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Use of a Tobacco Agar Medium (TAM) to Detect *Cryptococcus* and *Candida* Colonies Isolated from Tobacco*

by

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SUMMARY

The use of a tobacco agar medium (TAM) was investigated to visually differentiate *Cryptococcus* species from *Rhodotorula* and *Candida* species that can be isolated from tobacco. This study was first conducted with pure isolates of each of the major yeast species that have been isolated from tobacco. All *Cryptococcus* strains that were tested produced colonies with different degrees of pigmentation ranging from light to dark brown or black. All *Candida* and *Pichia* colonies were white to off-white. *Candida parapsilosis* colonies were easily differentiated since they had rough contours and surfaces. All *Rhodotorula* colonies were pink or orange. In order to validate the use of this medium, tobacco was spiked with a mixed culture of *Cryptococcus, Candida, Rhodotorula* and *Pichia*. TAM allowed visual detection and enumeration of the four yeast genera based on colony colour and/or morphology. [Beitr. Tabakforsch. Int. 22 (2006) 204–207]

ZUSAMMENFASSUNG

Die Verwendbarkeit eines Tabak-Agarnährbodens (TAM) zur visuellen Differenzierung der verschiedenen *Cryptococcus*-Arten von den *Rhodotorula*- und *Candida*-Arten, die man aus Tabak isolieren kann, wurde geprüft. Diese Untersuchung wurde zunächst mit reinen Isolaten der wichtigsten Hefearten, die aus Tabak isoliert wurden, durchgeführt. Alle untersuchten *Cryptococcus*-Stränge bildeten Kolonien von unterschiedlicher Pigmentierung, die von hellbraun bis dunkelbraun oder schwarz reichten. Alle *Candida* und *Pichia*-Kolonien waren weiß bzw. cremefarben. Kolonien von *Candida parapsilosis* waren aufgrund ihrer rauhen Konturen und Oberflächen leicht zu erkennen. Alle *Rhodotorula*-Kolonien waren rosa oder orangefarben. Um die Anwendung dieses Mediums beurteilen zu können, wurde Tabak mit einer Mischkultur aus *Cryptococcus, Candida, Rhodotorula* and *Pichia* versetzt. Der TAM-Nährboden erlaubte auf der Basis der Farbe und/oder Morphologie der Kolonien visuelles Erkennen und Auszählung der vier Hefearten. [Beitr. Tabakforsch. Int. 22 (2006) 204–207]

RESUME

L'utilisation d'un milieu gélosé à base de tabac (TAM) a été étudiée dans le but de différencier visuellement les espèces *Cryptococcus* des espèces *Rhodotorula* et *Candida* qui peuvent être isolées du tabac. Cette étude a d'abord été menée avec des isolats purs de chaque espèce de levures principalement isolées du tabac. Toutes les souches de *Cryptococcus* testées produisaient des colonies ayant différents degrés de pigmentation allant de brun pâle à brun foncé ou noir. Toutes les colonies de *Candida* et *Pichia* étaient dans les tons de blanc. Les colonies de *Candida parapsilosis* étaient facilement différenciées par leur contours et surfaces rugueux. Toutes les colonies de *Rhodotorula* étaient roses ou oranges. Afin de valider l'utilisation de ce milieu, le tabac a été enrichi d'une culture mixte de *Cryptococcus, Candida, Rhodotorula* et *Pichia*. Le milieu TAM a permis une détection et un décompte des quatre genres de levures en se basant sur la couleur et/ou la morphologie des colonies. [Beitr. Tabakforsch. Int. 22 (2006) 204–207]

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INTRODUCTION

A tobacco agar medium (TAM) supporting the growth of *Cryptococcus neoformans* and allowing its fast differentiation by formation of brown coloured colonies was recently described (8).The property of producing brown colonies on this media provided a definitive identification of *Cryptococcus neoformans.* It was attributed to the production of a black pigment from various substrates (caffeic acid, L-dopa, chlorogenic acid, dopamine, epinephrine, norepinephrine) which accumulated in the fungal cell due to laccase activity. *C. neoformans* has not been isolated from tobacco but other *Cryptococcus* considered as saprophytic species *(*e.g. *C. albidus, C. diffluens, C. magnus)* were isolated. Some of these *Cryptococcus* species can also produce laccase (2). Positive laccase activity was also recently reported in *Candida* species (6). The objective of this work was to validate the use of TAM to visually detect brown C*ryptococcus* colonies from other yeast species isolated from tobacco (e.g. *Candida parapsilosis*).

MATERIALS AND METHODS

Preparation of media and yeast strains

Tobacco Agar Medium (TAM): One litre distilled water was added to 50 g of flue-cured cut tobacco. The mixture was boiled for 30 min, filtered through cheese cloth and its volume was then adjusted to one litre. Twenty grams of agar were added to this infusion which was then autoclaved at 121 ˚C for 15 min. When TAM was used with antibiotic to inhibit bacterial growth, it was cooled at 45 ˚C prior to supplementation with chloramphenicol (100 µg/mL), chlorotetracycline (100 µg/mL) and streptomycin (30 µg/mL). The agar was translucent and brownish yellow in colour after setting.

Littman Oxgall Agar (LOA) (Difco no. 0294): One litre distilled water was added to 55 g of LOA powder. The medium was autoclaved at 121 ˚C for 15 min. The LOA was cooled at 45 ˚C prior to supplementation with streptomycin (30 µg/mL).

Yeast Malt Agar (YMA) (Difco no. 0711): One litre distilled water was added to 21 g of YMA powder and 20 g of Bacto agar *(Difco no. 0140).* The medium was autoclaved at 121 ˚C for 15 min. The YMA was cooled at 45 ˚C prior to supplementation with chloramphenicol (100 µg/mL) and chlorotetracycline (100 µg /mL).

Microorganisms: Except for *Candida albicans* ATCC strain no. 10231, the *Rhodotorula, Cryptococcus, Pichia* and *Candida* species selected for this work were isolated from uncured and flue-cured tobacco on either LOA, or YMA or potato dextrose agar (PDA) (Table 1). They were maintained at 8 ˚C on PDA slant. Their identification was confirmed by using the Biolog YT microplate (Biolog Inc., Hayward, CA, USA). Prior to streaking on tobacco agar and other media, all yeasts were cultured and maintained on PDA at 26 ˚C for 72 h. *Candida bombi* strain L010, *Pichia subpelliculosa* strain L015 and *Candida albicans* strain ATCC no. 10231 were used as negative controls because their colonies were white to off white after ten days of incubation.

Table 1. Colour of yeast colonies following incubation for ten days at room temperature (20–26 ˚C) on Tobacco Agar Medium (TAM) (non inoculated tobacco agar was translucent and brownish yellow in colour)

Strain name	Colour and appearance of colony [a]
Candida albicans	
L004-ATCC no. 10231	W to OW
Candida parapsilosis	
L079	Ww to OWw
L089	Ww to OWw
L094	Ww to Oww
Cryptococcus	
L072 *C. diffluens*	LB to B
L092 *C. albidus*	LB to B
L099 *C. oeirensis*	LB to BL
L103 *C. diffluens*	LB to BL
L104 *C. liquefaciens*	LB to B
L108 *C. magnus*	LB to BL
L119 *C. albidus*	LB to B
Rhodotorula	
L083 *R. mucilaginosa*	P
L087 *R. mucilaginosa*	P
L111 *R. minuta*	OWo to OB
L112 *R. minuta*	P
L113 *R. mucilaginosa*	P
L117 *R. slooffiae*	O
Others	
L010 *Candida bombi*	W to OW
L015 *Pichia subpelliculosa*	W

[a] w = wrinkled; W = white; OW = off-white; OWo = off-white orange; BL = black; B = brown; LB = light brown; O = orange; OB = orange brownish; P = pink.

Preparation of spiking yeast suspensions

Candida parapsilosis (L094), *Cryptococcus albidus* (L092), *Rhodotorula mucilaginosa* (L113) and *Pichia subpelliculosa* (L015) were grown on PDA for 48 h at 26 ˚C. Ten mL of yeast suspensions in 1X Ringer solution were prepared in a Biolog turbidimeter (590 nm) and adjusted to 32% ± 2% transmittance. Decimal dilutions (1:10 v/v) of each of these suspensions in 1X Ringer solution were prepared. A 0.5 mL portion of each dilution was delivered either onto TAM supplemented (TAMS) or not with antibiotics, or onto LOA or into YMA. For TAM and LOA, the diluted sample was surface spread and for YMA the pour plate method was used. The plates were incubated for ten days at room temperature (20–26 ˚C). Yeast counts were expressed in CFU/mL and transformed in log of CFU/mL.

Preparation of non inoculated control tobacco samples

An aliquot of 20 g of flue-cured cut tobacco was placed in a 19 × 30 cm stomacher bag (Nasco Whirl-Pak filter bags Cat No. B01318) containing 250 mL of 1X Ringer solution. The

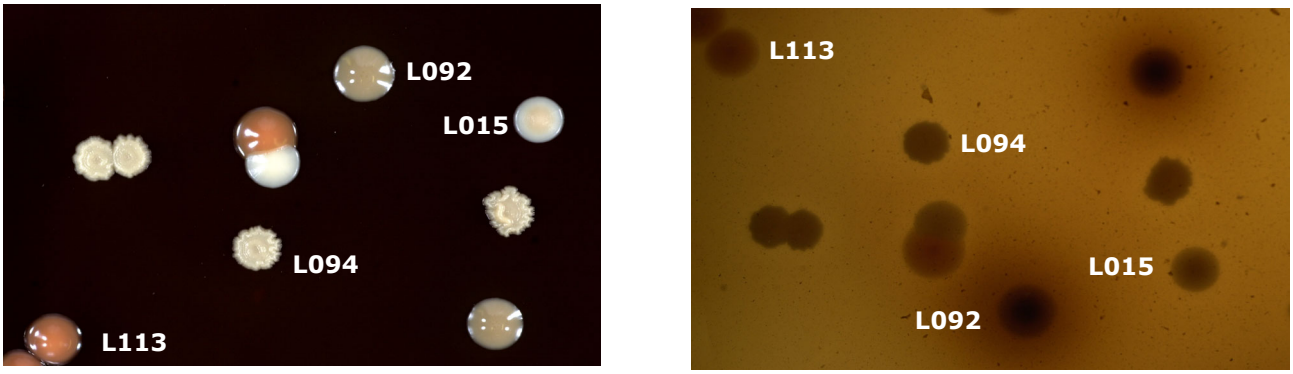

Figure 1. Yeasts grown for ten days on TAM at room temperature (20–26 ˚C) (L015 : *Pichia subpelliculosa*; LO92 : *Cryptococcus albidus*; L094 : *Candida parapsilosis*; L113 : *Rhodotorula mucilaginosa*)

bag was agitated at room temperature for 10 min in a Lab Blender Stomacher Model 400 (Seward Lab., London, SEI 9UG, UK). Four-fold dilutions (5/15 v/v) ranging from 1/4 to 1/16 were prepared using 1X Ringer solution. A 0.5 mL portion of the primary macerate and the prepared dilutions were delivered onto TAM, or onto LOA, into YMA in triplicate and onto TAMs in duplicate. The plates were incubated for ten days at room temperature (20–26 ˚C).

Preparation of spiked tobacco samples

A 0.75 mL portion of each yeast cell suspension were added to the stomacher bag containing the non inoculated control tobacco samples and agitated for 1 min. Dilutions up to 1/1024 and inoculations were performed as per control tobacco. The plates were incubated for ten days at room temperature (20–26 ˚C). Yeast counts were expressed as CFU/mL and transformed into log of CFU/mL.

RESULTS

Initially, the TAM was translucent and brownish yellow in colour. Following growth of all yeast strains, it became dark brown, except *Candida bombi* which did not darken the media. All *Cryptococcus* strains produced colonies with different degrees of pigmentation from light brown to brown or black. A dark brown halo was also visible around all *Cryptococcus* colonies on the reverse of the plate. All *Candida* and *Pichia* colonies were white to off-white and all *Rhodotorula* were pink or orange (Table 1). *Candida parapsilosis* colonies were easily differentiated from other *Candida* species since they had rough contours and surfaces at 20–26 ˚C (Figure 1).

No yeasts were detected in the control tobacco (Table 2). Yeast suspensions containing about 10^7 CFU/mL were used to spike the flue-cured cut tobacco sample. Yeast counts of the order of 10^7 CFU/mL were observed in spiked tobacco samples. The TAM supplemented or not with antibiotics, delivered yeast counts similar to the ones found in other media currently used to isolate and enumerate yeast (Table 2). The pH value of TAM was 5.0 ± 0.02 which rendered this medium selective enough to avoid using antibiotics to eliminate bacterial growth. The TAM did not inhibit mould growth.

DISCUSSION

This study was first conducted with pure isolates of each yeast genus predominantly isolated from tobacco, namely *Rhodotorula, Candida* and *Cryptococcus* species. Because the development of some *Cryptococcus* and *Candida* species could be harmful (2, 3, 4, 5, 6, 7), detection of *Cryptococcus* and *Candida* was investigated by using a tobacco agar medium (TAM). The yeast species were clearly differentiated following growth on TAM. The various degree of pigmentation of *Cryptococcus* strains has been proposed to divide the strains into four groups: true pigment, dark brown pigment, intermediate tonality and light pigment (1). We did not apply the above grouping because the pigmentation of *Cryptococcus* colonies was obviously different from the other genera tested i.e. *Candida and Pichia* (white to off-white) and *Rhodotorula* (pink or orange).

In order to validate the use of TAM to detect brown *Cryptococcus* colonies from other yeast species, a simulation of tobacco inoculated with mixed suspensions of *Candida, Cryptococcus, Pichia* and *Rhodotorula* was investigated. Detection of these yeasts on TAM supplemented or not with antibiotics and all yeast counts were comparable to the ones observed in culture media currently used for fungal growth, namely LOA and YMA. The addition of antibiotics to TAM was not essential because bacterial growth was inhibited most likely by selective pH values of 5.0 ± 0.02.

In conclusion, tobacco agar can be used to visually distinguish *Rhodotorula* and *Candida* genus from *Cryptococcus* species. *Candida parapsilosis* can be differentiated from other *Candida* species based on colony morphology. Yeast strains isolated on this medium can be re-grown and further purified on Potato Dextrose Agar (PDA), a recommended general purpose medium used for cultivating yeasts.

Table 2. Colour and appearance of yeast colonies and estimation of yeast spiking suspensions and in control tobacco on various media after ten days of incubation at room temperature (20–26 °C)

Media	Strain name [a]	Colour and appearance of the colony	Yeast – Log of CFU/mL			
			TAMs [b]	TAM	LOA	YMA
Control : Flue-cured cut tobacco (non inoculated)			not detected	not detected	not detected	not detected
			Yeast suspension (Log of CFU/mL) before spiking		Yeast recovery (Log of CFU/mL) after spiking	
TAMs	L094	White to off white wrinkled	7.1		7.2	
	L092	Light brown to brown	7.1		7.0	
	L113	Pink	7.5		7.2	
	L015	White	7.2		7.0	
TAM	L094	White to off white wrinkled	7.1		7.1	
	L092	Light brown to brown	7.1		7.0	
	L113	Pink	7.5		7.6	
	L015	White	7.2		7.2	
LOA	L094	Blue	7.0		7.1	
	L015		5.6			
	L092	Light grey	7.1		7.2	
	L113	Orange	7.5		7.4	
YMA	L094	White	7.0		7.7	
	L092		7.1			
	L015		7.2			
	L113	Orange	7.5		6.6	

[a] Strain name: L094 : *Candida parapsilosis*; L092 : *Cryptococcus albidus*; L113 : *Rhodotorula mucilaginosa*; L015 : *Pichia subpelliculosa*.
[b] TAMs: TAM supplemented with antibiotics.

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