) but after heat-treatment. Note orange *Neurospora* colonies (arrows) among fungal colonies. (e) Plating of field soil which had been mixed with *al* conidia of *Neurospora*. Note albino *Neurospora* colonies (arrow). (f) As (e) but after heating. Note recovery only of orange (*al*<sup>+</sup>) *Neurospora* colonies (arrow).

distributed. To verify this, field-grown sugar canes were cut and sorted into lower, middle and upper segments. These segments were burned and placed in trays. As seen in figures 4A-C, *Neurospora* appeared only in the lower segments of the canes to which soil adhered but not in the middle or upper segments. The results suggest that the inoculum is present in soil.

To determine whether sugar cane field soil harbours inoculum of *Neurospora*, the following experiment was carried out. Approximately 5 cm long pieces of sugar cane were dipped in 70% ethanol and flamed. The scorched pieces were placed in boxes (figure 4D) containing: (a) autoclaved soil from the sugar cane field: (b) unburnt soil; and (c) burnt soil. As seen in figure 4D(a), sugar cane segments placed over sterile soil did not show *Neurospora* infection. The substrate pieces placed on unburnt soil were overgrown by an unidentified mycelial growth which also spread over the soil. A little later *Neurospora* also grew in some of the sugar cane segments. By contrast, all sugar cane segments placed over burnt soil were overgrown by *Neurospora*. The experiment demonstrates that the inoculum is present in field soil. That burnt sugar cane pieces placed over unburnt field soil also became infected by *Neurospora* showed that heating of soil was not essential for infection of sugar cane by *Neurospora*. Visibly, burning reduced the potential competitors of *Neurospora* in the soil [figures 4D(b), (c)].

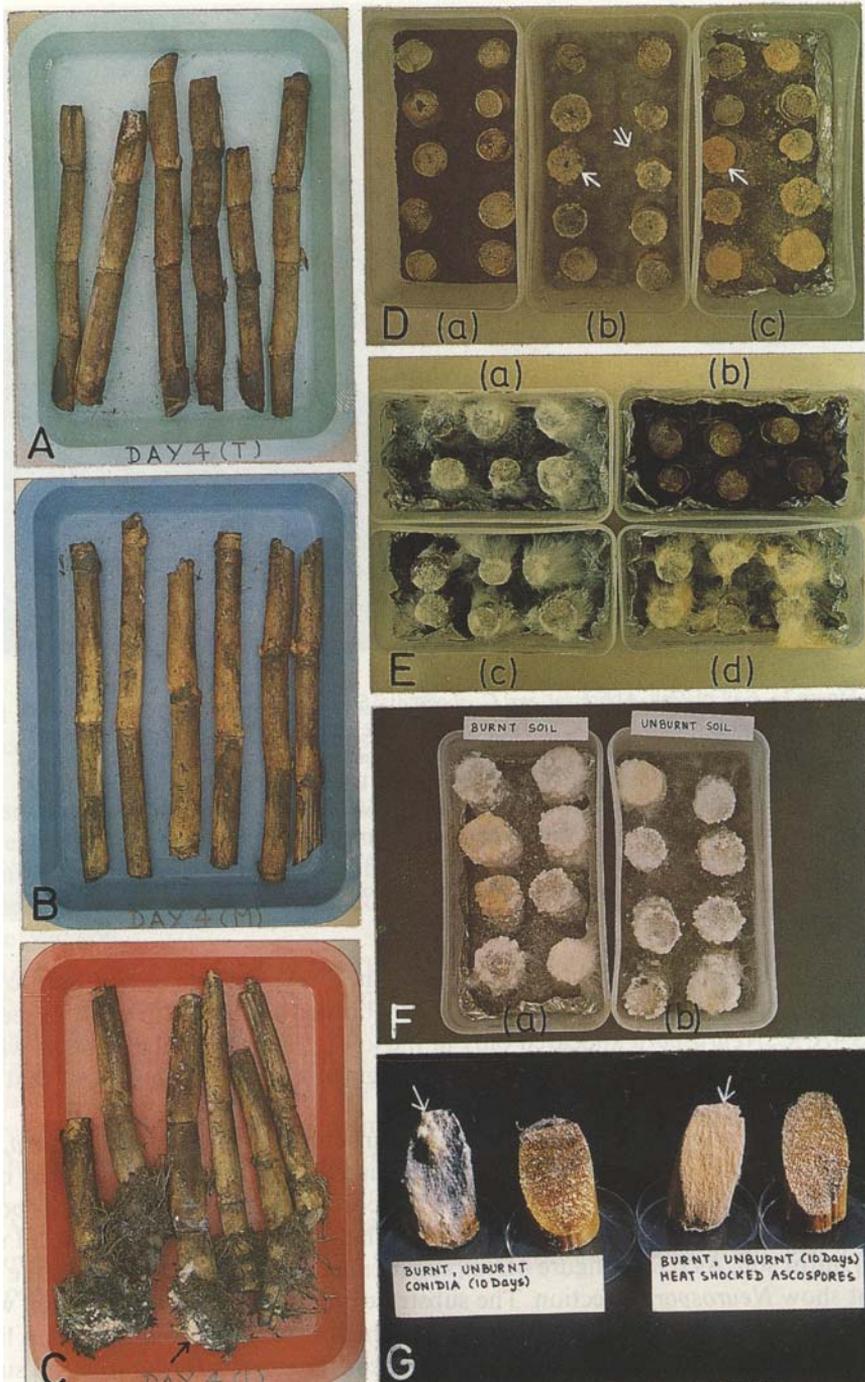


Figure 4.

3.2b *Inoculum in soil is ascospores*: To determine whether conidia or ascospores initiate infection of burnt sugar cane in the field, we used inocula carrying a genetic colour marker gene. Reciprocal mixtures of albino (*al*) and wild type (*al*<sup>+</sup>) ascospores and conidia were mixed into autoclaved soil. The soil and sugar cane pieces were burned and then put in separate boxes. As seen from figure 4E, *Neurospora* did not grow on sugar cane in soil into which only *al* conidia were added. By contrast, the phenotype of *Neurospora* which developed on the sugar cane pieces was that of the genotype of the ascospores that were mixed in soil. The experiment shows that thermoresistant ascospores in the soil and not the heat-vulnerable conidia initiate infection.

3.2c *Heating of soil is not essential for the activation of ascospores*: Hitherto, growth of *Neurospora* on burnt vegetation has been explained on the basis that heat from burning triggers the germination of the dormant ascospores, enabling the fungus to colonize the substrate. However, we have just shown that scorched sugar cane placed on unheated soil also is overgrown by *Neurospora*. Therefore, burnt sugar cane segments were allowed to cool for 15h before placing them over unburnt soil in which *al* ascospores had been mixed. As seen in figure 4F, albino *Neurospora* growth appeared on sugar cane. However, some orange-coloured *Neurospora* also appeared, apparently from the resident wild-type (*al*<sup>+</sup>) ascospores in soil. The results indicate that ascospores in soil are activated by a chemical which diffuses from the burnt (and cooled) sugar cane.

3.2d *Burnt sugar cane becomes a substrate for Neurospora*: In sugar cane fields *Neurospora* colonies were not found on unburnt stumps or on unburnt dry sugar cane leaves although potential inocula (ascospores in soil, conidia in air) were present. Since the previous experiment had shown that *Neurospora* colonized burnt substrate placed over unburnt soil, it appeared that scorching of plant tissue is a necessary requirement for growth of the fungus. The experiment whose results are shown in figure 4G shows that *Neurospora* conidia or heat-activated ascospores are able to infect burnt sugar cane tissue but not unburnt tissue. Therefore, *Neurospora* is essentially a saprophytic fungus and only the burnt sugar cane is a substrate.

How is it that burnt sugar cane is colonized by *Neurospora* to the exclusion of the soil fungi which also survive heat from burning? That *Neurospora* is not inhibited by the high sugar content of sugar cane (approx. 16% of fresh wt; Bull and Glasziou 1975)

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**Figure 4.** Development of *Neurospora* on burnt segments of sugar cane (four days after burning). (A) Upper segments. (B) Middle segments. (C) Lower segments. Note *Neurospora* (arrow). (D) Development of *Neurospora* on burnt cane pieces placed on: (a) Autoclaved field soil. (b) Unburnt field soil. Note *Neurospora* (arrow) and unidentified fungal mycelia over soil (double arrow), (c) Field soil burned in laboratory. Note profuse *Neurospora* growth (arrow). (E) Demonstration that ascospores in soil initiate infection. Cane pieces placed over soil were burned. (a) Soil contained *al* ascospores. (b) Soil contained *al* conidia. (c) Soil contained a mixture of *al* ascospores and *al*<sup>+</sup> conidia. (d) Soil contained a mixture of *al*<sup>+</sup> ascospores and *al* conidia. (F) Demonstration of chemical activation of ascospores in soil by the diffusate from burnt cane. Burnt cane pieces after cooling for 15 h were placed over (a) burnt soil, containing *al* ascospores (b) unburnt soil containing *al* ascospores. Note albino *Neurospora* in both cases (G) Experiment to demonstrate that burnt sugar cane is a suitable substrate for *Neurospora*. Note growth of *Neurospora* on burnt cane (arrow) and its absence on unburnt pieces.

appeared to be an important determinant of its selective colonization of sugar cane. To verify this, the growth rate of a field isolate of *N. intermedia* was compared with that of five unidentified soil fungi which were recovered from freshly burnt field soil. With the exception of one aconidiate fungus, the elongation rate of mycelium (approx. 4.3 mm/h) of *N. intermedia* on Czapek's medium in tubes (Ryan *et al* 1943) was some 11-40 times that of the other four fungi. More importantly, the rapid growth rate of *Neurospora* was virtually the same even in the presence of 15% sucrose (4.7mm/h). The results suggest that the very rapid growth rate of *Neurospora* contributes to its successful colonization of a sugary substrate in advance of other fungi surviving the burn.

3.2e *The chemical activator of Neurospora ascospores in soil is furfural*: As shown in table 2, the diffusate from the burnt sugar cane, but not from the unburnt sugar cane, is as effective as heat-treatment in breaking the dormancy of ascospores of *N. intermedia*. The active component was found to be volatile with steam, so we concentrated it by steam-distillation. The active component has the UV absorption spectrum given in figure 5. The biological activity of the fractions of the steam-distillate was correlated with the concentration of this component as determined by its UV absorption at 276 nm (data not shown).

Tests using a Conway diffusion cell showed that steam distillate activated the ascospores by diffusion through the vapour phase. The active steam-distillate was extracted with diethyl ether which on evaporation at room temperature yielded a small amount of a pale yellow oily liquid with a characteristic odour. The sample was dissolved in a minimum volume of water and purified by HPLC (figure 6). The material eluting at approximately 20 min (compound X) was biologically active.

The HPLC purified unknown compound (X) had a UV spectrum identical to furfural and both compounds had identical retention times on HPLC (data not shown). Co-chromatography of compound (X) with furfural by HPLC showed an enhancement in peak at the retention time of compound (X) (data not shown). The total ion

**Table 2.** Activation of *Neurospora* ascospores by sugar cane diffusate and furfural.

Species	Germination of ascospores (%) <sup>a</sup>						
	Control <sup>b</sup>	Heat-shocked <sup>c</sup>	Sugar cane diffusate <sup>d</sup>		Furfural (M)		
			Unburnt cane	Burnt cane	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
<i>N. crassa</i>	0	94-99	nd <sup>e</sup>	91	92	94	13
<i>N. intermedia</i>	0	96-99	1	93	100	62	2
<i>N. tetrasperma</i>	0	95-100	0	13	95	47	0
<i>N. sitophila</i>	0	87-93	nd	3	65	0	0
<i>N. discreta</i>	0	78	nd	nd	83	76	3

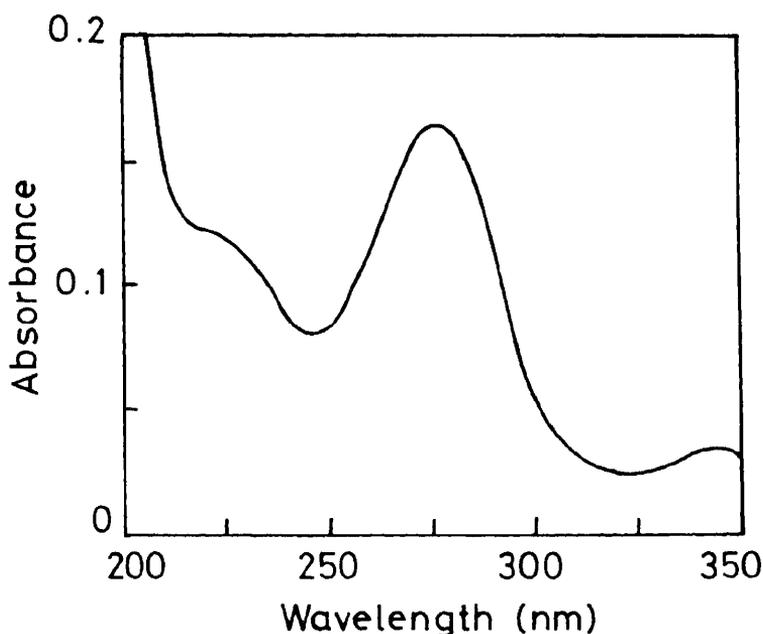
<sup>a</sup>Ascospores were harvested in 0.8% (w/v) Na<sub>2</sub> EDTA and washed with sterile distilled water before use.

<sup>b</sup>Incubated in distilled water containing chloramphenicol.

<sup>c</sup>As in control but given a heat shock at 60°C for 30 min.

<sup>d</sup>Ten, 2.5 cm pieces were soaked in 100 ml distilled water containing chloramphenicol for 24 h.

<sup>e</sup>not determined

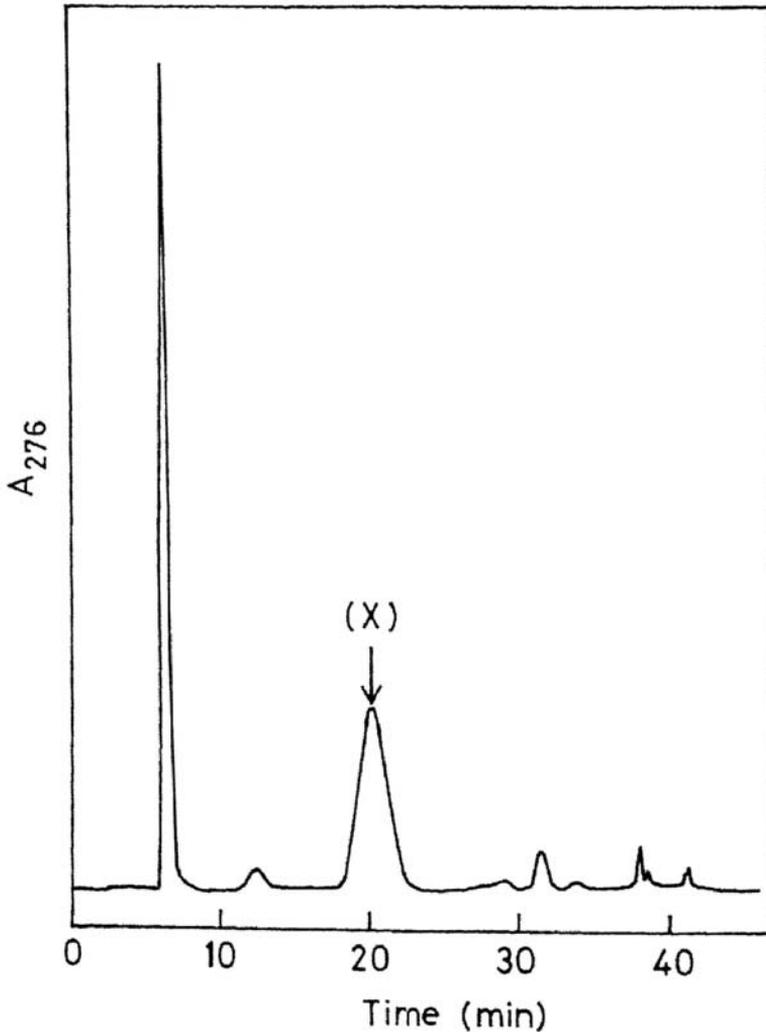


**Figure 5.** UV spectrum of burnt sugar cane diffusate after steam distillation.

chromatograph and mass spectrum of compound (X) purified by HPLC is shown in figures 7 and 8, respectively. The mass spectrum was similar to (94% homology) that of an authentic sample of furfural (data not shown). Thus, the chemical activator of *Neurospora* ascospores produced upon burning sugar cane was confirmed as furfural. As shown in table 2, furfural isolated from burnt cane and an authentic sample were active on ascospores of all known heterothallic and pseudohomothallic species of *Neurospora*.

To determine if furfural is released from burnt sugar cane into soil, 10 each of burnt and unburnt sugar cane segments were placed separately on sterilized moist soil (200 g) in separate boxes for 12 h following which the two samples were processed in identical manner. A slurry of the soil was made in water and steam-distilled. The steam-distillate (40ml) was extracted in ether and the two samples were subjected to HPLC as before. Both samples showed identical HPLC profiles except for a peak with a retention time of 20 min which was absent in soil in contact with unburnt sugar cane (data not shown). The compound represented by this peak co-chromatographed with authentic furfural as shown by enhancement of the peak at the same retention time. This experiment demonstrates that furfural produced in burnt sugar cane can diffuse into soil.

**3.2f Production of macroconidia on burnt substrate is dependent on supply of water, minerals and sugar:** In order that growth and reproduction of *Neurospora* could be examined closely, in particular with respect to the time of production of macroconidia and their role in the life cycle, conditions approximating that in a sugar cane field were reconstructed in the laboratory. Approximately 25 cm long segments of sugar cane were planted in field soil in pots and ascospores of *N. intermedia* were mixed into the soil. In some experiments *al(bino)* ascospores were used. The canes were covered with straw and burned (figure 9A). The experimental pots (figure 9B) were kept in the open



**Figure 6.** High performance liquid chromatography of steam distillate after ether extraction. Active fraction is indicated by arrow.

and watered regularly. The results of an experiment started in the first week of March (1993) are described below.

Three to four days after the burn, *Neurospora* mycelium was visible creeping over the soil towards the canes (figure 9C). Presumably, the mycelium entered into the cane through cracks induced by burning or through root openings because after 5-7 days of incubation, pustules of macroconidia appeared near the lower node of the canes. The colour of the conidia was characteristic of the genotype of the ascospores added into the soil. In two weeks, more pustules appeared at the upper internode (figure 9D). Watering increased macroconidiation. Inside the cane, a sheath of mycelium was concentrated a few cell layers below the epidermis (figure 10A). Hyphae also ramified into the vascular bundles (figure 10B). At places the weft of mycelium produced parallel, closely-packed hyphae comprising thin- and thick-walled cells.

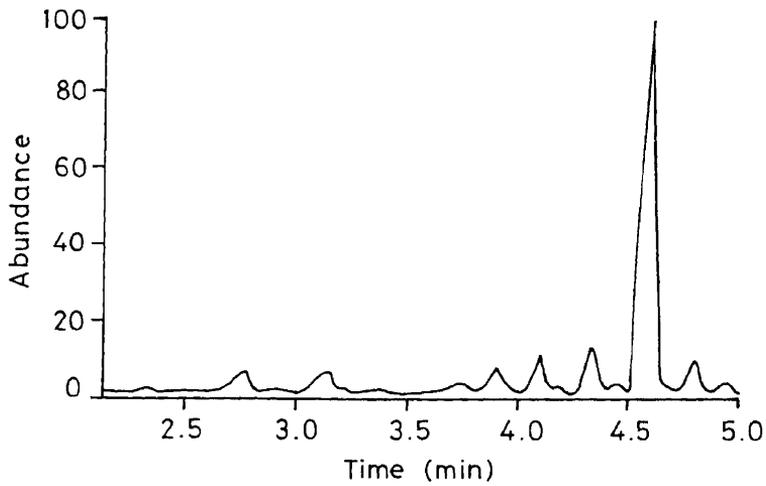


Figure 7. Total ion chromatograph (TIC) of active fraction obtained after HPLC.

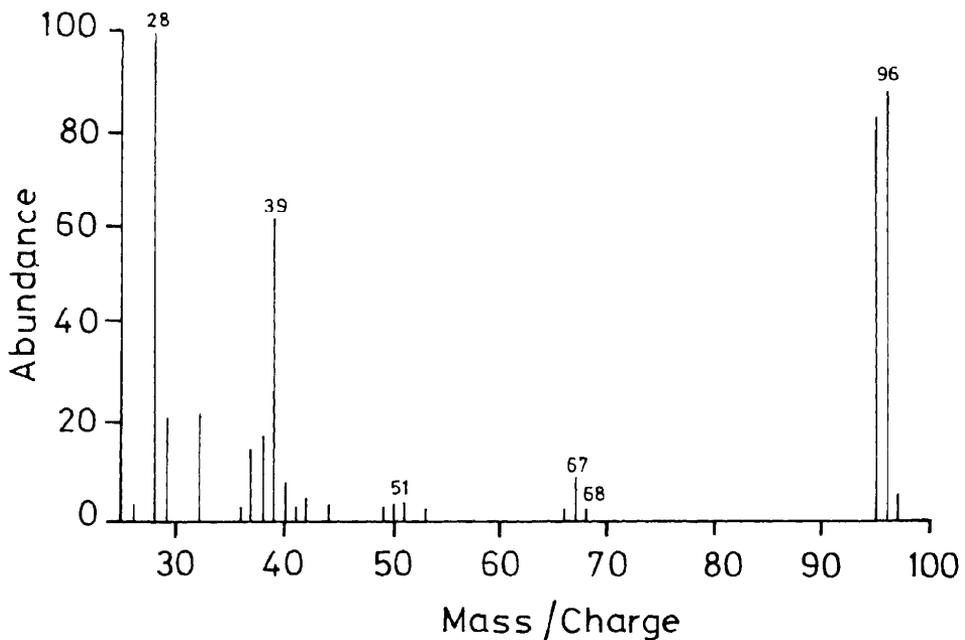


Figure 8. Mass spectrum of major peak from figure 7.

The sporodochia burst through the epidermal tissue and differentiated into conidiophores and produced macroconidia externally. Macroconidia were formed rapidly and dispersed constantly. The pustules commonly were infested by microarthropods which contributed to the disappearance of macroconidia. So long as the subepidermal absorptive mycelium supported conidiation, there was no development of protoperithecia.

After 3-4 weeks the production of macroconidia began to decline and by 7-8 weeks the pustules had withered. To determine the reason for the cessation of macroconidia

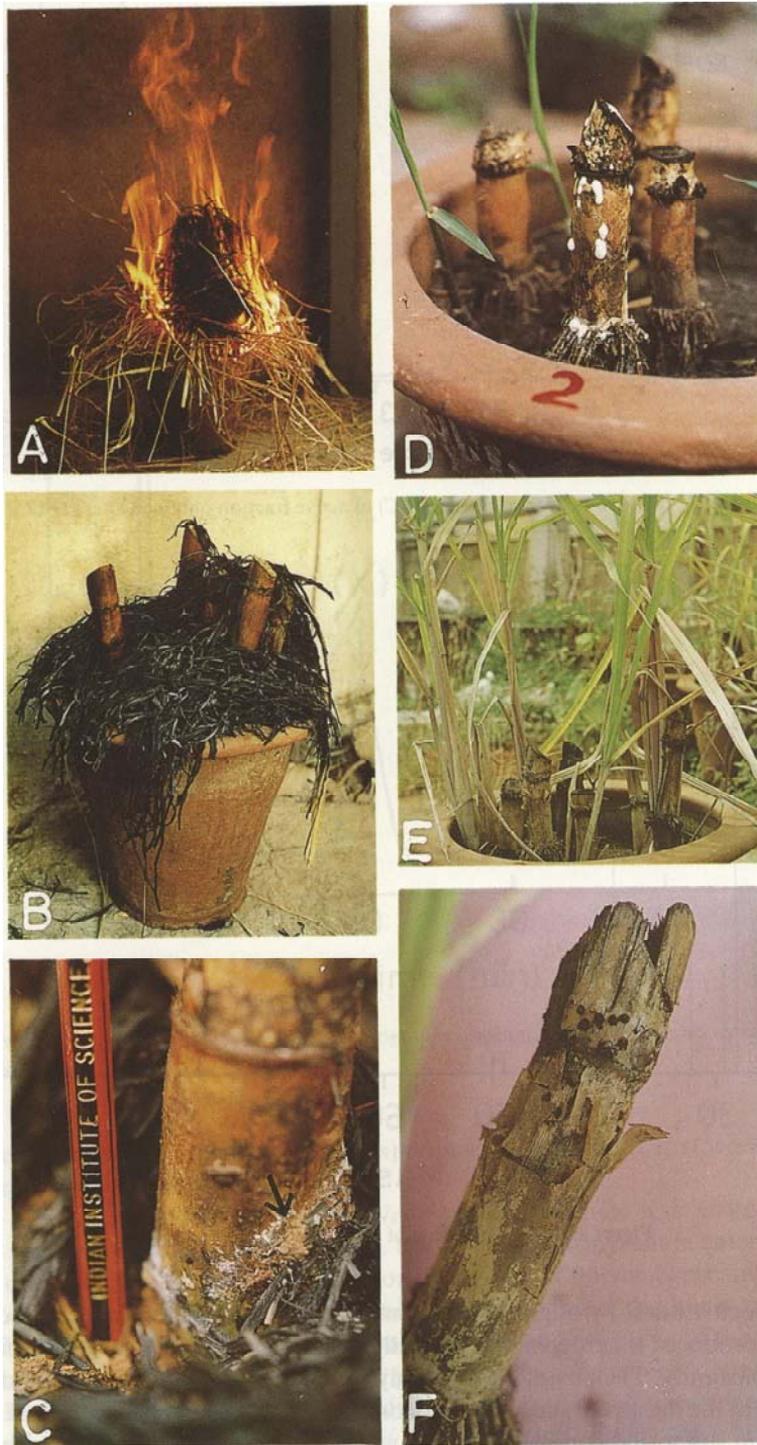
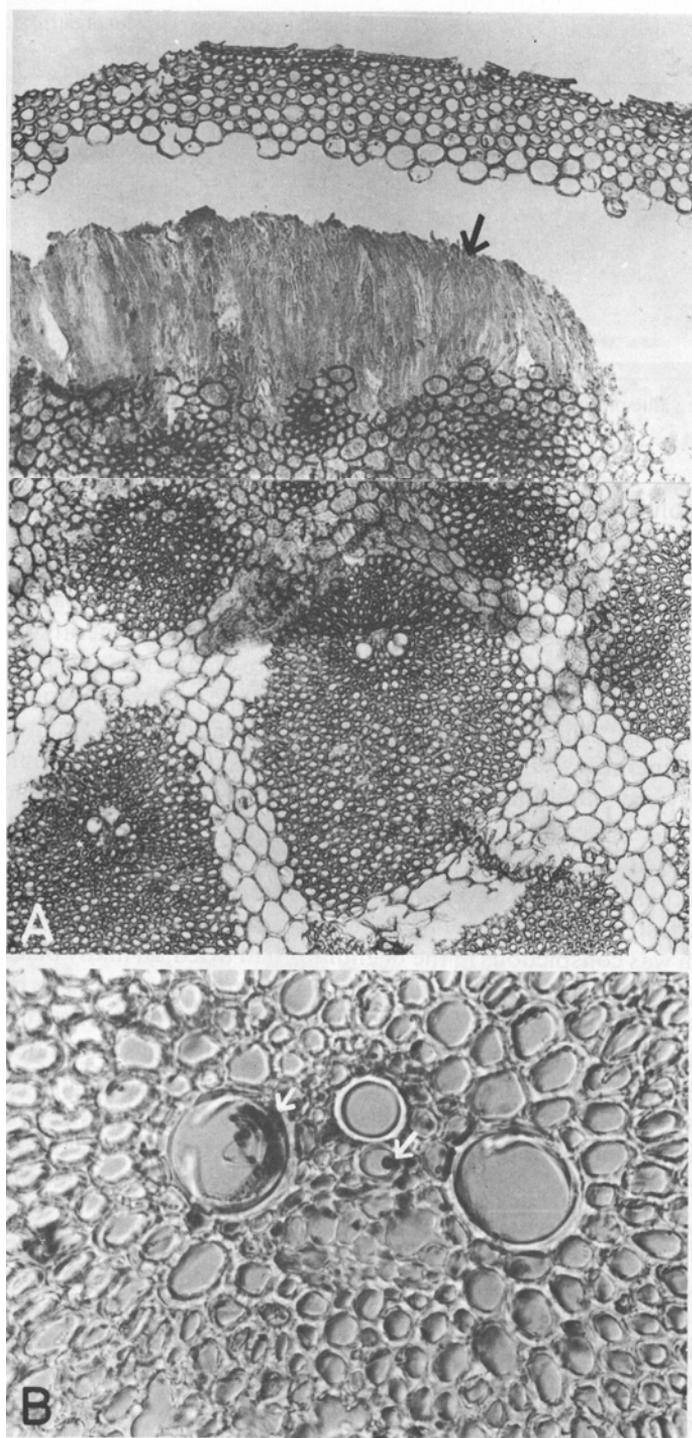


Figure 9. For caption, see p. 75.



**Figure 10.** For caption, see p. 75.

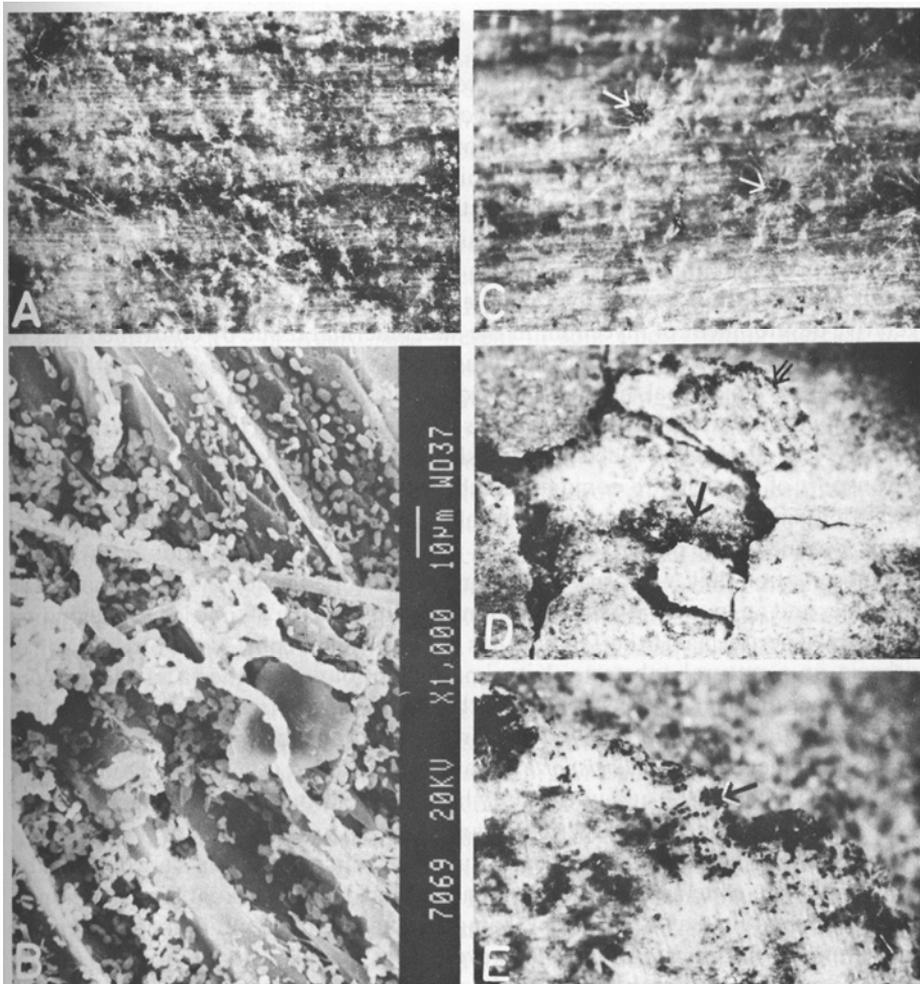
**Table 3.** Vigour of conidiation of *Neurospora* in relation to sugar content of burnt sugar cane stumps.

Time after burning and planting (week)	Soluble sugar (% of dry wt) <sup>a</sup>	Conidiation	Treatment which revived conidiation in tissue blocks taken from 'infected' sugar cane
0	59	Nil	
1	59	Distinct pustules	
2	36	Vigorous	Water
8	27	Ceased	Mineral salts or only a nitrogen salt
13	9	Ceased	Mineral salt + sucrose

<sup>a</sup> Average value of two pieces taken from lower and upper portions of the stumps.

production, soluble sugar content in the canes was monitored (table 3). In addition, conditions required for revival of conidiation were determined. To accomplish this, tissue blocks from old, infected sugar cane were cut, vacuum infiltrated with aqueous solutions and incubated in Petri dishes between moistened filter paper. Conidiation was revived in those tissue pieces which had been treated with mineral solution (Vogel's salt mixture) (Davis and de Serres 1970) or a mixture of minerals and sucrose (2%), but not in those which had been treated with sucrose alone (table 3). Among the elements, nitrogen was most critical for the revival of conidiation. The results showed that depletion of minerals was the chief reason for the cessation of macroconidiation. After 12 weeks, however, treatment of tissue pieces with both minerals and sugars was necessary for the revival of conidiation (table 3).

3.2g *Macroconidiation and sexual reproduction are separated in time. Fertilization is presumably effected by microconidia:* After 16 weeks, the subepidermal web of mycelium, which was conspicuous in the beginning, had dried. In many places it appeared to have been consumed by mites which were common in the tissue. The monsoon rains had begun but the production of macroconidia was not revived. The stumps were now only a fibrous mass of cellulose and hemicellulose and were surrounded by a ratoon crop (figure 9E). The epidermal tissue had begun to flake off at some places (figure 9F). In the rain-soaked canes a fine growth of *Neurospora* mycelium was revived which produced microconidiophores (figures 11 A, B) and protoperithecia (figure 11C). These were produced in little pockets created between the ground and the epidermal tissue. A variety of reproductive structures of various fungi were present in several places. By 20 weeks, the perithecia of *Neurospora* had formed in the pockets in the stumps. Presumably, microconidia were carried to protoperithecia by nematodes which were active in the rain-soaked tissue. The perithecia tended to be clustered (figure 11D) and some had discharged ascospores (figure 11E). Compared with the material from the field, perithecia were common on sugar cane in our reconstruction experiments, probably because the number of ascospores that were added to soil ensured that mycelia of both mating types grew in the canes. Some ascospores were isolated on an agar medium and heat-shocked. As expected from the genotype of the ascospores used as inoculum, these produced albino *Neurospora*. During this time, air sampling in the vicinity of the experimental pots did not reveal the presence of *Neurospora* ascospores.



**Figure 11.** Sexual reproduction by *Neurospora* in burnt sugar cane in reconstruction experiments. **(A)** A light micrograph of microconidiophores on cortex of sugar cane. The epidermal tissue was removed for observation of fungus. **(B)** Scanning electron micrograph of microconidiophores (scale bar 10  $\mu\text{m}$ ). **(C)** Light micrograph of protoperithecia (arrows) and microconidiophores on exposed sugar cane cortex. **(D)** A cluster of perithecia (arrow) formed below the epidermal tissue of sugar cane. Note ascospores on underside of epidermal tissue (double arrow). **(E)** A magnified view of ascospores shot on the underside of epidermal tissue (arrow).

**Figure 9.** Development of *Neurospora* in sugar cane in reconstruction experiments **(A)** Burning of sugar cane segments in laboratory. **(B)** Fire-scorched sugar cane. **(C)** Visible growth of *Neurospora* on scorched sugar cane on the third day (arrow). **(D)** Albino (*al*) *Neurospora* pustules two week after burning. **(E)** Old stumps after 16 weeks surrounded by ratoon shoots. **(F)** A close view of old stump after 16 weeks showing cracked epidermal tissue.

**Figure 10.** *Neurospora* in sugar cane. **(A)** A transverse section of sugar cane showing a subepidermal sporodochium (arrow). **(B)** A magnified view, showing *Neurospora* mycelium in cells of vascular bundle.

## Discussion

The extensive blooms of *Neurospora* observed on burnt sugar cane in several countries attests to the successful genetical and biochemical adaptation of the fungus to this substrate. Therefore, the present observations on growth and reproduction of *Neurospora* on sugar cane, a crop with long history, are especially relevant and fill in a neglected area of *Neurospora* biology. Further, the present work should also be useful in furthering our understanding of the life history of related ascomycetous fungi, some of which are important plant pathogens.

A noteworthy feature of the growth of *Neurospora* in the burnt cane is the extensive vegetative development of mycelium in the subepidermal tissue by controlled lysis of cells. This could serve the following functions: first, the invasive mycelial growth causes a separation of epidermal tissue from ground tissue, followed by rupture by the sporodochium-like structures that produces conidiophores and liberate macroconidia externally; second, the embedded mycelial sheath apparently serves as a food base for the transport of nutrients to conidia, thereby drawing nutrients through the hyphae which ramify in the sugar containing cells of the ground tissue; third, when epidermal tissue is loosened, little pockets are created wherein sexual reproduction occurs.

The observation that *Neurospora* mycelium is embedded inside the epidermal tissue of the canes and an inquiry into its condition with the passage of time led to the finding of submerged perithecia. Hitherto, there has been only one report of the observation of perithecia on a natural substrate. Kitazima (1925) found perithecia of *Neurospora* under a rather unusual circumstance—on the bark of a pine tree in Tokyo which had been burnt in the fire which followed an earthquake! All subsequent attempts to find the teleomorphic state of *Neurospora* in nature have failed (Shear and Dodge 1927; Prakash 1965; Shaw 1990) and uncertainty has persisted concerning the occurrence of its sexual life cycle under natural circumstances.

From the limited number of sugar cane stumps in which perithecia were detected by us, the immediate conclusion was that the actual incidence of sexual reproduction in *Neurospora* in nature may be quite low, being dependent on the simultaneous presence of mycelium of both mating types. However, there is reason to believe that sexual reproduction in nature is more common than suggested by our experience in observing perithecia. The reason is that the perithecia are embedded in sugar cane tissue. Moreover, *Neurospora* perithecia are found in proximity to those of other saprophytic ascomycetes, rendering their recognition very time-consuming. However, the ease with which we are able to isolate *Neurospora* from sugar cane field soil is consistent with the prevalence of sexual reproduction and the liberation of ascospores into the soil.

Our finding of sexual reproduction of *Neurospora* in nature supplements the strong evidence for the generality of this process in nature obtained through population genetic analysis. Perkins and Turner (1988) reviewed studies with a number of different genetic markers in *Neurospora* collected from nature. These data revealed a high degree of variability in the population, consistent with their prediction that a system for outbreeding must exist in nature. Our reconstruction experiments with genetically marked ascospores mixed into soil provide clear evidence that ascospores initiate infection of burnt sugar cane. The pattern of *Neurospora* development on sugar cane in laboratory reconstruction experiments was exactly as in the fields—a clear demonstration that soil-borne ascospores are the inocula for freshly burned sugar cane. It is, perhaps, significant that among some 116 species tested, the only spores which

germinated without any restraint when placed directly on natural soil were the activated ascospores of *N. tetrasperma* (Lingappa and Lockwood 1963). Presumably other species of *Neurospora* also behave similarly. The germinability of *Neurospora* ascospores in addition to the rapid growth rate of its mycelium, despite the high sugar content of the plant tissue, might contribute to this organism's ability to selectively colonize burnt sugar cane.

The development of *Neurospora* colonies on burnt vegetation has been explained on the basis that dormant ascospores require a heat-treatment for activation. We have assessed the role of burning in terms of its effects on microbial population in soil and on the substrate itself. It is clear from figure 4D, that burning kills the propagules of many potential competitors of *Neurospora* present in soil. The resulting increase in the inoculum potential (Garrett 1963) of *Neurospora* would enhance its competitive ability. Although we did not measure the temperature of soil during and after a burn, we have felt intense heat on the surface of the soil soon after a burn in the field. Dix and Webster (1995) mention that at bonfire sites the temperatures of the soil surface may reach 500°C or more. Therefore, it appears likely that protection from heat, rather than activation by it, is of crucial importance to the fungus. Presumably, the ascospores of *Neurospora* survive because of their high content of trehalose (Sussman and Lingappa 1959)—a disaccharide that has been shown to play a role in thermoprotection (Weimken 1990; Colaco *et al* 1992). We believe that activation of ascospores by furfural (Emerson 1948; Sussman 1953), which is derived from hemicellulosic material upon heating (Blatt 1946) may be more important in the field conditions than heat-activation. In the present study it has been demonstrated that furfural is the active component of the diffusate from burnt sugar cane and that it activates ascospores of all heterothallic and pseudohomothallic species of *Neurospora*.

In the field as well as in our reconstruction experiments, perithecia were produced 4-6 months after the burn when the monsoon had begun and the nutritional environment in the burnt sugar cane was no longer favourable for mycelial growth and the associated production of macroconidia. During this time, the plant tissue had been transformed from a chiefly sugary environment into a sugar-depleted cellulosic substrate. The latter condition is known to favour the production of protoperithecia and microconidiophores (Westergaard and Mitchell 1974; Ricci *et al* 1991; Pandit and Maheshwari 1993). Not only are macroconidiation and sexual reproduction separated in time but also in space. As described earlier, macroconidiophores are produced externally whereas perithecia and microconidiophores are produced in tissue pockets – a biological solution for the need to ensure humid conditions for the development of reproductive structures and fertilization (Pandit and Maheshwari 1993). These features of development in *Neurospora* strongly suggest that, under the natural conditions, microconidia play the sexual role in fertilization.

This raises the question of the role of macroconidia which are produced in all heterothallic and pseudohomothallic species of *Neurospora*. Considering that: (i) soil-borne ascospores, and not air-borne conidia, initiate primary infection in sugar cane, (ii) the production of macroconidia and of perithecia are well separated in time, (iii) the nutritional conditions favouring the production of macroconidia and perithecia are quite opposite, and (iv) viable macroconidia are unlikely to gain access to protoperithecia which are formed in the tissue pockets, our data do not support a role of macroconidia in the dispersal or cross-fertilization of *Neurospora* in the ecosystem represented by the sugar cane fields. However, in this habitat, it is very likely that they

do perform a different and a crucial physiological role: their prolific production and dispersal into air are mechanisms for the removal of sugar from plant tissue and create nutritional conditions favourable for sexual reproduction.

This information on the life-history of *Neurospora* in nature is important to studies on ecology and speciation. Thus, the present work extends the pioneering study of population biology initiated by David Perkins (Perkins *et al* 1976). Our work not only provides direct evidence for the prevalence of sexual reproduction by *Neurospora* in nature, but also for the origin of colonies on burnt sugar cane from ascospores. The collection of fresh visible *Neurospora* colonies from burnt plant material will continue to be valuable for evaluation of genetic variation within populations.

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Corresponding editor: M S SHAILA