

Relations Among Sorghum Ergot Isolates from the Americas, Africa, India, and Australia

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ABSTRACT

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Sorghum ergot, initially restricted to Asia and Africa, was recently found in the Americas and Australia. Three species causing the disease have been reported: *Claviceps sorghi* in India, *C. sorghicola* in Japan, and *C. africana* in all ergot-positive countries. The objective of our study was to study the intraspecific variation in *C. africana* isolates in the Americas, Africa, India, and Australia. We confirmed *C. africana*, *C. sorghi*, and *C. sorghicola* as different species using differences in nucleotide sequences of internal transcribed spacer 1 and 5.8S rDNA regions. Sequences of this region obtained from the representative American, Indian, and Australian isolates of *C. africana* were identical. In addition, random amplified polymorphic DNA (RAPD) banding patterns of sorghum ergot pathogen isolates from the United States, Mexico, Puerto Rico, Bolivia, Australia, and India were evaluated with nearly 100 primers. A total of 65 primers gave identical patterns for all isolates, which confirmed that all were *C. africana*. The identity of RAPD pattern and rDNA sequence of Indian isolates with those of *C. africana* confirmed that the species is now present in India. Only 20 primers gave small pattern differences and 7 of them were used for routine testing. All of the American isolates were identical and three isolates of the same type were also found in South Africa, suggesting Africa as the origin of the invasion clone in the Americas. Australian and Indian isolates were distinguishable by a single band difference; therefore, migration from the Asian region to Australia is suspected. Another distinct group was found in Africa. Cluster analysis of the informative bands revealed that the American and African group are on the same moderately (69%) supported clade. Isolates from Australia and India belonged to another clade.

Additional keywords: *Sphacelia sorghi*, sugary disease

Ergot disease of sorghum recently gained prominence due to sudden expansion in its geographical distribution (5). The disease is a serious constraint in the production of F₁ hybrid seed, an essential input in productive sorghum cultivation systems worldwide. Crop losses are caused by reducing the quantity and quality of seed, seed predisposition to seedling diseases, and difficult harvesting and threshing. The presence of ergot bodies in seed

creates serious problems in the international trade of sorghum produce.

Sorghum ergot was first detected in India in 1917 (24) and, subsequently, in Kenya in 1923 from its co-occurrence with the specific hyperparasite *Cerebella andropogonis* Cesati which overgrows sphacelia (23). The ergot pathogen colonizes ovaries, replacing them individually with the sphacelial mass. Sphacelia (anamorphic conidiogenous form of *Claviceps* spp.) exude sweet, sticky honeydew in which millions of conidia are released. Later, the fungal tissues may differentiate to form sclerotia. Initial research on sorghum ergot, conducted in Samaru, Nigeria in the mid-1960s by a team of researchers from the Agricultural Research Service of the United States Department of Agriculture, illustrated the high susceptibility of male-sterile parents of F₁ hybrids (16).

Three *Claviceps* spp. have been reported to cause ergot in different parts of the

world. These are *C. sorghi* Kulkarni, Se-shadri, and Hegde, *C. africana* Frederickson, Mantle, and de Milliano, and *C. sorghicola* Tsukiboshi, Shimanuki, and Uematsu. *C. sorghi* was first reported as the perfect stage in India in 1976 (20), and for the next 15 years was also assumed to represent the perfect stage of the sorghum pathogen in Africa. *C. africana* was formally described as the cause of sorghum ergot in Zimbabwe in 1991 (12), and *C. sorghicola* in Japan in 1999 (33).

C. africana, *C. sorghi*, and *C. sorghicola* are distinguished as different species by their teleomorph morphology (features of stipe, capitulum, asci, and ascospores), morphology of sclerotia (small and rounded in *C. africana*, elongated and protruding in *C. sorghi*), dihydroergosine content of sclerotia in *C. africana*, and ability to produce secondary conidia (11,12,20,33). The imperfect stages of *C. sorghi* and *C. africana* are similar and are both named *Sphacelia sorghi* McRae (24). Conidia of *C. sorghicola* are much smaller in size than those of the other two species.

The global distribution of sorghum ergot has been recently summarized (5). Introduction of F₁ hybrid seed technology is linked to the enhanced importance and wider distribution of ergot in Africa and Asia (3,8,11). After being restricted to Asia and Africa for nearly 80 years, sorghum ergot was detected in Brazil in mid-1995 and identified as *C. africana* (26). *C. africana* spreads most efficiently by wind-borne secondary conidia (11,13) and, by mid-1996, the disease was reported from Argentina, Paraguay, Uruguay, and Bolivia, countries close to ergot-affected areas in Brazil. Subsequently, the disease continued to spread northeastwards, covering Colombia, Venezuela, and Honduras by the end of 1996, and the Caribbean, Mexico (35), and the continental United States by the end of March 1997 (17). *C. africana* was reported from Australia in April 1996. The source country of inoculum introduction into the Americas and Australia is not clearly known. However, an African country was initially assumed to be the source of inoculum introduced into Brazil and Australia because *C. africana* was known

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as prevalent only in Africa before ergot spread to the Americas. Recent alkaloid analyses revealing the presence of dihydroergosine in the Indian isolates proved that *C. africana* is present in India, too (7).

In India, smaller sclerotia resembling the ones of *C. africana* frequently occurred naturally in the field together with the elongated sclerotia typical only for of *C. sorghi* (4,28). However, the second author has not seen typical sclerotia of *C. sorghi* naturally occurring in the field during the last 12 years. The ergot pathogen in India also produces secondary conidia (6), a character associated with *C. africana* and not *C. sorghi* (12). Therefore, the need to determine the identity and distribution of the ergot pathogen in India was stated earlier (14).

Sclerotia of *C. africana* do not germinate readily (12). Therefore, traditional methods of identifying *Claviceps* spp. using teleomorph characters are impractical. DNA-based methods are good molecular tools to resolve problems of identification of plant pathogens (19) as demonstrated in several pathogens including species of *Mycosphaerella* causing Sigatoka disease of banana and plantain (18).

The objective of our research was to study the intraspecific variability of the *C. africana* isolates recently collected in the United States, Mexico, Puerto Rico, Bolivia, India, South Africa, and Australia with respect to the possible origin of invasion clones in the Americas and Australia. An authenticated isolate each of *C. sorghi* from India and *C. sorghicola* from Japan

were also included in the study for comparison. We used random amplified polymorphic DNA (RAPD) and sequencing of internal transcribed spacer (ITS) 1 and 5.8S rDNA regions to substitute for traditional mycological methods of germinating sclerotia of sorghum-infecting *Claviceps* spp. for pathogen characterization.

MATERIAL AND METHODS

A total of 28 isolates of *C. africana* from the United States, Mexico, Puerto Rico, Bolivia, Australia, India, and South Africa (Table 1) were used in the RAPD study. Isolates from the United States, Mexico, Puerto Rico, and Bolivia are together termed "American" isolates in this paper. The DNA from *C. sorghicola* (Japan), *C. sorghi* (India), and one representative ergot isolate each from Bolivia, Australia, and India were used in the nucleotide sequence work. DNA of *C. sorghi* and *C. sorghicola* was isolated from sclerotia. Sclerotia of *C. sorghi* were formed in England in 1987 on male sterile sorghum line 2219A inoculated by an isolate originating from Akola, Maharashtra, and India (identical to that described in 12). Sclerotia of *C. sorghicola* were similarly derived in England in 1996 from male-sterile sorghum inoculated with conidia of the pathogen provided by T. Tsukiboshi.

Isolation and cultivation of the mycelial cultures. Indian isolates were isolated from infected panicles collected at different locations and sent to the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). Panicles were soaked

in water to release ergot conidia from the honeydew. Male-sterile sorghum panicles in the greenhouse were immersed in the resultant conidial suspension and bagged to induce infection on fresh stigmas. Young (5 days after inoculation) sphaecelia were dissected from infected flowers before honeydew exudation, surface sterilized in 1% sodium hypochlorite for 3 min, rinsed five times in sterile water, and plated on potato dextrose agar (PDA) medium. Passage of the fungus through the plant ensured a higher success rate for obtaining pure cultures than isolation from the field-collected samples that were often heavily contaminated with saprophytes.

Australian isolates were obtained as pure cultures authenticated as *C. africana*. The American and African isolates were cultivated directly from infected plant material. Young sclerotia or sphaecelia were surface-sterilized for 15 min in 1.3% sodium hypochlorite, washed for 1 to 2 min in 95% EtOH, and rinsed three times in distilled water. Sterilized sclerotia were placed on T2 agar plates (30) supplemented with 100 µg/ml of ampicillin. T2 medium (per liter of distilled H₂O) is composed of sucrose, 100 g; L-asparagine, 10 g; yeast extract, 0.1 g; KH₂PO₄, 0.25 g; MgSO₄·7H₂O, 0.25 g; FeSO₄·7H₂O, 0.02 g; ZnSO₄·7H₂O, 0.015 g; KCl, 0.12 g; Ca(NO₃)₂·4H₂O, 1 g; agar, 20 g; pH 5.2. The isolates were maintained on T2 or PDA agar slants at 4°C and subcultured every 3 to 6 months.

Preparation of genomic DNA. Spore or mycelium suspensions of the isolates were plated on cellophane discs laid on T2 or PDA plates and grown for 2 to 3 weeks at 24°C. Mycelium was scraped to liquid nitrogen and ground to a fine powder. To 0.5 g of mycelium, 3 ml of extraction buffer (2% Triton X-100; 1% sodium dodecyl sulfate; 0.25M NaCl; 0.1 M Tris-HCl, pH 7.8; 0.1 M EDTA, pH 8.2) and 3 ml of phenol-chloroform were added. The suspension was gently agitated for 15 min, centrifuged at 10,000 × g, and the supernatant precipitated for 2 h with 0.7 volume of isopropanol at 0°C. The pellet was dissolved in 3 ml of 1× Tris-EDTA, pH 8.2, treated with 50 µg of RNase, extracted two to three times with chloroform, precipitated with 0.7 volume of isopropanol/0.3M sodium acetate, and dissolved in 200 to 300 µl of sterile H₂O. Polysaccharides were then partially removed by the addition of 150 µl of Nucleon Phyto-Pure DNA extraction resin (Amersham Life Science, Amersham, UK) and 400 µl of chloroform cooled to -20°C, shaken for 10 min at room temperature, and centrifuged at 10 000 × g for 10 min. The DNA was precipitated from the water phase with 0.7 volume of isopropanol/0.3M sodium acetate.

DNA from *C. sorghi* was isolated from 0.3 g of sclerotia by the above method without Nucleon treatment. The purified DNA was degraded to a smear, but amena-

Table 1. Identity of *Claviceps africana* isolates, their origin (location), year of collection, and collector's name

Isolate ^a	Origin	Collected	Source
Dharwar 2	Dharwar, Karnataka, India	1997	S. Indira
ICRISAT 2	Patancheru, Andhra Pradesh, India	1997	R. Bandyopadhyay
Unknown	India ^b	1997	S. Indira
Akola 2	Akola, Maharashtra, India	1997	C. Sangitrao
Indore	Indore, Madhya Pradesh, India	1997	S. Indira
Australia	Mutchilba, Queensland, Australia	1996	J. L. Alcorn
T10 763	Norwin, Queensland, Australia	1996	M. J. Ryley
T10 764	Norwin, Queensland, Australia	1996	M. J. Ryley
T10 765	Mutdapilly, Queensland, Australia	1996	M. J. Ryley
T10 777	Kingaroy, Queensland, Australia	1997	M. J. Ryley
Bolivia 2	Bolivia ^b	1996	P. G. Mantle
E 627	Kelly Green, Texas	1997	R. A. Frederiksen
A 623/1 ^c	College Station, Texas	1997	R. A. Frederiksen
A 623/2 ^c	College Station, Texas	1997	R. A. Frederiksen
A 357/1 ^c	Texas A&M Research Farm, College Station, Texas	1997	R. A. Frederiksen
TAMU	Texas A&M Research Farm, College Station, Texas	1997	R. A. Frederiksen
MX 2.1	Celaya, Mexico	1998	R. Bandyopadhyay
MX 2.2	Celaya, Mexico	1998	R. Bandyopadhyay
MX 6	Celaya, Mexico	1998	R. Bandyopadhyay
MX 8	Celaya, Mexico	1998	R. Bandyopadhyay
MX 9.2	Celaya, Mexico	1998	R. Bandyopadhyay
PR CI	Puerto Rico	1998	R. Bandyopadhyay
Africa 1	Potchefstroom, Northwest, South Africa	1998	N. W. McLaren
Africa 2/1	Hazyview, Mpumalanga, South Africa	1999	N. W. McLaren
Africa 2/2	Hazyview, Mpumalanga, South Africa	1999	N. W. McLaren
Africa 3/3	Hazyview, Mpumalanga, South Africa	1999	N. W. McLaren

^a Host of all isolates is *Sorghum bicolor*, except T10 764 which was isolated from *Sorghum* spp.

^b Place of collection is unknown.

^c Isolated from greenhouse-grown infected plants.

formed moderately supported (69%) clade with groups consisting of American isolates, Africa 2/1, Africa 2/3, and Africa 3/3. The second clade was formed by Indian and Australian isolates.

DISCUSSION

We expected that the Indian isolates may be different from the American and Australian isolates because only *C. sorghi* had been reported in India so far. However, three representative isolates from Bolivia, Australia, and India had identical nucleotide sequences in the ITS1 and part of the

5.8S rDNA regions. The Australian isolates had been confirmed as *C. africana* (27) after comparison with the type *C. africana* isolate from Zimbabwe (12). Our conclusion that *C. africana* is present in India is further supported by similarity in the RAPD patterns. The same conclusion was also recently arrived at by Bogo and Mantle (7) using biological and biochemical data.

C. sorghi was undoubtedly present in India, but the presence of *C. africana* went undetected earlier. A comparative study of *C. africana* and *C. sorghi* (12) used only

the large sclerotia of *C. sorghi* from Akola, India, which germinated readily. However, problems with sclerotial germination in *C. africana* were highlighted and it was the main factor delaying the proper description of *C. africana*. All attempts to germinate the smaller sclerotia of size and shape similar to *C. africana* have been futile in India.

A literature search and its interpretation in the present context suggest that there may have been a gradual change in the species causing sorghum ergot in India to the possible predominance of *C. africana* over *C. sorghi* at present. Sclerotia of different sizes have been seen in India. Early Indian observations during 1914 to 1917 (1) and 1946 (25) on ergot in Karnataka and Andhra Pradesh States, as well as a survey in Akola (Maharashtra state) and Dharwar (Karnataka state) during the early 1960s (29), mention mostly large elongated sclerotia found on sorghum. Both long and short sclerotia were observed in the late 1970s (20,28) to mid-1980s (Fig. 3), which might have been due to variability in the size of sclerotia of *C. sorghi* or to a start of *C. africana* infections. In any case, since the late 1980s, the typical long and protruding sclerotia of *C. sorghi* have not been observed in the fields in Karnataka, Andhra Pradesh, and Maharashtra states (Fig. 4). However, an analysis of population structure with more isolates from each location is necessary to determine the relative preponderance of the two sorghum ergot species in India.

The RAPD patterns of a limited number of recent isolates from Akola and Dharwar, where only *C. sorghi* had been reported earlier (12,20), show that there has been a change in the species composition of sorghum ergot in India. There may not be a way to precisely document when the changes began. We hypothesize that the process started with the introduction of hybrid seed technology in India in the 1970s.

Reproductive potential is an important determining factor for the relative predominance of two pathogen species competing for the same niche in an ecosystem. Production of secondary conidia and their spontaneous wind dispersal are most likely the key characters of pathogenic fitness in sorghum ergot. The ability of *C. africana* to rapidly produce wind-dispersible secondary conidia (11) offers the pathogen a remarkable potential to spread quickly over time and space (13). Also, environmental conditions (high humidity, dew, or rain) favorable for the production of secondary conidia occur frequently, allowing the continual presence of secondary conidia in the air for a long period. In contrast, *C. sorghi* does not produce secondary conidia in vivo (12). The absence of secondary conidia in *C. sorghi* makes it rely primarily on rain splash for spread. Rain-splash dispersal is periodic because it is dependent on rain

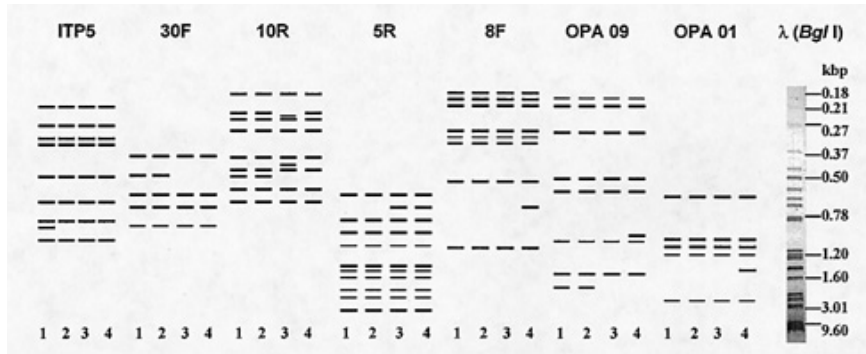


Fig. 2. Schematic random amplified polymorphic DNA patterns of the groups and isolates of *Claviceps africana*. 1 = Australian isolates; 2 = Indian isolates; 3 = African isolates Africa 1 and Africa 2/2; and 4 = American isolates and African isolates Africa 2/1, 2/3, and 3/3. Only the reproducible bands, regardless of their intensity, were scored. No other bands that would discriminate inside the groups were found.

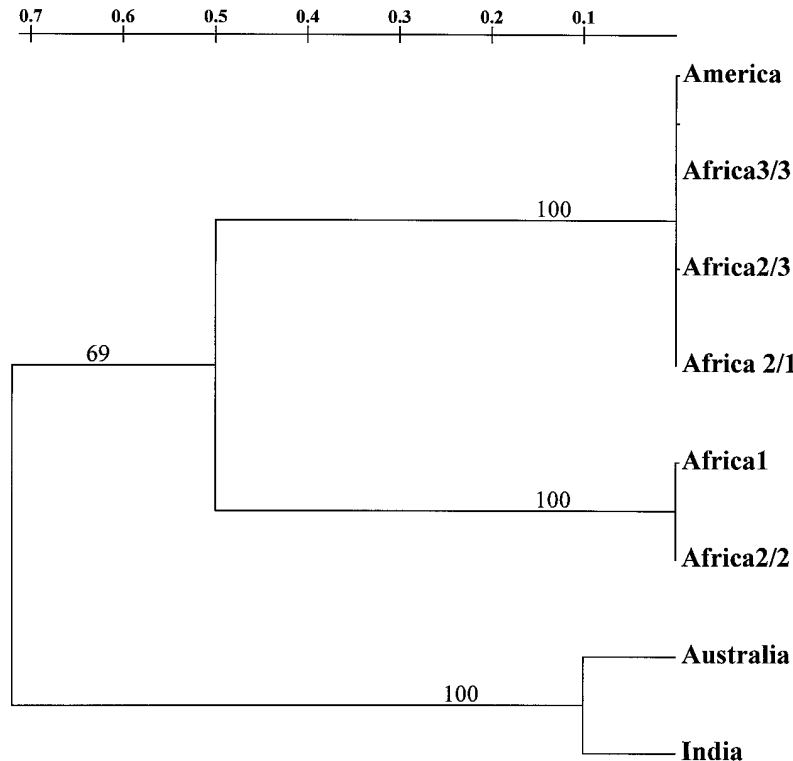


Fig. 3. Dendrogram constructed from random amplified polymorphic DNA data showing the relatedness of *Claviceps africana* groups. Matrix of pairwise distances was calculated using simple matching coefficient (distances shown above the dendrogram). The dendrogram was generated using unweighted pair group method with arithmetic mean. Bootstrap values are given on appropriate branches.

events that are not frequent in the semi-arid tropics where sorghum is grown. Therefore, *C. africana* has greater potential for spread, making it epidemiologically more fit than *C. sorghi*. After the appearance of *C. africana* in India, greater dispersal efficiency and potential to spread are most likely the major factors related to its predominance over *C. sorghi*. In retrospect, the isolate used in the study that demonstrated the production of secondary conidia in *C. sorghi* (6) most probably belonged to *C. africana*, because the production of secondary conidia is a typical feature of this species and morphology of the conidia was identical to the species described in Zimbabwe (11,12).

The ITS-rDNA sequence of the three species causing sorghum ergot differed substantially, confirming species differentiation determined on morphological characters. Both *C. africana* and *C. sorghicola* are present in Japan (22,33) and *C. sorghicola* does not produce secondary conidia in nature; therefore, it would be interesting to study the changes in the population structures of the two sorghum ergots in Japan over a period of time.

Partitioning of the 28 *C. africana* isolates into four geographically distinct groups suggests that pathogen introduction into the Americas and Australia may have occurred from two different sources. India,

or a Southeast Asian country such as Thailand, where *C. africana* is also present (12), is the likely origin of ergot introduction into Australia. Similarities in the RAPD pattern of isolates from India and Australia support this assumption. On the other hand, the presence of isolates with identical patterns in South Africa and in the Americas suggests that Africa is the source of ergot pathogen introduction in the Americas.

There is little likelihood of the occurrence of sexual recombination as a mechanism to generate variability in *C. africana* because sclerotia germinate inefficiently in vitro and germination has not been reported in nature. Moreover, *C. purpurea* was found to be homothallic (9); therefore, the same mechanism could apply for *C. africana*. The lack of a sexual cycle combined with homothallism will no doubt lead to a clonal character for *C. africana* populations. Also, repeated recovery of uniform patterns in the isolates over several years and long distances suggests predominant clonal reproduction (2), at least inside the rapidly invading populations. There is a need to analyze the population variability of *C. africana* isolates from across Africa to elucidate the spreading patterns and population structure of *C. africana*. Our preliminary result with isolates from just two localities have shown

that representatives of different groups occurred in the same field, which may suggest greater variability.

Intensive research on late blight of potato and tomato has shown that maximum diversity in *Phytophthora infestans* occurs in the highlands of Mexico (15). Taking into account the spreading pattern of *C. africana* in Africa during the past 40 years, we suggest that the highlands of eastern Africa, a region close to the center of origin of sorghum and where ergot continues to be endemic, is the most likely geographic area for the center of diversity for *C. africana*. Analysis of isolates from this region could give clues to the origin of the aggressive clones that have begun to threaten sorghum worldwide during the past 10 years.

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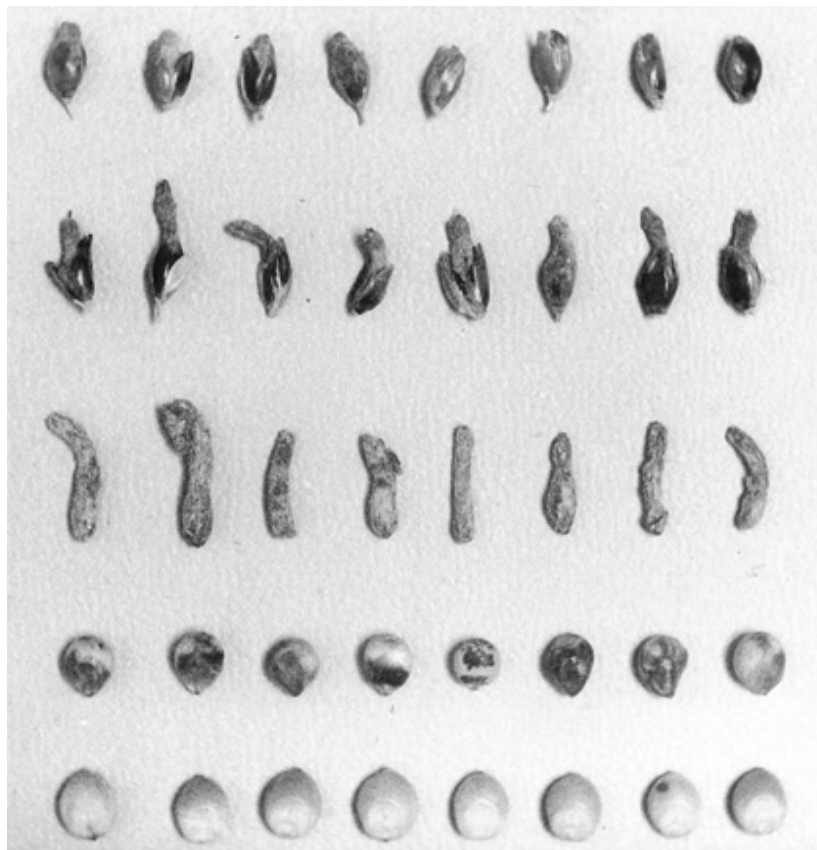


Fig. 4. Differences in the size of sorghum ergot sclerotia and grain discoloration because of honeydew. Top to bottom: small sclerotia concealed inside glumes, long sclerotia with glumes, long sclerotia removed from glumes, discolored seed, and healthy seed. Sclerotia were collected in 1985 from the same sorghum field in Akola, Maharashtra State, India.

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