Volume 5 Número 3 Jul. - Set. 1974

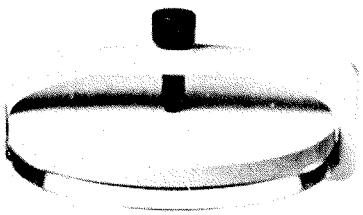
Revista de Microbiologia

Órgão Oficial da Sociedade Brasileira de Microbiologia
São Paulo – Brasil

ESMORECER PARA NÃO DESMERECER NÃO

Jarras GasPak

nova dimensão no sistema BBL para anaeróbios



JARRA ANAERÓBIA "100"

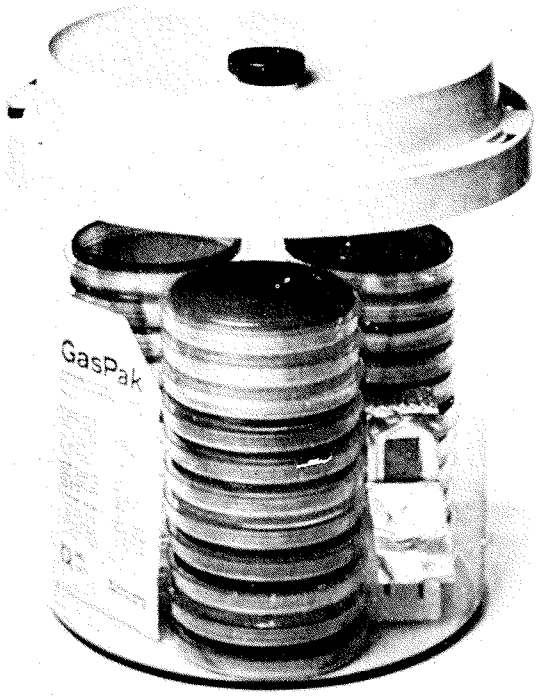
As infecções devidas aos germes anaeróbios são muito mais frequentes do que deixam supor os exames de laboratório. De fato, estes germes foram mal conhecidos durante muito tempo, por ser bastante complicada a sua identificação nas condições utilizadas até agora.
Hoje, o sistema BBL oferece simplicidade de trabalho e segurança nos resultados desses exames, imprescindíveis em todos os laboratórios pela multiplicidade de aplicações que representam, sejam na clínica, no ensino, na pesquisa ou na indústria.

PRINCÍPIO

As jarras GasPak permitem a obtenção de uma anaerobiose perfeita, numa hora, sem auxílio de qualquer aparelho anexo. Em policarbonato, material transparente, autoclavável, realizam as condições ótimas de higiene e visualidade.
Os envelopes GasPak são geradores de hidrogênio e de gás carbônico que permitem realizar, sem bomba de vácuo, uma atmosfera inerte no interior da Jarra Anaeróbia.
Os dois componentes de base são:

- Um gerador de hidrogênio + um gerador de gás carbônico.
- Um catalisador de paládio agindo à temperatura ambiente.

Por adição de 10 ml de água no envelope GasPak, há desprendimento de hidrogênio e gás carbônico. Sob o efeito do catalisador, o hidrogênio reage com o oxigênio do ar presente na jarra, para formar água; cria-se assim uma atmosfera anaeróbia.
O gás carbônico produzido favorece a multiplicação de certos microorganismos que se desenvolveriam mal ou seriam inibidos na ausência deste gás. Um indicador constituído por uma banda de papel de filtro embebida numa solução de azul de metileno, permite controlar visualmente a anaerobiose.



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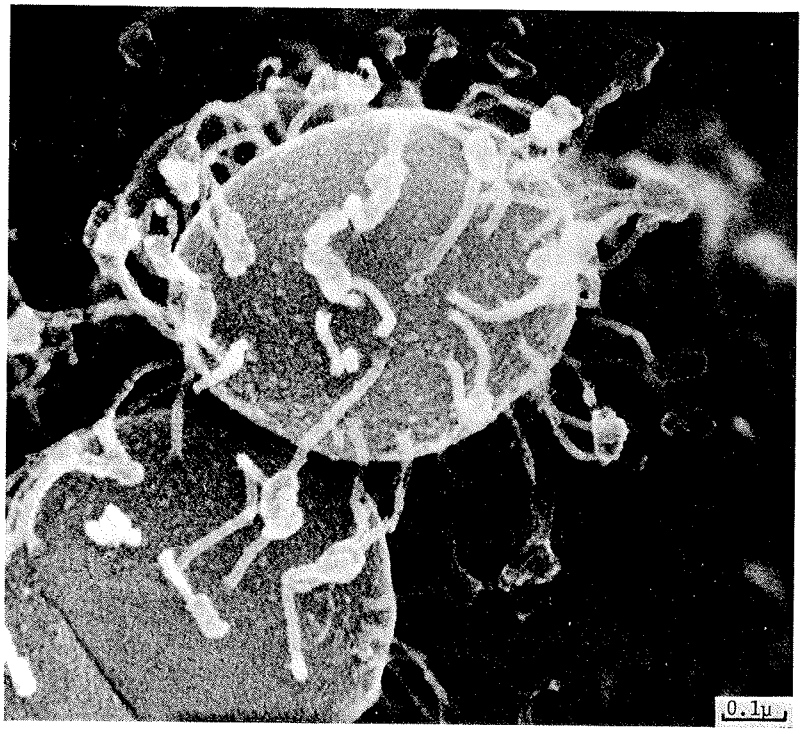

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Yeasts associated with cacao (*Theobroma cacao*) pods

S.I. Faparusi*

Summary

Yeasts associated with cacao pods were isolated from randomly selected material from five plantations of two varieties of *Theobroma cacao* located in two divisions of Western State of Nigeria. Yeast substrates consisted of cacao flowers, epidermis and tissues of both unripe and ripe pods. The isolates were grouped into major, minor and trace yeast flora components on the basis of their percentage incidence. Among the yeasts found on fermenting cacao beans by previous workers some of the species of *Candida*, *Kloeckera*, *Torulopsis* and *Pichia* appeared to be derived from the cacao flowers.

Resumo

Leveduras associadas ao fruto do cacao (Theobroma cacao)

Isolamento de leveduras associadas a frutos de cacao, coletados ao acaso em cinco plantações de duas variedades de *Theobroma cacao*, localizadas em duas divisões do Estado Ocidental de Nigeria. Os substratos consistiram de flores, epiderme e tecidos de frutos maturos e imaturos. As leveduras isoladas foram agrupadas em componentes superiores, inferiores e raros, conforme seu porcentual de incidência. Entre as leveduras já mencionadas por outros autores na fermentação das sementes do cacao, algumas espécies de *Candida, Kloeckera, Torulopsis* e *Pichia* parecem derivar de flores da planta.

Introduction

Cocoa is the chief agricultural product of Western State of Nigeria. Recently, efforts have been made at diversifying the products from cacao fruits. For example, cacao wine and jellies have been produced from cacao juice and husks respectively, and fodder production from the pods is being considered at the Cocoa Research Institute of Nigeria.

A smell of alcohol emanating from freshly fermenting cacao beans soon after they have been heaped up is very striking. Therefore, it is not surprising that it was early assumed that alcohol was produced by yeast fermentation of sugars in the seed pulp.

Rombouts[15] critically reviewed the yeast flora of fermenting cacao. Yeasts isolated during the first 24 to 36 hours included species of *Saccharomyces*, *Candida*, *Kloeckera* and *Pichia*.

Microfloral analyses of cocoa wine performed in the writer's laboratory yielded yeasts similar to those responsible for cacao beans fermentation.

Local variations of yeast flora of fermenting cacao have been recognised by numerous workers[15] because of the dissimilarities in the conditions under which the cacao beans are fermented.

The yeasts of fermenting cacao have not been identified for Nigeria. The present study of yeasts associated with development of cacao fruits may contribute to the knowledge of the important subject of cacao fermentation in this country. During the study yeast flora associated with cacao flowers, unripe and ripe pods were isolated and identified.

Material and Methods

Flowering cacao trees were randomly chosen from five cacao plantations in Ibadan and Ekiti Central Districts of Western State of Nigeria. Three plantations from Ibadan District are designated as "Plantations, A, B & C"; while "Plantations D & E" are in Ekiti Central District. Plantations A, B & E contain the Amazon variety of *Theobroma cacao,* while C & D are of a local variety.

Flowers were cut from experimental trees as soon as they bud in September. The flowers were put in sterilised polythene bags with several sheets of sterile

* Department of Biochemistry, University of Ibadan, Nigeria.

filter paper to prevent the accumulation of moisture, which otherwise would enhance mould growth[3]. Bags were sterilised by immersion into 80% ethanol, rinsed with sterile distilled water, and stored in a refrigerator at 1-2°C until required. Yeast culture was done within a week of flower collection. Unripe pods were collected after the fruits have developed for a period of 3 to 4 weeks. Fruits were considered ripe when their colour changed from bluish-green to orange.

The following media were used for yeast isolation: yeast extract-malt extract agar (MYGP agar)[16]; acidified yeast nitrogen base agar containing 2% (w/v) glucose[8] and sometimes glucose-peptone-beef extract agar.

Aureomycin and actinomycin were added to MYPG agar[1] to inhibit bacterial growth; 0.5% ferric ammonium citrate was added to MYPG agar to allow full pigmentation of *Metschnikowia pulcherrima*[2,3]. When necessary 0.25% (w/v) sodium propionate was added to restrict the development of fungal colonies[8]. Stock cultures were maintained on MYPG agar at 5°C.

To isolate yeasts from flowers samples were disintegrated in a sterile Waring blender. Sterile dilutions made by one quarter Ringer's solution[6] were streaked and plated on the various media. The colonies were purified by subsequent subculturing.

To culture yeasts on fruit epidermis combined swabrinse and rinse methods were employed[9]. Sterile one quarter Ringer's solution[6] was used for rinsing and successive dilutions were prepared conventionally.

To culture yeasts from fruit tissues, the epidermis was flamed off and seeds and pulp treated using the pulping technique of Marshall & Walkley[13]. Yeast flora in the husks of the fruits were also cultured.

For the identification of the yeast samples in general the methods given by Lodder[12] were used, and only brief descriptions are given below. Yeast nomenclature was also based on Lodder[12]. Cultural characteristics and morphology of vegetative cells were studied with both liquid and solid Wicherham's malt extract medium; examinations were made on the third day and after four weeks. Cell size and shape were determined by measuring an average of 15 cells from a 3-day broth.

Pseudomycelium or mycelium formation was examined on Dalmau plates with corn meal agar. Examination was made after one week's incubation.

Ascospore formation was tested on Fowell's acetate agar[10], gypsum block, and Wickerham's sporulation agar. Glucose yeast agar (GYA) was used as a presporulation medium. Sporulation was checked fortnightly for 8 weeks.

Fermentation tests with Einhorn tubes were carried out on the folowing carbon sources: glucose, galactose, lactose, maltose, sucrose, melibiose, raffinose and trehalose. Examination was made after 3 days, them at 7-day intervals for 4 weeks. The basal medium without sugar was inoculated with yeast as a control.

Carbon assimilation tests were carried out in liquid medium using Wickerham's nitrogen base (Difco) and Seitz-sterilized sugar solutions; starch and inulin were autoclaved; and 0.1ml of a standard inoculum (2+ on Wickerham's Density Card) was used. Carbon compounds tested were glucose, galactose, inositol, trehalose, maltose, lactose, sucrose, melibiose, raffinose, starch, inulin, D- and L-arabinose, L-rhamnose, DL-lactic acid, D-mannitol and D-glucitol. Controls were set up in the same way as for the fermentation tests. Tubes were examined weekly for 4 to 6 weeks using a Density card.

Arbutin-splitting was tested on arbutin agar and examined after 7 days. The presence of extracellular amyloid products was tested by streaking yeasts on the agar medium of Lodder and Kreger van Rij and testing with iodine after 14 days incubation. Nitrate assimilation was tested auxanographically using Lodder's auxanographic agar and yeast water. These were examined after 2 and 7 days. Growth at 37° was tested on incubated streak plates of GYA. Unless otherwise stated all tests were incubated at 28°C.

Results and Discussion

Table 1 shows the yeast flora associated with the flowers of the two cacao varieties. The yeasts were divided into three groups on the basis of their percentage incidence. The major yeast components of the flora had at least 20% incidence. Those with 10-20% incidence were designated "minor yeast components"; "trace components" had less than 10% incidence.

In all five plantations, *Pichia kluyveri, Hanseniaspora uvarum, Metschnikowia pulcherrima, Kloeckera apiculata*, and *Candida guilliermondii* var. *carpophilla* could be regarded as the major yeasts of cacao flowers. The minor yeast components consisted of *Torulopsis candida, Rhodotorula piliminae, Rh. glutinis* var. *glutinis, Candida krusei* and *Kloeckera corticis. Pi. membranaefaciens, Rh. piliminae, Rh. glutinis* var. *glutinis* and *C. valida* were among those grouped as trace yeast components. A species of *Torulopsis* (*Torulopsis* sp. *X*) was found in less than 10% of the cacao flowers.

Cells of *Torulopsis* sp. *X* are eliptical, 2.0-2.5 x 4.0-6.0 μm when grown on glucose yeast extract peptone water for 3 days. Streak culture after one month on GYA was cream-coloured, glossy and smooth. Dalmau plate cultures on corn meal agar yielded branched chains of oval cells, but no pseudomycelium was formed. The yeast failed to assimilate KNO_3; it fermented no sugar, but assimilated glucose,

Table 1

Yeast flora of flowers from two cacao varieties

Cacao variety	Source: Plantation	Source: Location	Yeasts constituting the: Major component (20-100% incidence)	Minor component (10-20% incidence)	Trace component (< 10% incidence)	Population per flower
1. Amazon	A	Ibadan District	*Pichia kluyveri, Hansoniaspora uvarum, Metschnikowia pulcherrima, Kloeckera apiculata, Candida krusei, Candida guilliermondii* var. *carpophilla* and *Klockera cortisis*	*Torulopsis candida, Rhodotorula glutinis* var. *glutinis* and *Candida valida*	*Torulopsis* sp., *Pichia membranaefaciens,* and *Rhodotorula piliminae*	13,644
2. Amazon	B	Ibadan District	*Pi. kluyveri, Hans. uvarum, C. gulliermondii* var. *carpophilla, Met. pulcherrima, K. apiculata* and *K. corticis*	*T. candida, Rh. glutinis* var. *glutinis, C. valida* and *C. krusei*	*Rh. piliminae*	14,525
3. Local	C	Ibadan District	*Pi. kluyveri, Hans. uvarum, C. guilliermondii* var. *carpophilla, Met. pulcherrima, K. apiculata* and *C. krusei*	–	*T. candida, Rh. glutinis* var. *glutinis, Torulopsis* sp. *X, K. corticis* and *Pi. membranaefaciens*	8,780
4. Local	D	Ekiti Central District	*Pi. kluyveri, Hans. uvarum, C. guilliermondii* var. *carpophilla. Met. pulcherrima, C. krusei* and *K. apiculata*	–	*T. candida, Torulopsis* sp. *X, Rh. piliminae, K. corticis, Pi. membranaefaciens* and *C. valida*	10,600
5. Amazon	E	Ekiti Central District	*Pi. kluyveri, Hans. uvarum, C. guilliermondii* var. *carpophilla, Met. pulcherrima* and *K. apiculata*	*T. candida, C. krusei* and *K. corticis*	*Rh. piliminae, Rh. glutinis* var. *glutinis, Torulopsis* sp. *X, Pi. membranaefaciens* and *C. valida*	16,975

galactose, D-mannitol, sucrose, raffinose and glycerol.

Torulopsis X: morphological features are similar to those of *Torulopsis halonitraphila*. However, they differed physiologically; *T. halonitraphila* fermented glucose weakly, and failed to assimilate galactose, sucrose, raffinose and KNO_3[12].

From Table 1 it appears the yeast types from the cacao flowers are uncorrelated with cacao variety. The location of the plantation apparently played a greater influence on the yeast flora. It is probable that insects visiting the flowers influence the yeast composition. A number of ants, bees and drosophila species were observed on the flowers. It has been shown that apiculate yeasts including *Pichia* species are associated with drosophila species[4,5]. Species of *Candida* have been reported on bees and ants[7].

Yeast population figures showed that flowers of the Amazon variety had a higher number of yeasts than those of the local variety (plantations C & D). This difference could be due to ease of access to the flowers by visiting insects. Cacao trees from plantations A, B & E were unshielded by other trees, and became, therefore, clearly exposed to insects' attention.

Table 2 shows that four yeasts constituted the major flora on the epidermis of unripe cacao fruit. The total yeast species were *M. pulcherrima, C. krusei, Pi. kluyveri, K. apiculata, Rh. glutinis* var. *glutinis* and *Saccharomyces delbrueckii.*

A yeast which has been designated *Torulopsis* sp. *Y.* was found in various percentage of incidence in different plantations. This yeast differs from *Torulopsis* sp. *X* described above in being able to ferment glucose, sucrose and raffinose weakly. It assimilated glucose, galactose, sucrose, maltose, melezitose, D-mannitol, ethanol and glycerol. It failed to assimilate KNO_3. This yeast seems similar to *Torulopsis candida* excepting its failure to assimilate cellobiose, xylose and L-arabinose[12].

Sporobolomyces roseus occurred in less than 5% of the samples tested.

Of the yeasts isolated from epidermis of unripe cacao fruits, *C. krusei, M. pulcherrima, Rh. glutinis* and *K. apiculata* could have originated from the fertilized flowers.

Yeast population figures per cm^2 of the pods showed a variation similar to that of the flowers; that is, population density from the Amazon plantations (A, B & E) being higher than that of the other local variety (plantations C & D).

Table 3 shows that the epidermis of a ripe cacao fruit was very rich in yeasts in comparison to the epidermis of an unripe pods.

Saccharomyces species (*S. delbrueckii,* and at times, *S. cerevisiae*) were among the major yeast components. Other dominant yeasts were *C. krusei,*

Table 2

Yeast flora of epidermis of unripe cacao fruits

Cacao variety	Source: Plantation	Source: Location	Yeasts constituting the: Major component (20-100% incidence)	Minor component (10-20% incidence)	Trace component (< 10% incidence)	Total yeasts per cm^2
1. Amazon	A	Ibadan District	*Met. pulcherrima, C. krusei, K. apiculata, Pi. kluyveri* and *Torulopsis* sp. *Y*	*Rh. glutinis* var. *glutinis*	*Saccharomyces delbrueckii* and *Sporobolomyces roseus*	400
2. Amazon	B	Ibadan District	*Met. pulcherrima, C. krusei, S. delbrueckii, K. apiculata* and *Pi. kluyveri*	*Rh. glutinis* var. *glutinis, Sp. roseus* and *Torulopsis* sp. *Y*		425
3. Local	C	Ibadan District	*Met. pulcherrima, C. krusei, Sp. roseus, K. apiculata* and *Pi. kluyveri*	*Rh. glutinis* var. *glutinis* and *S. delbrueckii*	*Torulopsis* sp. *Y*	256.5
4. Local	D	Ekiti Central District	*Met. pulcherrima, C. krusei, S. delbrueckii, K. apiculata* and *Pi. kluyveri*	*Rh. glutinis* var. *glutinis*	*Torulopsis* sp. *Y*	220
5. Amazon	E	Ekiti Central District	*Met. pulcherrima, C. krusei, S. delbrueckii, Rh. glutinis* var. *glutinis, K. apiculata* and *Pi. kluyveri*	*Torulopsis* sp. *Y*	*Sp. roseus*	378

Table 3

Yeast flora of epidermis of ripe cacao fruit

Cacao variety	Source: Plantation	Source: Location	Yeasts constituting the: Major component (20-100% incidence)	Miner component (10-20% incidence)	Trace component (< 10% incidence)	Total yeasts per cm^2
1. Amazon	A	Ibadan District	*S. delbrueckii, C. krusei, C. tropicalis, K. apiculata* and *Hans. uvarum*	*Pi. pastoris,* and *Sp. roseus*	*Rh. glutinis* var. *glutinis, S. rouxii* and *S. cerevisiae*	1,345
2. Amazon	B	Ibadan District	*S. delbrueckii, K. apiculata, C. krusei, C. tropicalis* and *Pi. pastoris*	*Sp. roseus* and *Rh. glutinis* var. *glutinis*	*S. cerevisiae, Hans. uvarum* and *S. rouxii*	1,830
3. Local	C	Ibadan District	*S. delbrueckii, C. krusei, C. tropicalis* and *K. apiculata*	*S. cerevisiae, Pi. pastoris, Hans. uvarum* and *S. rouxii*	*Sp. roseus* and *Rh. glutinis* var. *glutinis*	1,008
4. Local	D	Ekiti Central District	*S. cerevisiae, S. delbrueckii, C. tropicalis, C. krusei* and *K. apiculata*	*Pi. pastoris.*	*Sp. roseus, Rh. glutinis* var. *glutinis, Hans. uvarum, S. rouxii, Schizosaccharomyces pombe* and *Hansenula anomala* var. *anomala*	360
5. Amazon	E	Ekiti Central District	*S. delbrueckii, C. krusei, C. tropicalis, K. apiculata* and *Pi. pastoris*	*Rh. glutinis* var. *glutinis* and *Hans. uvarum*	*S. cerevisiae, S. rouxii* and *Sp. roseus*	1,105

C. tropicalis, K. apiculata, and at times, *Pi. pastoris.*

Sp. roseus, Rh. glutinis var. *glutinis* and *Hanseniaspora uvarum* in addition to *Pi. pastoris* constituted the minor component of the yeast flora. Some of these yeasts were also found as trace component in some plantations.

S. rouxii, Schizosaccharomyces pombe, and *Hansenula anomala* var. *anomala* were isolated from less than 10% of the samples.

Population figures show that on the average there were more than three times yeasts per cm^2 on a ripe fruit epidermis than on an unripe fruit epidermis. The richness of ripe fruits in both the yeast types and population could be due to changes in sugar content as the fruit ripens[11] and/or changes in animal visitors to the fruit. Apart from ants and

Table 4

Yeasts isolated from the tissues of cacao fruits

Yeast		*Unripe* fruit incidence in plantations (%)					*Ripe* fruit incidence in plantations (%)				
		A	B	C	D	E	A	B	C	D	E
Torulopsis sp. *X*	(a)	5.2	3.3	7.5	18	4.0	17.5	8.3	5.0	10	12.5
	(b)	6.0	4.0	7.5	20	5.0	25	12.5	10.0	15	33.3
Pichia pastoris	(a)	36	15	30	20	15	30	25	17.5	12.5	22.5
	(b)	0	0	4.0	2.0	2.5	2.0	0	1.5	3.3	2.5
Pichia membranaefaciens	(a)	33.3	25	10	20	25	40	45	25	50	33.3
	(b)	0	3.0	2.5	0	5.0	5.0	10	7.5	10	5.0
Saccharomyces sp.	(a)						7.5	6.0	5.0	4.0	2.0
	(b)						7.5	5.0	5.0	5.0	4.0
Total yeast population (per g)	(a)	165	120	144	105	132	250	185	205	295	345
	(b)	50	60	55	72	67	111.3	122	88	102	150

a = seeds and pulp; b = husk.

other insects, rats and other mammals are usually attracted by the beautiful colour of a ripe fruit. *Candida* species have been reported on some rats and other mammals[5].

Only *C. krusei, K. apiculata, Hans. uvarum* and *Rh. glutinis* var. *glutinis* were found in the cacao flowers and fruits. Thus, these four yeasts could probably have originated from the flowers.

The results of yeast flora analyses from the internal tissues are shown in Table 4. The four yeasts isolated from the tissues were *Pi. membranaefaciens, Pi. pastoris, Saccharomyces* sp. and *Torulopsis* sp. *X*. *Torulopsis* sp. *X* was earlier cultured from the fruit epidermis.

Saccharomyces sp. grown on malt extract at 28°C for 3 days, forms ovoid cells, mainly singly but at times in pairs; ranging 3.0–5.4 X 4.8–10.5 μm. After four weeks at room temperature (22°C) the culture is light brown and dull with crenate margin. In Dalmau plate cultures on corn meal agar pseudomycelium is formed. Two to four ascospores were formed per ascus. The yeast fermented glucose, sucrose, raffinose and maltose slowly. Glucose, sucrose, raffinose, trehalose, D-mannitol were assimilated. It failed to assimilate KNO_3, and did not split arbutin. This yeast is similar to some strains of *Saccharomyces cerevisiae* which form small cells[12].

Comparing the percentage incidence of the yeasts found in the husks and seeds plus pulp tissues of unripe and ripe fruits, it seems that *Pi. pastoris* and *Pi. membranaefaciens* were the usual inhabitants of the internal tissues. The other two yeasts (*Torulopsis* sp. *X* and *Saccharomyces* sp.) might probably have originated from epidermal infection. These two yeasts were found in greater numbers in cacao fruits which appeared to have suffered fungal attack, than in those fruits that appeared quite sound.

Saccharomyces sp. was not found in the tissues of unripe fruits. Ripe fruits which usually suffer high fungal attack could provide more favourable environment for this particular yeast growth.

It has been explained by Marshall & Walkley[13] that internal infection of fruits could be due to contamination of the gynaecium by yeasts from either air-born infection or insect carrier during pollination. During fruit growth and development, the microorganisms become sealed in. *Pi. membranaefaciens* could have originated in this way from the cacao flowers.

Rombouts[15] and Roelofsen[14] reviewed the microfloral studies of cacao fermentation. Among the yeasts found on fermenting cacao on the first day were *C. krusei, C. valida, K. apiculata, H. anomala, Schizosacc. pombe, Rhodotorula* spp., *Torulopsis* spp., *Pichia* spp. and *Saccharomyces* spp. Most of these were isolated from the flowers and epidermis of ripe and unripe cacao fruits.

Roelofsen[14] named three possible sources of early microfloral inoculation of the cacao seeds, viz: hand inoculation of microorganisms on the pods, contamination by basket microorganisms and inoculation of microorganisms carried by drosophila flies. Thus, considering the similarity of the epidermal

yeasts and those of fermenting cacao seeds it appears a substantial proportion of yeast types responsible for fermenting cacao could have originated from epidermal yeasts. These yeasts probably had transferred into the seeds when the cacao pods were broken open.

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Pruebas de imunodifusión para identificación de sangre ingerida por garrapatas*

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Resumo

A técnica de gel-difusão em agar (Ouchterlony), foi usada para a identificação da fonte de alimentação de carrapatos. Foram analisados 348 artrópodos capturados na República Dominicana e os resultados encontrados mostraram que a técnica oferece excelente rendimento, possibilitando o estudo da participação desses vetores na transmissão de várias zoonoses, notadamente algumas causadas por riquetsias e vírus.

Summary

Immunodiffusion tests for identification of blood ingested by ticks

The agar gel-difusion technique (Ouchterlony) was applied to the definition of blood meals of blood--sucking ticks. Blood-samples from 348 arthropodes trapped in the Domenican Republic were studied and the results emphasized the reliability of the method to study the participation of these vectors in many zoonosis.

Introducción

Entre los muchos aspectos que se consideran en el estudio de la ecologia de molestias transmisibles, el conocimiento de los hábitos alimentarios de artrópodos hematófagos que participan en la cadena epidemiológica de algunas de ellas es de suma importancia. Por esta razón, la investigación del origen de la sangre ingerida por vectores ha sido, desde el princípio del siglo, objeto de muchos trabajos en todo el mundo[1,4-6,8,10-13].

Gozony[4], en 1914, fué el primero en envestigar en ese campo proponiendo, la técnica de fijación de complemento. Poco después, Bull & King[1] reportaron la importancia de esos estudios con fines epidemiológicos, introduciendo la prueba de precipitación en tubo capilar, método que durante muchos años fué el preferido por varios investigadores, principalmente Weitz[11-13], seguramente el más fecundo pesquisador en ese campo. Trabajando en el Instituto Lister, en Londres, Weitz mantuvo durante muchos años un laboratório de referencia donde identificaba muestras de sangre procedentes de varios países. Utilizaba, para eso la precipitación en tubo capilar e inhibición de hemoaglutinación. Este último recurso, permitia evitar los casos de reacciones cruzadas que frecuentemente ocurrían cuando los vertebrados dadores estaban estrechamente relacionados en la escala zoológica. De este modo, la identificación era especie-específica.

Recientemente, surgieron nuevos métodos para la identificación de sangre ingerida por artrópodos[2,3,8]. Destacamos aquí, el propuesto simultaneamente por Ricciardi & Mello[8] y Crans[2], ambos basados en la técnica de doble difusión en gel de agar (Ouchterlony). Tanto los anos como el otro, demonstraron que la difusión en gel de agar se presenta como procedimiento más simple y más seguro que la tradicional prueba de precipitación.

Nuestra intención en este trabajo es, fundamentalmente, analizar la eficiencia de esa técnica en la identificación de la sangre ingerida por garrapatas,

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artrópodos que tienen una participación activa en la transmisión de virosis y rickettsiosis, principalmente encefalitis y fiebres hemorrágicas[7].

Material y Metodos

Fué utilizada la técnica de inmunodifusión en agar, distribuido en cajas de Petri. El procedimiento para la inmunodifusión y preparación del agar, siguió los moldes convencionales usados por Ricciardi & Mello[8]. Los antisueros fueron obtenidos segun la técnica utilizada por Weitz[11].

Para hacer la aplicación de la técnica de gel-difusión en agar, fueron examinados los contenidos estomacales de 348 garrapatas capturadas en caballos, bueyes, perros y mulos. Esos artrópodos fueron analizados en 106 reacciones, algunas hechas con macerado estomacal de un ejemplar y, otras, con macerado de varios ejemplares reunidos.

Fueron analizados los repastos sanguíneos de tres especies de garrapatas: *Boophilus microplus, Dermacentor nitens* y *Rhipicephalus sanguineus.* Las cantidades de muestras analizadas y su procedencia, figuran en la Tabla 1.

Tabla 1

Ejemplares de garrapatas estudiadas; referencias de captura

Espécie	Procedência (*)	Nº de Ejemplares
Boophilus microplus	Bayona D.N.	180
Boophilus microplus	San Cristobal	36
Bloophilus microplus	Engombe D.N.	9
Dermacentor nitens	Higuey	21
Dermacentor nitens	San Cristobal	18
Dermacentor nitens	Engombe D.N.	9
Dermacentor nitens	Sto.Domingo	15
Rhipicephalus sanguineus	Sto.Domingo	60

(*) Distrito Nacional, Provincia Altagracia y Provincia San Cristobal, Republica Dominicana.

Resultados

En la Tabla 2 estan referidos los resultados de las 106 reacciones ejecutadas, en las cuales se obtuvieron 83 resultados positivos, lo que representó 78.3% de positividad. En la misma tabla, llama la atención la ausencia de resultados positivos en los artrópodos capturados en mulos, aun considerando lo pequeño de la muestra. Por otro lado, es de destacarse la elevada positividad encontrada en ejemplares capturados en perros.

Tabla 2

Resultados obtenidos en el analisis del contenido estomacal de las garrapatas

Procedencia de la garrapatas	Cantidad de Macerados	Reacción con antisuero especf. (*)		
		Positivos	Negativos	% Positividad
caballo	36	25	11	69,4
buey	45	39	6	86,6
perro	20	19	1	95,0
mulo	5	0	5	0
TOTAL	106	83	23	78,3

* Antisueros usados: caballo, buey y perro

Discusión

Diversos métodos han sido puestos en práctica para identificar la sangre ingerida por artrópodos hematófagos. Algunos muy sencillos pero poco eficientes; otros, algo trabajosos aunque ofreciendo resultados compensadores.

Entre los primeros, se destacan la técnica de identificación por microscopía directa de los hematíes encontrados en el tracto digestivo del artrópodo[6] y la técnica de aglutinación de los mismos, con inmunosueros preparados contra hematies de diversas especies animales[5]. Ambos son de eficiencia discutible, principalmente porque el proceso digestivo destruye rapidamente las caracteristicas morfológicas diferenciales existentes en los eritrocitos de algunos grupos animales (reptiles, aves, algunos mamiferos). Por la misma razón, las características antigénicas también son modificadas, interfiriendo en la aglutinación.

En nuestro trabajo, nos inclinamos por los métodos de Ricciardi & Mello[8] y Crans[2] este último método ya que ofrecen grandes ventajas sobre la técnica de precipitación en tubo capilar[4], ventajas estas ya demonstradas experimentalmente[8], a saber: 1) mayor sensibilidad, principalmente cuando se hace, previamente a la lectura, vaporización con ácido acético[9]. 2) Las cajas de Petri, con el agar ya listo para la reacción, puede ser guardado en cámara húmeda, a temperatura ambiente, sin perjuicio de los resultados, por más de 6 meses. Esta es uma ventaja fundamental,

ya que permite la realización de reacciones en el campo. 3) No da margen a variación de resultados, entre uno y otro lector, hecho que suele ocurrir con otras técnicas, principalmente cuando la muestra sanguínea está acompañada de detritus. 4) Mayor rapidez, ya que la muestra a ser analizada puede ser colocada, de una sola vez, frente a varios inmunosueros.

Las 106 reacciones ejecutadas, evidenciaron 23 resultados negativos (Tabla 2). Entre estos, es especialmente interesante la ausencia de reacciones positivas para artrópodos capturados en mulos. Aunque la muestra fué mínima, se puede especular acerca de la gran especificidad que presenta la técnica escogida, viéndose que no ocurrieron reacciones cruzadas con inmunosuero de caballo.

Los demás resultados negativos pueden ser explicados, en parte, por el tiempo que transcurrió entre la captura del artrópodo y el procesamiento de la reacción, ya que no fué usada la técnica de transporte de la muestra sanguínea en papel de filtro[10,11]. Este argumento se refuerza si se observa el elevado porcentaje de positividad encontrado en perros (95%), una vez que la obtención de garrapatas de estos animales siempre ocurrió cerca del local del análisis, en contraposición a lo que ocurría con garrapatas capturadas en locales más distantes, ya sea en caballo, buey o mulo (Tabla 2).

Se puede pues concluir, que es perfectamente posible definir los hábitos alimentarios de garrapatas, por la técnica de doble difusión en gel de agar, método que se presenta más seguro y sencillo que la prueba de precipitación en tubo capilar.

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Isolamento de micobactérias atípicas a partir de gânglios linfáticos de suínos*

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Resumo

Isolamento de 79 amostras de micobactérias atípicas a partir de 200 gânglios linfáticos cervicais e mesentéricos de suínos aparentemente sadios. A maioria pertence ao grupo IV de Runyon (82%), seguindo-se micobactérias dos grupos III (13%) e II (5%). Entre as amostras do grupo II, foram identificadas *M. gordonae, M. flavescens* e *M. scrofulaceum.* As do grupo III apresentaram comportamento similar ao do complexo *avium-intracellulare* e *M. terrae.* No grupo IV, houve predominância de *M. fortuitum.* O significado de tais isolamentos é discutido.

Summary

Isolation of atypical mycobacteria from lymph nodes swines.

Seventy-nine strains of atypical mycobacteria were isolated from two hundred mesenteric and cervical lymph nodes of apparently healthy swines. Most of the strains belonged to the Runyon's group IV (82%) and some to groups III (13%) and II (5%). The strains of group II were identified as *M. gordonae, M. flavescens* and *M. scrofulaceum.* The strains of group III displayed all caracteristics of *avium-intracellulare* complex and *M. terrae.* Most of strains belonging to group IV were *M. fortuitum.* The significance of the results is discussed.

Introdução

O interesse pelas micobactérias atípicas cresceu consideravelmente na última década, sobretudo em decorrência do significado clínico de muitos desses microrganismos[4,7,24]. Embora já se tenha conseguido uma classificação que permite identificar as espécies com potencialidade patogênica para o homem, muito pouco é conhecido sobre a epidemiologia das micobacterioses produzidas por tais microrganismos[5,17,19]. O reservatório permanece desconhecido, mas os animais constituem um dos candidatos potenciais, sobretudo da espécie *M. intracellulare*[3,5,7,19]. Há comunicações, na literatura, do isolamento desse e de outros microrganismos do gênero *Mycobacterium* a partir de suínos, gado bovino e outros animais[3,6,20,25].

Neste trabalho é relatado o isolamento de micobactérias atípicas de gânglios linfáticos de suínos aparentemente sadios.

Material e Métodos

Foram utilizados 200 gânglios linfáticos mesentéricos e cervicais de suínos aparentemente sadios, provenientes do "Frigorífico Diniz" (Contagem, Minas Gerais), colhidos em fins de 1971. Os gânglios foram embalados em sacos plásticos, imediatamente após a coleta, e transportados em caixa de isopor contendo gelo. No laboratório, foram transferidos, sob condições assépticas, para placa de Petri estéreis e conservados em "Freezer" a -20°C. Antes da manipulação, as placas foram mantidas à temperatura ambiente, durante 1 a 4 horas. Os glânglios foram então triturados em gral estéril, com auxílio de areia, adicionando-se 10ml de solução fisiológica. Aproximadamente 1,0ml da suspensão foi retirada com pipeta Pasteur com bulbo.

Foi empregado o método de Loewenstein adicionando-se, à suspensão, volume a volume, ácido sulfúrico a 6%. Após 15 minutos, o material foi centrifu-

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gado a 3.000 r.p.m., durante 30 minutos e ressuspenso em solução salina estéril. Seguiu-se nova centrifugação e o sedimento resultante, foi suspenso em 0,5ml de solução salina tamponada a pH 7,0.

A partir de cada gânglio, foram inoculados três tubos de Loewenstein-Jensen, um dos quais contendo 0,5% de piruvato para isolamento de *M. bovis.* Os tubos foram incubados a 37°C durante 6 semanas, sendo examinados ao final de cada semana. As colônias visualizadas foram caracterizadas como constituídas por microrganismos álcool-ácido resistentes, de acordo com o método de Ziehl-Neelsen.

As amostras isoladas foram identificadas através das seguintes características: pigmentação em presença e ausência de luz, velocidade de crescimento em meio de Loewenstein-Jensen, fermentação de sacarose e manitol[8], atividade arilsulfatásica em três dias[18], redução do telurito de potássio em três dias[10], crescimento em agar MacConkey[12], atividade catalásica à temperatura ambiente[15] e a 68°C[1], redução do nitrato[13], hidrólise do "Tween 80"[26] e crescimento às temperaturas de 40°C, 45°C e 52°C.

Resultados

Em apenas 29 (14,5%) dos 200 gânglios linfáticos examinados obtiveram-se culturas positivas para micobactérias. De acordo com a morfologia colonial, foram selecionadas 79 amostras, sendo 63 provenientes do meio de Loewensteins-Jensen e as restantes do meio adicionado de piruvato. Somente dois gânglios renderam crescimento apenas no último meio (amostras números 49 e 50).

Todas as micobactérias isoladas foram classificadas em três dos grupos de Runyon: 4 (5%), 10 (13%) e 65 (82%) amostras, pertenceram aos grupos II, III e IV, respectivamente. Não foi constatada a presença de micobactérias fotocromogênicas (grupo I).

Os isolamentos, com a respectiva identificação das espécies, estão referidos nas Tabelas 1 e 2. Na Tabela 1, observa-se a identificação das amostras de crescimento lento. As escotocromogênicas (grupo II) comportaram-se como *M. gordonae*[2], *M. flavescens*[1] e *M. scrofulaceum*[1]. A reação de redução do telurito de potássio em três dias foi a única exceção observada; foi positiva para *M. gordonae* e *M. scrofulaceum.* No que se refere às 10 micobactérias não cromogênicas, de crescimento lento (grupo III), cinco foram identificadas como do complexo *avium-intracellulare* e cinco restantes como *M. terrae*. Saliente-se que *M. terrae* não reduz o telurito de potássio no tempo indicado, como fizeram as amostras isoladas.

Na Tabela 2, estão relacionados os isolamentos e a identificação de micobactérias de crescimento rápido (grupo IV). Entre estas, a grande maioria foi constituída por *M. fortuitum* (74%). Foram ainda identificadas amostras de *M. peregrinum*[3], *M. vaccae*[3] e *M. diernhoferi*[2]. Houve duas exceções quanto ao comportamento esperado: as amostras de *M. peregrinum* não fermentaram o manitol e as de *M. diernhoferi* cresceram a 40°C.

Considerou-se como pertencente a uma dada espécie a amostra que variou apenas em uma característica do comportamento esperado para a mesma[14,16]

Tabela 1

Identificação de micobactérias dos grupos II e III de Runyon

Grupo	Nº de Amostras	TESTES							Espécies
		Presença de Pigmento	Redução de Nitrato > +++	Hidrólise Tween 80 em 5 dias	Redução do telurito em 3 dias*	Ati. Catalásica. < 45mm	> 45mm	+ 68°C	
II	2	+	-	+	+	+	+	+	*M. gordonae*
	1	+	+	+	+	+	+	+	*M. flavescens*
	1	+	-	-	+**	+	+	+	*M. scrofulaceum*
III	5	-	-	-	+	+	-	+	*M. avium M. intracellulare*
	5	-	V*	+	+**	+	+	+	*M. terrae*

* Variável
** Comportamento divergente do esperado

Tabela 2

Identificação de micobactérias do grupo IV de Runyon

Presença de Pigmento	Nº de Amostras	TESTES								
		Aril-Sulfatase 3 dias	Crescimento MacConkey	Redução de Nitrato >+++	Fermentação		Crescimento			Espécie
					Sacarose	Manitol	40°C	45°C	52°C	
Ausente	50	+	+	+	-	V	+	-	-	*M. fortuitum*
	3	+	+	+	+	-**	-	-	-	*M. peregrinum*
	2	-	+	+	-	+	V*	-	-	*M. diernhoferi*
	4	+	+	+	V	-	V	-	-	Não identificada
Presente	3	-	-	V*	+	+	+**	-	-	*M. vaccae*
	3	V*	-	V*	-	-	V*	-	-	Não identificada

V* Variável ** Comportamento divergente do esperado

Discussão

Micobacterioses ocasionadas por micobactérias atípicas têm sido registradas em todos os países[22]. Na maioria, são causadas pelas espécies *M. kansasii* e *M. intracellulare*[17]. No entanto, o reservatório natural destes microrganismos permanece desconhecido até o momento[19]. As fontes ambientais (solo, água, etc) e animais parecem ser as causas mais prováveis de infecção[11].

A maioria das micobactérias isoladas a partir de gânglios linfáticos de suínos pertence às espécies *M. intracellulare* e *M. gordonae*[3,20,21,23,25]. Há dados, não confirmados na literatura, da presença de *M. kansasii* e *M. xenopi* neste tipo de material[9,25].

O presente trabalho relata o isolamento de 79 amostras de bacilos álcool-ácido-resistentes, na sua maioria (82%) de crescimento rápido. Entre as de crescimento lento, foi encontrada uma amostra de *M. scrofulaceum* e cinco do complexo *avium-intracellulare,* que representam problema potencial ao homem[17].

A alta freqüência de micobactérias do grupo IV, pertencentes sobretudo à espécie *M. fortuitum* (74% dentro do grupo), parece sugerir uma contaminação externa, dada a distribuição ubiquitária deste microrganismo.

Entre as amostras que cresceram somente em Loewenstein-Jensen com piruvato (números 49 e 50), uma pertence ao complexo *avium-intracellulare,* enquanto a outra, não identificada, fornece reações mistas.

O papel dos suínos, nas infecções por micobactérias atípicas, sobretudo as causadas por *M. intracellulare,* tem sido sugerido como possível, em virtude de inúmeros isolamentos desse microrganismo relatados entre os suínos[3]. O animal poderia ser intermediário na cadeia epidemiológica ou, juntamente como o homem, infectar-se a partir de uma fonte comum[11].

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The production of soil pores by bacteria*

D.B. Arkcoll**

Summary

The application of fermentable substrates to soils increases the aggregation of particles because of the cohesive properties of the polysaccharide gums produced by soil bacteria. Under anaerobic conditions *Chlostridia* also produce acid and gas and fix nitrogen. When diffusion is poor this gas can expand the soil volume as much as threefold. Some contraction occurs on drying but the porous structure is maintained on rewetting because the individual pores appear to be surrounded by polysaccharides.

Resumo

Produção de poros no solo por bactérias

A aplicação de substratos fermentáveis ao solo aumenta a agregação de partículas devido às propriedades coesivas de mucilagens polissacarídicas produzidas por bactérias do solo. Sob condições anaeróbicas, espécies de *Clostridium* também produzem ácido e gás e fixam nitrogênio. Quando a difusão é pobre, o gás pode expandir o volume do solo até três vezes. Podem ocorrer contrações com a dessecação, mas a estrutura porosa é mantida com o umidecimento, pois cada poro parece estar circundado por polissacarídio.

Introduction

Fodder crops are fractionated commercially now in several parts of the world[1]. Apart from lipid-protein complexes for monogastric animals and the fibrous residue for ruminants, a liquor is also produced that contains most of the heat soluble plant compounds. This seems likely to create a waste disposal problem at least during early production until the feasability of fermenting it to microbial protein and a beverage has been established. Consequently it has been suggested that the liquor should be irrigated back on the land to return soil nutrients. Some effects on soil structure are reported here.

Methods

Different quantities of liquor from several plant species were applied to a number of soil types in plant pots or 250 ml graduated beakers. Changes in the macrostructure were observed together with the microstructure of thin sections cut after impregnation with resin. Volumetric changes were recorded continuously and densities were also measured on extracted cores. The stability on slaking 2 to 6 mm particles with water was determined by the method of Williams & Cooke[6]. Further experimental details are given in the following text.

Results and Discussion

In the first experiment, pots were filled with a mixture of three parts of soil to one of sand and mustard seeds sown in a layer of sand placed on the surface. The pots were irrigated with 200 ml of either water or Lucerne liquor twice a week. After two weeks it was noticed that the sand on the top of the liquor pots had stuck together and was impeding the penetration of liquor into the soil. The experiment was stopped and two months later the dry soil was

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carefully turned out of the pots. Soil from the watered pots fell apart readily and free sand particles were clearly visible. The soil treated with liquor came out in a single clod and it was difficult to make out individual grains of sand with the naked eye because they had become intimately bound into soil aggregates. Presumably microbes had grown on the liquor and produced the polysaccharide gums that are thought to play an important part in the stabilization of soil aggregates[4]. In addition, the liquor itself contains many plant polysaccharides as well as phenolic compounds that may enhance this effect[3].

In the next experiment Saxmundham soil which appears to have an inherently poor structure[2] was stirred in a bucket with either water or liquors from different plants and left to dry. The soils were then carefully removed undisturbed at about field capacity. The liquor had clearly increased the volume and had produced a crumb structure as good as that from the best of soils. Cores were taken for measurement of bulk density and slaking instability and also for plant growth experiments. The density had been lowered by between 10 and 20% because of the presence of numerous pores up to 3 mm in size. Slaking tests on aggregates showed an increase in stability in the soil treated with Lucerne but not with Cocksfoot liquor. This was explained by the presence of a large amount of fungal mycelium inundating the Lucerne treatment. However the 10 cm cores taken for plant growth studies showed no volumetric change after watering all treatments for a month. Other cores showed that the control shrank more on drying and swelled more on rewetting than the treated soils because of the stability conferred by both liquors.

Thin sections of treated soil showed that most of the pores were spherical in shape and that the soil particles surrounding them were coated in an unfamiliar layer. Staining with acidic aniline revealed the presence of large and almost spherical bacteria usually in a single chain along this layer. The individual cells were well separated by typical polysaccharide capsules. Thus a continuous layer of bacterial gum appears to surround the pores and give them stability. When small aggregates are produced for the slaking tests the pore coat is ruptured in many places which may explain the loss of stability in the Cocksfoot treatment. This suggests that pores rather than aggregates may be the stable units in some soils. The predominant bacteria producing the ferment were shown to be of the *Clostridium butyricum* or *C. pasteurianum* type[5].

Subsequent experiments showed that it is possible to obtain up to a threefold increase in soil volume in 250 ml beakers with 1% by weight of fermentable solids under optimum conditions. Such a ferment would contract on drying to give as much as double the original volume. Typically the phenomenon involves an initial lag followed by a rapid expansion during the growth of the organism, a peak on the exhaustion of the substrate and then a gradual collapse into a stable porous clod. The peak occurs between twelve hours and ten days after treatment depending on the type of substrate, the temperature and the initial anaerobic count. These and the moisture content also affect the degree of expansion. Some effect was seen in all the soils studied without innoculation. Even fine sands and clays responded, although different quantities of liquor were needed to create the correct waterlogged conditions. Too small a volume inhibits the growth of *Clostridium spp.* and allows gas to diffuse away. Similarly, if too much is present, most of the ferment takes place in the surface liquid and the gas escapes readily.

In addition to the gas production about 20 mg of N are fixed per gram of carbohydrate and suficient organic acid produced to lower the soil pH about one unit. A secondary fermentation also occurs if the soils are left under waterlogged conditions for several months. This also takes place in untreated soils and depends on the quantity of organic matter present. It is slow because the compounds are more complex. The organisms involved are thought to be methane producers and the strongly anaerobic conditions created around their gaseous pores often precipitated dissolved iron compounds as black ferric sulphide. This ring of sulphide then oxidized as the soil dried out leaving an oxide coat around the pores.

The practical significance of these effects is now being studied. It seems likely that they already take place in nature to some extent when the right conditions prevail because of the widespread occurence of the organisms involved and the presence of root exudates and decaying vegetation in soils. However the practical use of adding fermentable wastes to soils will depend not only on the ease with which the conditions of poor diffusion can be produced in the field but also on the subsequent effect on plant growth. Whilst we have had improved growth resulting from better root penetration in some preliminary trials, in others there has been inhibition of germination and phytotoxicity. It seems that much research is needed to benefit from the advantages of increased aggregation, porosity, nitrogen fixation, mineral release and the destruction of some pathogens, without suffering from the problems of increased acidity, microbial competition for and binding of nutrients and pathological attack by the increased microbial population.

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Monitoring for the kerosene fungus *Amorphotheca resinae**

J.E. Sheridan**

Summary

Microbial contamination of jet fuel was world-wide in the 1960's and the most frequently isolated organism was the fungus *Amorphotheca resinae (Cladosporium resinae)*. While early attempts to isolate *C. resinae* from soil and air failed due to lack of reliable techniques, during the last few years techniques have been devised for successful monitoring of soil and air for this fungus. These have not yet been widely used. Jet fuel, soil and air have been monitored in New Zealand and results show that *C. resinae* is widespread forming a component of the airspora. Results are presented and the need for establishing global monitoring stations for this fungus stressed.

Resumo

Monitoração do fungo querosenófilo Amorphotheca resinae

Contaminação microbiana de combustível de jatos ocorreu amplamente na década de 1960 e o principal microrganismo isolado foi o fungo *Amorphotheca resinae (Cladosporium resinae)*. Não havia sido possível isolar o fungo do solo e ar em vista à falta de técnicas adequadas. Nos últimos anos, todavia, o desenvolvimento de técnicas mais específicas permitiu a monitoração do organismo no solo e ar, embora isso não tivesse sido utilizado mais amplamente. Combustível de jatos, solo e ar têm sido monitorados na Nova Zelândia e os resultados revelam que *A. resinae* é elemento bastante disseminado e componente normal da biota aérea. Resultados da monitoração são apresentados e a necessidade de detecção do fungo de modo mais global é enfatisada.

Introduction

Microbial contamination of kerosene-type jet fuel caused problems, i.e. blockages and corrosion, in aircraft in the early 1960's, was world-wide and was worst in aircraft operating in the tropics[8]. The organism most frequently isolated was the fungus *Cladosporium resinae* (Lindau) de Vries, perfect state *Amorphotheca resinae* Parbery. Hazzard[5] found it in 78% of fuel samples from aircraft in Australia in 1961 and 1962, Gutheil[4] found it in fuel taken from a number of aircraft in Brazil in 1963 and about the same time Engel and Swatek[3] found it in 80% of fuel samples from aircraft in California. A few years later Darby & col.[2] found it to be the dominant fungus in a military distribution system in New Hampshire, U.S.A. It has also been isolated from aviation fuel, tank filters or aircraft fuel tanks in Denmark, England, India, Nigeria, New Zealand, Syria[1] and Japan[7]. This fungus has, therefore, been found to occur in aircraft fuel around the world and it is considered to be the most important fungus in contamination and corrosion[9]. After steps were taken to reduce growth of micro-organisms in jet fuel and the introduction of "good housekeeping" little damage was reported. However, *C. resinae* was not eliminated from fuel and still occurs with high frequency in jet fuel in New Zealand as will be seen in the work reported here.

Until recently attempts to isolate this fungus from nature failed because of inadequacies of techniques. Reliable methods are now available for selectively isolating *C. resinae* from soil[10,17] and air[12] and it has been found to be widespread in soils in Australia, Britain, Europe[10] and New Zealand[17]; it is probably a natural component of the soil mycoflora[10]. During the last few years viable spores have been trapped consistently over Wellington, New Zealand[11] and

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there is little doubt that these form a significant component of the airspora in this country. Although previous attempts to correlate the occurrence of *C. resinae* in nature with incidence of fuel contamination failed, the application of new techniques and build up of knowledge on *C. resinae* in the airspora should make it possible to solve this problem. Attempts are being made to do this in New Zealand and findings are presented and discussed in this paper. Since this fungus usually occurs in Jet fuel and in nature in the imperfect state *C. resinae f. avellaneum*[2,10,15] it will be referred to throughout this paper as *C. resinae*.

Materials and Methods

Microbiological records collected between 1967 and 1971 relating to military installations and aircraft in New Zealand were examined. Between September, 1972 and March, 1973, my colleagues, J.J. Soteros and N. Grbavac, and myself initiated a survey of fuel installations, tankships and aircraft for fungal contamination using Hazzard's method[6] and methods developed in our laboratory[16]. Fuel from military installations and aircraft contained the anti-icing additive ethylene glycol monomethyl ether (EGME, Icinol) at 0.10 to 0.15 per cent. Methods for examining soil and air were those described by Sheridan, Steel & Knox[17] and Sheridan & Nelson[12] respectively.

Results

Occurrence of C. resinae in jet fuel — Results indicated that 50% of fuel samples were contaminated with *C. resinae* prior to the introduction of the anti-icing additive EGME in 1968. Thereafter contamination fell to 9% in fuel containing EGME but *C. resinae* was apparently not eliminated (Fig. 1). In the 1972-73 survey, *C. resinae* was found in 93% of all samples without EGME and 28% of samples containing EGME. Fig. 2 shows the origin of the samples (tankships, ground installations, aircraft) and the levels of contamination. *C. resinae* was found in 86% of samples from ground installations and all samples from aircraft in the absence of EGME. Samples were collected from internally operating Boeing 737's, Friendships and Viscounts and DC.8 aircraft operating between New Zealand and Australia. Mean counts/litre were highest in internally operating aircraft (55/litre); in one aircraft counts of 234 and 303/litre were obtained for starboard and port tanks respectively. Levels in tankships, ground installations and aircraft operating overseas were very similar. The temperature of fuel

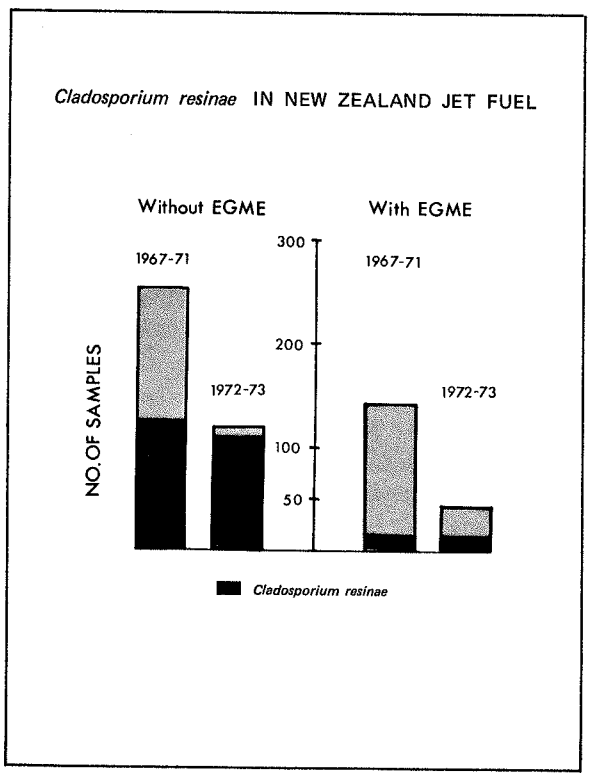

Fig. 1. — *Cladosporium resinae* in New Zealand jet fuel: frequency of contamination during 1967-71 and 1972-3.

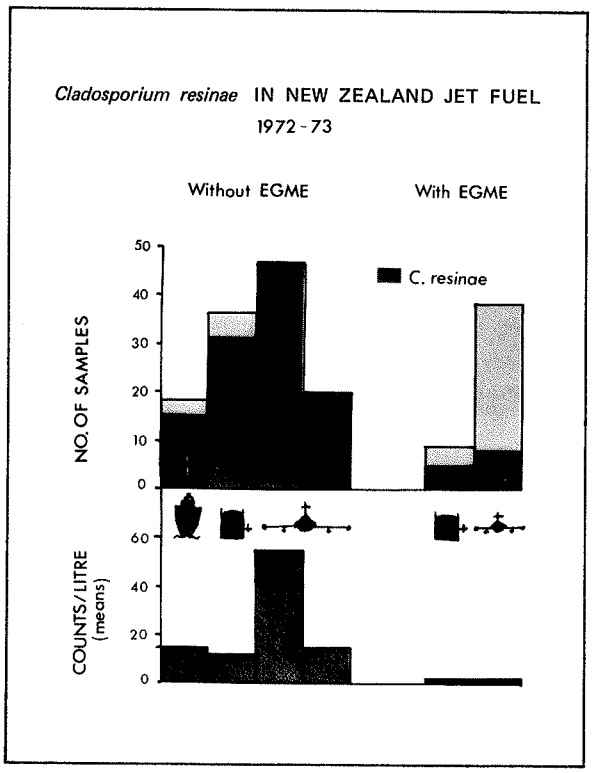

Fig. 2. — *C. resinae* in New Zealand jet fuel: source of samples and mean counts/litre.

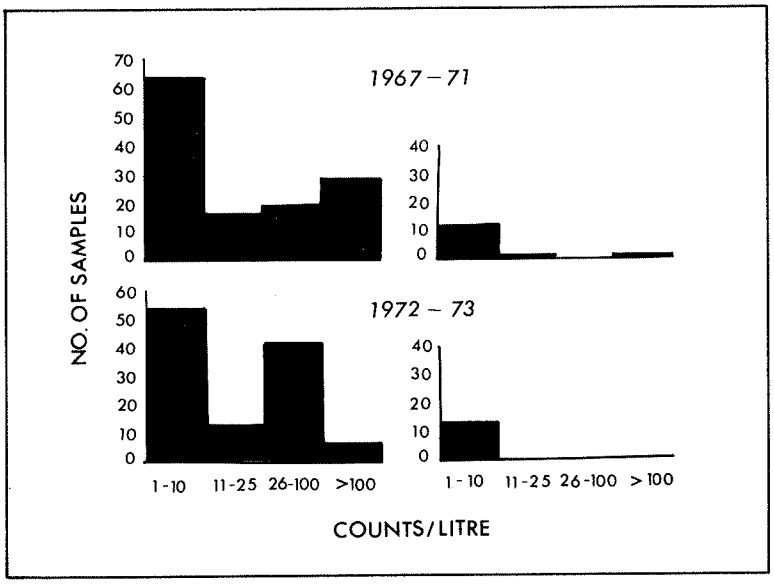

Fig. 3. — Levels of *C. resinae* in New Zealand jet fuel with (right) and without (left) EGME 1967-71 and 1972-3.

collected from these aircraft ranged from 1–6°C while in internally operating aircraft it was around 20°C. In the latter case *C. resinae* could grow if other conditions were suitable and this may explain the higher counts. *C. resinae* occurred less frequently in fuel containing EGME and levels were low being 2/litre in both ground installations and aircraft. Where contamination occurred it was possible in some cases to trace the cause to refuelling of aircraft with a fuel to which EGME had not been added. However, the possibility of EGME tolerance cannot be ruled out and should be investigated.

In an attempt to locate the source of contamination tankships were surveyed and five out of eight tested were found to be contaminated. There is also some evidence that where *C. resinae* was not detected this was due to causes such as delay in testing or effect of transportation. Fig. 3 shows a breakdown of contamination levels; only two samples containing EGME had counts greater than 10/litre.

While *C. resinae* was the most frequently isolated organism and the only fungus which grew vigorously in jet fuel, over thirty other fungi were isolated including *C. cladosporioides, C. herbarum* and *C. sphaerospermum.*

Many of these are components of the airspora. They probably enter jet fuel as airborne spores and although most of them do not grow in kerosene-type fuel and none of them grow as vigorously as *C. resinae* the spores remain viable for some time.

This survey shows clearly that *C. resinae* is present in most fuels in New Zealand at the present time, that the micro-filters and coalescers do not remove it from the system, and that it still poses a potential threat. The addition of icinol (EGME) at 0.10–0.15 per cent greatly reduces incidence but does not eliminate the fungus.

Occurrence of C. resinae in soil and air — A survey of soil showed that *C. resinae* is widespread in New Zealand[17]. It is apparently active in the soil throughout the year because it was isolated from soil, and airborne spores were trapped above a cultivated soil, at all seasons of the year. The air over Wellington, New Zealand, has been monitored for *C. resinae* since March, 1971 and for total airspora since August, 1972, using two Hirst spore traps. Viable

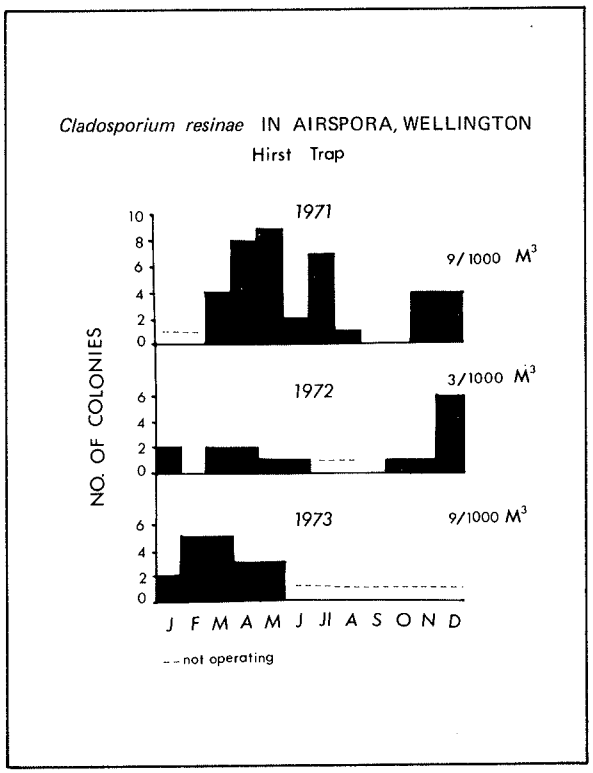

Fig. 4. — *C. resinae* in airspora over Wellington, New Zealand 1971-3 (Hirst spore trap).

airborne *C. resinae* was trapped during all the months of the year except September but more frequently during the Summer and Autumn (Fig.4). The mean concentration is low (less than 10/1000 M^3) compared to that of all *Cladosporium* species (Fig.5).

Since trappings began in March 1971, more spores have been trapped in early morning and in the evening than at other times (Fig.6). The indications are that *C. resinae* follows a seasonal and circadian rhythm but as yet too few trappings have been made and the work has not continued long enough to confirm this. The concentration of spores in the air probably varies from place to place and year to year.

Trappings have been made up to two metres above a cultivated soil but it is not known how high spores rise after release, how long they remain viable, how far they travel, nor whether they come together to form "spore clouds". All of these aspects are currently under investigation.

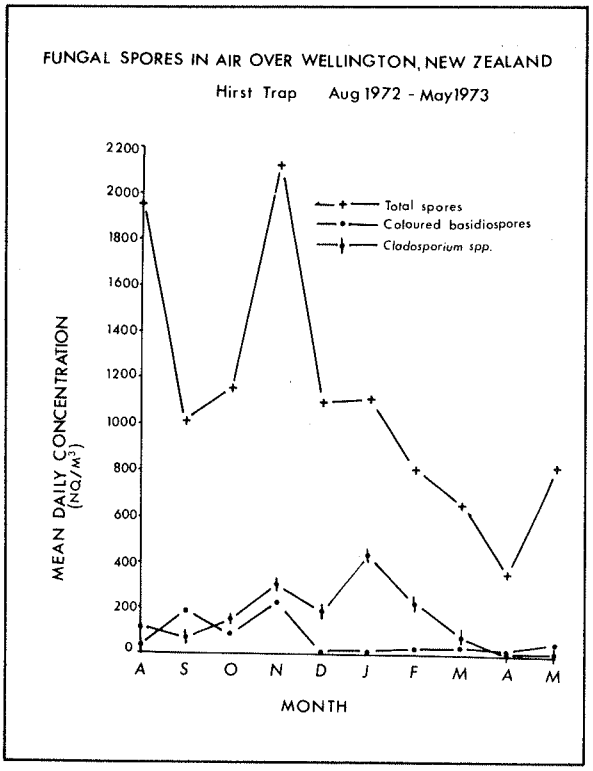

Fig. 5 — Mean daily concentration of fungal spores in the air over Wellington, New Zealand from August, 1972 until May, 1973 (Hirst spore trap).

Discussion

In New Zealand there are three possible sources of contamination of jet fuel: tankships, soil and air. *C. resinae* has been found in all three. Because the majority of fungal contaminants are components of the airspora it is considered that *C. resinae* also enters the fuel from the air. However, since most of the fuel brought to this country is apparently already contaminated, airborne *C. resinae* probably plays an insignificant role in contamination at the present time. Where fuel supplies are initially free of *C. resinae* or where *C. resinae* forms a greater component of the airspora, airborne spores could possibly contribute significantly to contamination.

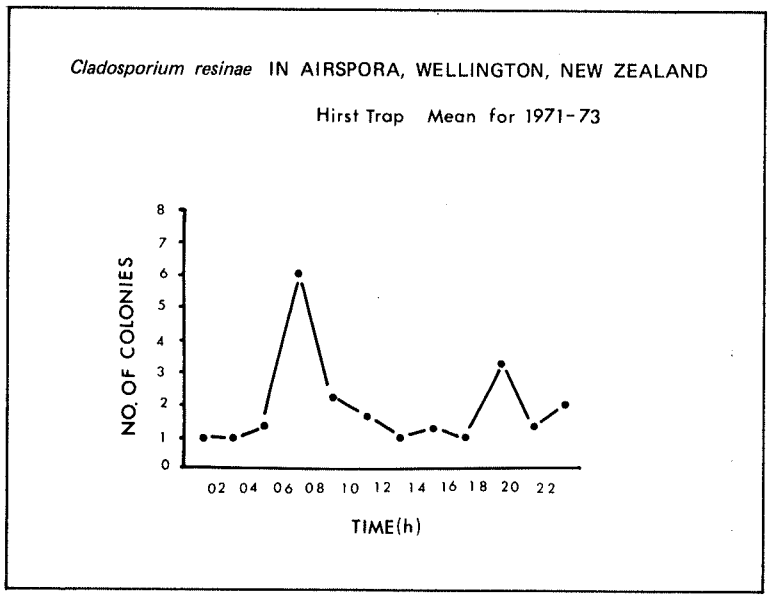

Fig. 6. — Relation between time of day and number of spores of *C. resinae* trapped 1971-3 (Hirst spore trap).

Although there is some evidence for seasonal and circadian rhythms these are not yet firmly established and until such times as this is done they cannot be related to seasonal incidence of aviation fuel contamination.

In addition to its occurrence in aviation fuel, soil and air, *C. resinae* has been isolated from creosoted timbers such as telegraph poles and railway sleepers; tarred wood, asphalt and resins; bituminised cardboard; a cosmetic face cream; the female sex hormone, progesterone; methyl-p-hydroxybenzoate, and bird feathers[14].

Apart from New Zealand little is known about the occurrence of *C. resinae* in soils and air. It is not known for example, whether this fungus is present in soil or air in tropical countries nor whether soil there will support its growth. Studies on the geographic distribution of *C. resinae* are, therefore, urgently needed in order to determine the role of airborne spores in fuel contamination. It would be desirable to see Global Monitoring Stations set up for *C. resinae* from which results could be sent back to New Zealand for collation. This fungus is so well suited to environmental studies and it is trusted that this paper may stimulate interest researchers in this unique, intriguing and important organism.

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