Preservation of Fungal Culture with Special Reference to Mineral Oil Preservation

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Abstract

It is very important to preserve fungi so that they can be studied and utilized in future. Fungal resource centres play a key role in conservation of fungi for research pertaining to diversity, taxonomy, epidemiology, biotechnology, biosafety, biosecurity and IPR issues. Preserved cultures of fungal strains are utilized by all stakeholders associated with agriculture, pharmaceutical, brewery and industry for developing new technologies and products for all the sections of the society. Various preservation methods of fungi are given in this chapter with special reference to mineral oil preservation method which is easy, simple and cost-effective as it does not require any sophisticated tool and material. This is the simplest method for preservation of both sporulating- and non-sporulating fungi in any small laboratory and small-scale industry where infrastructure is less.

Keywords

Fungi · Biosafety · Mineral soil preservation · Culture collections · Microorganisms

21.1 Introduction

Fungi, integral component of the Earth, are associated with various ecosystem functions and services. Over the years, our understanding about fungi has developed for their utilization as biological 'tools' in biotechnology. Fungal-based fertilizers,

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growth stimulators and bioinsecticides have the potential to enhance production and to ameliorate barren and degraded lands. In medical sciences, novel pharmaceutical products, blood proteins, hormones, interferon, cell growth stimulators, insulin, therapeutic products, etc. are being produced by employing fungi and other microbes (Blanch et al. 1985). Gases, power-alcohols, petroleum substitutes and other renewable energy sources can be produced using fungi. Recycling processes relying on fungal based biotechnology are cost-effective by means of disposing effluent (Robinson and Howell 1985). Besides, authentic and viable fungal cultures are needed to use as reference for identification of human, plant and animal pathogens and also for carrying out taxonomic and ecological studies. Scientific development in the field of mycology is incumbent upon the availability of authentic cultures defined in various publications and patent applications for independent study. Fungal studies are based on the availability of quality cultures that are well defined and this can be taken care of by their long-term storage. That is why culture collections are important for the preservation of diverse fungi and also serve as a source from which material can be had for teaching, research and other purposes (Onions 1971). The upkeep of fungal cultures in collections is a tardy task. It is advantageous to have a procedure that ensures the viability of valuable strains. One of the methods which is very simple and widely used for extending the ordinary stock cultures is mineral oil method (Buell and Weston 1947; Hartsell 1953). It is, therefore, of paramount importance to preserve cultures of high quality for use in agriculture, medicine, industry, etc. and also in the area of taxonomy (Malik and Claus 1987). This chapter will discuss about fungal culture collection or resource centres as well as methods for preservation of fungi.

21.2 Fungal Culture Collections

Fungal culture collections have an important place in microbiology and biotechnology. Their primary role is to preserve cultures and competent to provide information on the availability, identification, maintenance, nomenclature of cultures and regulations pertaining to transportation and patent regulations. Specialized culture collections give advice and provide services to industry. To characterize and identify fungal cultures is a time-consuming task. These cultures are invaluable and should be preserved and deposited at Culture Collection Centres at national and international level (Tables 21.1 and 21.2). At these centres, the experts properly maintain the cultures that are subjected to rigorous rules and regulations to safeguard the intellectual property rights of the depositors. The culture can be procured from these centres on payment. Therefore, these cultures are very valuable from commercial point of view (Anonymous 2008). As per World Data Centre for Microorganisms, total 3,119, 654 microorganisms comprising1,341,588 bacteria, 824,696 fungi, 38,622 viruses and 32,220 cell line are preserved in 781 culture collections in 76 countries and regions are registered in CCINFO (http://www.wfcc.info/ccinfo/. Accessed as of 13th June, 2019).

Table 21.1 National status of fungal culture collections: 15 out of 32 culture collections in India are fungal collection centers

Culture collection center	No. of fungal strains
1. Culture Collection, Department of Microbiology, Bose Institute	40
2. Culture Collection, Microbiology and Cell Biology Laboratory, Indian Institute of Science	78
3. Food and Fermentation Technology Division, University of Mumbai	20
4. Goa University, Fungus Culture Collection and Research Unit	1000
5. Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute	3800
6. MACS Collection of Microorganisms, Agharkar Research Institute	08
7. Microbial Culture Collection, National Centre for Cell Science	15,338
8. Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH)	1245
9. National Agriculturally Important Microbial Culture Collection, National Bureau of Agriculturally Important Microorganisms (NBAIM), Indian Council of Agricultural Research (ICAR), Kushmaur, Uttar Pradesh	3828
10. National Collection of Dairy Cultures, National Dairy Research Institute (Karnal)	15
11. National Collection of Industrial Microorganisms National Chemical Laboratory (NCL) (CSIR)	950
12. National Fungal Culture Collection of India MACS' Agharkar Research Institute, Pune	3050
13. NII Microbial Culture Collection NIICC National Institute for Interdisciplinary Science and Technology (CSIR) Trivandrum, Kerala	78
14. North Maharashtra Microbial Culture Collection Centre, Jalgaon	
15. Fungal Culture Collection, New Delhi	60

Status of Fungal Culture Collection Centers. (http://www.wfcc.info/ccinfo/collection/by_country/i/ dated 10/04/2019)

21.3 Preservation Techniques

Various techniques are available to preserve microorganisms (Table 21.3). These can be classified into three categories:

1. Continuous growth.
2. Dehydration.
3. Frozen storage.

The preservation methods aim to keep the viability and genetic stability of the culture through reduction in metabolic rate of microbes. This helps to prolong the period between subcultures. Continuous growth is achieved through various

Table 21.2 Global status of fungal culture collections: 4 out of 5 regions in world have fungal culture collections as follows

Name	Acronym	Country
I. Africa		
1. National Collections of Fungi: Culture Collection	PPRI	South Africa
2. Suez Canal University Fungarium	SCUF	Egypt
II. America		
3. ARS Collection of Entomopathogenic Fungi	ARSEF	U.S.A.
4. Culture Collection of Fungal Pathogens Strains from the Basic Mycology Laboratory of the Department of Microbiology and Parasitology, Faculty of Medicine, UNAM	BMFM-UNAM	Mexico
5. Entomopathogenic Fungal Culture Collection of Argentina	CEP	Argentina
6. Invertebrate-Associated Fungal Collection of Embrapa	CG	Brazil
7. Chilean Fungal Collection	CHFC-EA	Chile
8. Culture Collection of Phytopathogenic Fungi Prof. Maria Menezes (Colecao de Culturas de Fungos Fitopatogenicos Prof. Maria Menezes	CMM	Brazil
9. Canadian Collection of Fungal Cultures	DAOMC	Canada
10. Culture collection of biomedical interest fungal	DMic	Argentina
11. Fungal Genetics Stock Center	FGSC	U.S.A.
12. Colecao de Culturas de Fungos Filamentosos	Fiocruz/CCFF	Brazil
13. Coleção de Fungos da Amazônia	Fiocruz/CFAM	Brazil
14. Colecao de Fungos Patogenicos	Fiocruz/CFP	Brazil
15. Coleção de Fungos de Referência em Vigilância Sanitária	Fiocruz/CFRVS	Brazil
16. FUNCTIONAL FUNGI	FUNCTIONAL FUNGI	U.S.A.
17. IIB-INTECH Collection of Fungal Cultures	ICFA	Argentina
18. Pathogen Fungi and Actinomycetes Collection	INDRE	Mexico
19. INIFAT Fungus Collection	INIFAT	Cuba
20. International Culture Collection of Arbuscular Mycorrhizal Fungi	INVAM	U.S.A.
21. Culture Collection of Histoplasma capsulatum Strains from the Fungal Immunology Laboratory of the Department of Microbiology and Parasitology, Faculty of Medicine, UNAM	LIH-UNAM	Mexico
22. The Fungus Culture Collection of the Northern Forestry Centre	NoF	Canada
23. UAMH Center for Global Microfungal Biodiversity	UAMH	Canada
III. Asia		
24. Center for Fungal Genetic Resources	CFGR	Rep. of Korea
25. First fungal culture bank of Pakistan	FCBP	Pakistan
26. Fungal Molecular Biology Laboratory Culture Collection University of Agriculture, Faisalabad	FMB-CC-UAF	Pakistan

(continued)

Table 21.2 (continued)

Name	Acronym	Country
27. Goa University Fungus Culture Collection and Research Unit	GFCC	India
28. Iranian Fungal Culture Collection	IRAN	Iran
29. Kasetsart University Fungus Collection, Department of Plant Pathology, Faculty of Agriculture	KUFC	Thailand
30. Mongolian Cultur Collection of Fungal	MCCF	Mongolia
31. mycolo fungal culture collection	mccf	Israel
32. Marine-derived Fungi Collected from South China Sea	MFCSCS	China
33. National Culture Collection of Pathogenic fungi	NCCPF	India
34. National Fungal Culture Collection of India	NFCCI	India
35. National Fungal Culture Collection of Pakistan	NFCCP	Pakistan
36. Research Center on Entomogenous Fungi	RCEF	China
37. Fungal Pathogens of Hevea Rubber in Sri Lanka	RRIASR	Sri Lanka
38. Fungal isolates	RRISL	Sri Lanka
39. Fungal Culture Collection	TAUFCC	Israel
40. Fungal Culture Collection	VPCI	India
41. Yew Endophytic Fungus	YEF	Iran
IV. Europe		
42. ATHens University Mycetotheca—Culture Collection of Fungi	ATHUM	Greece (Hellenic Rep.)
43. BCCM/IHEM Fungi collection: Human and Animal Health	BCCM/IHEM	Belgium
44. Centraalbureau voor Schimmelcultures, Filamentous fungi and Yeast Collection	CBS	Netherlands
45. Culture Collection of Fungi	CCF	Czech
46. EX Culture Collection of extremophilic fungi	EX	Slovenia
47. Culture Collection of Fungi at Kyiv University	FCKU	Ukraine
48. Fungal Cultures University of Goteborg	FCUG	Sweden
49. IBT Culture Collection of Fungi	IBT	Denmark
50. Fungal Strain Collection, Laboratory of Cryptogamy	LCP	France
51. Belgian Coordinated Collections of Microorganisms/MUCL Agro-food and Environmental Fungal Collection	MUCL	Belgium
52. National Collection of Pathogenic Fungi	NCPF	U.K.
53. Swiss Collection of Arbuscular Mycorrhizal fungi	SAF	Switzerland
54. Tartu Fungal Culture Collection	TFC	Estonia
55. UOA/HCPF University of Athens/Hellenic Collection of Pathogenic Fungi	UOA/HCPF	Greece (Hellenic Republic)
56. Uppsala University Culture Collection of Fungi	UPSC	Sweden

Status of Fungal Culture Collection Centers. (http://www.wfcc.info/ccinfo/collection/by_country/i/ dated 10/04/2019)

Table 21.3 Methods of preservation of fungal cultures

Method of preservation	Preservation procedure
Periodic transfer	Transfer of microbial culture to new media at periodic interval depend on several factors viz. medium used, periodicity of transfer, temperature at which cultures are stored. These factors determine the rate of mutation and appearance of variants
Mineral oil slant	The fungal culture slant is immersed in sterilized mineral oil and stored at low temperature
Distilled water or water agar	Water is used to preserve cultures under refrigeration. This helps to maintain the viability of cultures for three to five months or longer
Freezing in growth media	Microbial structures can be damaged by freezing cultures in growth media and, therefore is not a good storage method. However, it can be used for maintenance of cultures
Drying	Sterile filter paper disks, sterile soil or gelatin drops are used to dry microbial cultures which can be frozen or stored at low temperature to enhance their viability
Lyophilization	The water content in spore-forming fungal cultures is reduced by sublimation in the presence of cryoprotectant. The cultures are sealed in an ampoule. They can remain viable for up to 30 years
Ultrafreezing	Fungal cultures can be stored in vapour phase of liquid nitrogen at −156 °C. By this method, the viability of cultures can be maintained for more than 15 years

techniques that permit the fungi to grow and metabolize during period of storage. Various elements, namely, controlling growth conditions by restricting carbon, nitrogen and energy sources, decreasing the temperature, preventing dehydration, etc. step up the time period between subcultures (Anonymous 2008). Fungi can also be preserved by dehydration or drying techniques that comprise air-drying, drying in a vacuum either from the liquid or frozen phase, desiccation in or above a desiccant. In frozen storage, the fungal culture is stored at a temperature which freezes the culture, its metabolic rate is reduced and there is no physical change in it. The success of the preservation depends on medium and cultivation method used and the age of the culture at the time of preservation. There are two preservation methods: Short term and long term. Short-term methods of preservation are serial transfer of fungi to fresh medium followed by low temperature storage and keeping spores in dry sterile soil. Long-term methods include freeze-drying or ultra-freezing in vapour phase liquid nitrogen (−156 °C). There is no one method that can be used to preserve all types of fungi (Nakasone et al. 2004). Different fungal taxonomic groups react differently to different methods of preservation. Biological properties of fungi and their reaction to changes in their environment determine the type of methods for success of their preservation (Fennell 1960; Lloyd 1994; Simione and Brown 1991).

Different preservation methods withhold availability of nutrients, water and oxygen to fungi leading to reduction in their metabolic rate. This is achieved by reduction in storage temperature or by a combination of these. The method

of preservation to be used is determined by a number of factors, viz. nature of fungi, objective of preservation, availability of equipment and skilled manpower, probable preservation time frame, culture number and their use in future, ease of carrying them, frequency of use of cultures and keeping costs (Collee et al. 1996). All preservation methods have more or less similar protocol with distinct stages, viz. checking culture purity, preparing ampoules, growing the culture, cells suspension in preservation medium, putting cell suspension into ampoules, preservation, storage of ampoule stocks, updation of ampule stock records and testing viability, purity and genetic stability of preserved culture stocks.

21.3.1 Serial Transfer

Fungal cultures can be maintained by periodic transfer on fresh and sterile medium. Alternate cycles of active growth and storage periods are achieved by a series of subcultures. Periodicity of transfer differs with the kind of fungi.

21.3.2 Storage in Soil

Dry, sterile soil or sand is a very good medium for storage of some fungi for many years. This method is cheap and suitable for fungi such as *Rhizoctonia* (Sneh et al. 1991), *Septoria* (Shearer et al. 1974) and *Pseudocercosporella* (Reinecke and Fokkema 1979). Dryness induces dormancy over a period of time. Changes in morphology of some fungi due to it have been reported. The available moisture is used by the fungi during their growth and then they reach dormant state. The bottles are kept in refrigerator. The culture can be regrown by putting few soil particles on a suitable medium.

21.3.3 Storage in Silica Gel

Perkins (1962) developed this method for preservation of *Neurospora* species. He observed that viability of sporulating fungi can be maintained by using skimmed milk to protect them and then storing on silica gel. Silica gel powder can be used to store bacteria and yeast for a period of 1–2 years at low temperature. In this method, fine powder of silica is made followed by heat sterilization, cooling and then mixing with a thick suspension of cells and storing at a low temperature. This technique relies on quick desiccation at low temperature, and this helps to keep the cells viable for a long period.

21.3.4 Liquid Nitrogen

Liquid nitrogen can be used to preserve many fungi. The fungi which are not amenable to lyophilization can be preserved in liquid nitrogen. It is a costly method of preservation because continuous supply of liquid nitrogen has to be maintained. Dictyostelids (Raper 1984), amoebae (Davis 1956; Evans et al. 1982), zygomycetes including Entomophthorales (Humber 1994), oomycetes (Nishi and Nakagiri 1991), phytopathogenic fungi (Dahmen et al. 1983) and yeasts (Kirsop 1991) can be preserved using liquid nitrogen. Cell division and metabolic rate determines the mutation rate of cultured fungi. Therefore, any storage method that prevents division of cells and slows down metabolism while retaining viability is considered as the best method. Freezing fungal cultures at or below −139 °C prevents growth of ice crystals and slows down biophysical processes considerably, thereby ensuring cell survival.

21.3.5 Storage by Freeze-Drying

Spore-forming fungal cultures can be preserved by freeze-drying or lyophilization. In this method, cultures are rapidly frozen and a cryoprotectant is added to dissolve ice crystals and minimize their growth. Skimmed milk powder (sterile 5% or 10% solution) and filter-sterilized bovine serum are the commonly used cryoprotectants besides the proteinaceous materials.

21.4 Mineral Oil Overlay

A mineral oil is a petroleum distillate and contains a light mixture of higher alkanes. It is colourless and odourless. Mineral oil is also obtained as a liquid by-product while refining crude oil to make gasoline and other petroleum products. This liquid by-product is transparent, colourless and contains alkanes and cycloalkanes, related to petroleum jelly. It has a density of around 0.8 g/cm^3. It is also known as liquid paraffin and white oil.

This method is simple, cost-effective and preserves fungal cultures for long duration time at ambient temperature (Fig. 21.1). Slant culture of microbes is immersed in mineral oil and the tubes are stored in upright position at room temperature.

Points to be taken into account while preserving the microbial cultures in oil**:**

1. The medium dries out and separates from the tube wall and floats to the wall surface if the medium is not completely immersed in oil leading to death of fungal culture.
2. The oil must be of good quality. The fungi are harmed if it is rancid or contains any toxic substance.

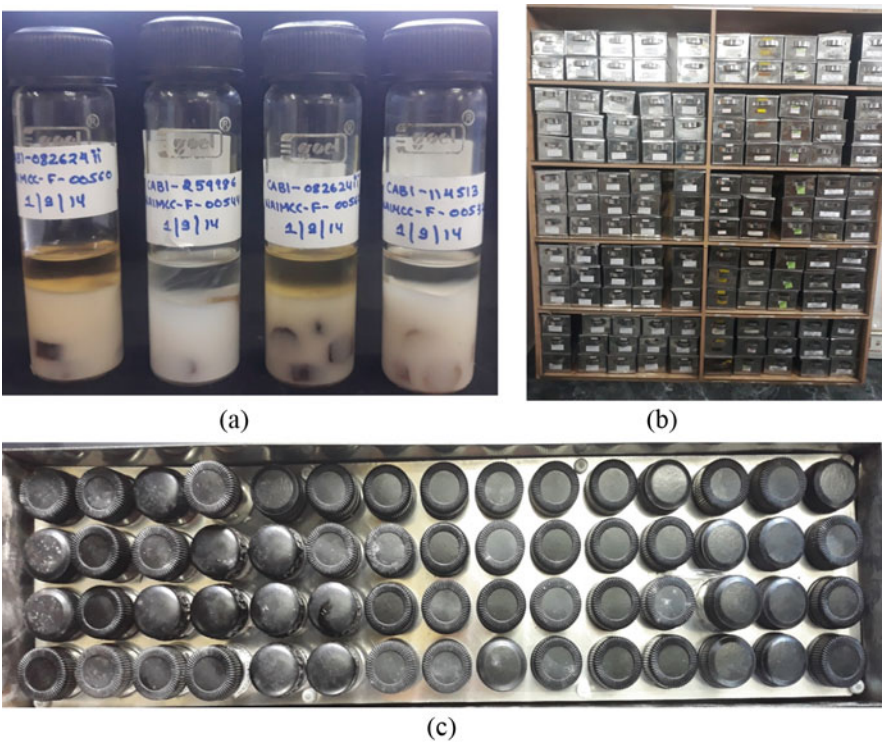

Fig. 21.1 Mineral oil storage of fungal cultures at NAIMCC, ICAR-NBAIM, Maunath Bhanjan, U.P. (**a**) Tubes containing fungal culture in mineral oil; (**b**) Rack to place the box in store room; (**c**) Box containing tubes

3. During autoclaving, the oil becomes milky due to mixing of moisture with it. Therefore, it is advisable that the oil be sterilized in the hot air oven at 150–170 °C for 1 h to remove milkiness.

21.4.1 History of Mineral Oil Preservation

Professor Frantisek Kral (1846–1911) of Prague was the first person to understand the importance of Culture Collections. He collected cultures and made these available to other workers by charging a fee. Professor Ernst Pribram later shifted this Collection to the University of Vienna in 1915. The other very old Collection, Centraalbureau voor Schimmelcultures (CBS), was founded in 1906 and is still in existence at Baarn, the Netherlands (Malik and Claus 1987). Mineral oil was used to preserved bacterial cultures about 100 years ago by Ungermann in 1918. He conserved bacterial cultures in dilute sera by overlaying them with oil. M. Michelle in (1921) used this method to preserve *gonococci, meningococci* and *pneumococci* in broth (Krasilnikov 1967). He slightly modified the method by using solid media. Morton and Pulaski (1938) used this method to maintain 45 cultures of bacteria.

He compared this method with other storage methods and found mineral oil method to be useful to maintain viability of bacterial cultures for longer duration (Uzunova-Doneva and Donev 2005; Hartsell 1953).

Sherf (1943) used this method to conserve filamentous fungi, viz. *Fusarium* and *Alternaria,* and found cultures to be viable even after 6 months. In Australia, Norris (1944) preserved fungi by the said method and published a note on reporting cultures of seven genera of plant pathogens still viable after 18 months under oil (Hartsell 1956).

There are reports mentioning that fungi depending on their properties can be conserved without cultivation for 1–12 years under Vaseline oil. Optimal time limits have been worked out for cultivation of different taxonomical groups (Uzunova-Doneva and Donev 2005).

21.4.2 Process of Preservation of Fungi in Mineral Oil

1. The culture slants in glass tubes should be fresh and vigorously grown.
2. Take good quality mineral oil of low specific gravity, i.e. 0.830–0.890 g/cm^3.
3. Heavy mineral oil should be autoclaved twice. First autoclaving may cause activation of bacterial spores followed by keeping the vials at room temperature for 24–48 h for their germination. Second round of autoclaving would kill germinated spores presented in oil.
4. Mineral oil is dried in oven at 170 °C for 1–2 h to remove entrapped moisture. It is an important step to remove water molecules from oil water.
5. The culture slant should be covered with sterile oil up to the depth of 1 cm.
6. Tightly cap tubes and put paraffin film to act as a vapour barrier.
7. Tubes should be stored in upright position. The cultures remain viable for a longer time if stored at low temperature as compared to ambient temperature.
8. Periodically check oil level in the tubes and add oil if need be.

21.4.3 Process of Culture Revival

1. Remove a part of culture immersed in mineral oil with a sterile needle/loop.
2. Excess oil should be drained from the explants by keeping on sterile filter paper and then placing it on fresh medium.
3. Afterwards, the tube should be resealed and returned for long-term storage.
4. Cultures should be monitored for viability and contamination.
5. The culture may be required to be subcultured several times to remove oil from it.

Mode of action of mineral oil preservation.

The mode of action of mineral oil is yet to be fully unravelled. The evidence points to its action via checking dehydration of the cultures, retarding metabolic activity and growth of the fungi and by slowing down the gases exchange within fungi and surrounding (Table 21.4).

Table 21.4 Maximum survival time of *Aspergillus* species under mineral oil (Christina 1989)

Name of *Aspergillus* species	Duration of survival (years)
Aspergillus fumigatus	11.9
A. niger	11.9
A. candidus	11.8
A. flavus	11.8
A. clavatus	6.8
A. versicolor	11.9
A. sydowii	11.7
A. nidulans	11.8
A. unguis	11.10
A. melleus	13.2
A. ustus	11.6
A. terreus	11.9

21.4.4 Advantage and Disadvantage of Mineral Oil Preservation

The mineral oil method has merits such as:

1. The procedure is simple.
2. Preserved cultures can be easily transferred.
3. The medium does not dry due to presence of paraffin oil.
4. All fungi including non-sporulating ones can be preserved.
5. The cultures do not get contaminated with mites.
6. Cultures remain in dormant state due to aerobic conditions.
7. Is useful for laboratories with limited amenities.

The mineral oil method has some disadvantages also

1. Changes in characterization of fungal cultures still occur due to growth of fungi and there can be selection of mutants capable of growing under adverse conditions.
2. Slow growing on retrieval.
3. Contamination of microbial spores from air.

21.5 Conclusion

Utmost care has to be exercised while preserving and maintaining fungal cultures in fungal culture collections. A number of fungal collections are available in India and abroad. Quality control is also important so that the revived cultures are true to type when compared with the original cultures. One has to be conversant with various methods of preservation so that suitable preservation method may be chosen keeping in view the characteristics and requirements of the microorganisms. Various factors,

namely, the strain, culture medium, storage temperature, the sub-culturing medium, and the periodicity and technique of transfer determine viability duration and the maintenance of cultural characteristics of the strain. Of these methods, the mineral oil method appears to be an easy, convenient, economical and effective tool for the preservation of fungal strains for their long-term preservation under low temperature.

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