ASCOCARP PRODUCTION BY NANNIZZIA OTAE ON KERATINOUS AND NON-KERATINOUS AGAR MEDIA AND MATING BEHAVIOR OF N. OTAE AND 123 JAPANESE ISOLATES OF MICROSPORUM CANIS

M. HIRONAGA, K. NOZAKI & S. WATANABE

Department of Dermatology, Shiga University of Medical Science, School of Medicine, Seta Tsukinowa-cho, Otsu, 520–21 Japan

Abstract

Ascocarp production by *Nannizzia otae*, VUT 77054'+' x VUT 77055'−', was compared on 8 different (1 keratinous and 7 non-keratinous) agar media.

Oatmeal salts agar and diluted Sabouraud dextrose salts agar with or without yeast extract were found to be unsuitable for ascocarp production in this species. In contrast, three different variants of oatmeal salts agar enriched with yeast extract proved to be satisfactory for the same purpose, while oatmeal salts agar with both yeast extract and horsehair powder was not necessarily superior to the former three media in this regard. Niger seed salts agar enriched with yeast extract was the most superior to any other seven media in all of the following respects; i.e., the number of gymnothecia produced per plate, the germination rate of ascospores, and the suppression of sporulation.

Asci from the cross VUT 77054'+' x VUT 77055'−' that yielded abundant fertile gymnothecia on niger seed salts agar with yeast extract were dissected and 51 ascospores were randomly isolated. Of the 51 ascospores, 47 (92 %) germinated to form mature colonies. Of the 47 monoascospore F_1 progeny back crossed to the parentals, 21 (45 %) mated or reacted with the '+' mating type, 18 (38 %) did with the '−' mating type, and the remaining 8(17 %) were sexually nonreactive.

One hundred and twenty-three Japanese isolates of *Microsporum canis*, obtained from human and animal ringworms for the past 12 years, were also crossed with the tester strains of *N. otae* on selected 3 of the 8 media to determine their mating type. Out of these 123, 113 (92 %) produced fertile gymnothecia in crosses with VUT 77054 +', 9(7 %) were non-reactive, and the only one isolated from human in Osaka city produced fertile gymnothecia in crosses with VUT 77055'−'. The data suggest that *M. canis* (*N. otae*) exists predominantly as '−' mating type in Japan. A possible explanation for this unequal distribution of mating type is presented.

Introduction

Recently Hasegawa and Usui (2) obtained the perfect state of *Microsporum canis* Bodin by crossing two isolates, originating from feline ringworms, on soil and hair and on oatmeal salts agar. Their mating study revealed this fungus to be heterothallic (3). Subsequent mating of additional Japanese isolates of *M. canis* revealed that only 10 of their 21 isolates paired with the tester strains of *Nannizzia otae* (VUT 74037'+' and VUT 74039'−') derived from the original cross VUT 73015 × VUT 74001 reproduced sexually (3).

More recently Weitzman & Padhye (11) presented their paper dealing with the mating behavior of *N. otae* and of 198 isolates of *M. canis* obtained in 12 countries. Their mating experiments were all carried out on either oatmeal salts agar or Pablum cereal agar. The striking aspects of their results were poor ascospore germination and a high percentage of non-reactors in F_1 generations from the strongly reactive crosses of *N. otae*, and the absence of sexual reactions in crosses of a number of isolates of *M. canis* with Japanese and also their additional tester strains of *N. otae*.

Weitzman & Padhye suggested several possible explanations for the poor ascospore germination and the non-reactors, such as an associated lethal factor, some incompatibility factors in the strains, or the rapid spontaneous reteriodation of sexual power. Their further genetic analysis of the F_1 generations have precluded the likelihood of the first possibility, however the latter two remain to be elucidated. In addition, our recent preliminary

Mycopathologia 72, 135-141 (1980). 0301-486x/80/0723-0135 $ 1.40.

mating study of *N. otae* revealed that oatmeal salts agar, apart from Pablum cereal agar, was too insufficient to support ascocarp production in this species, thus suggesting another possibility that the two media that they used for crosses in their study might partially account for such their results of mating.

The present study was undertaken; 1) to clarify the influences of media used for crosses on ascocarp production in *N. otae*, 2) to select out the most reliable medium for this purpose, and 3) to elucidate the mating behavior of *N. otae* and 123 Japanese isolates of *M. canis*.

Materials and methods

Tester strains and isolates

A pair of sexually reactive tester strains of *N. otae* (VUT 77054'+' and VUT 77055'−') derived from the original cross of two feline isolates (VUT 73015 × VUT 74001) were provided through the courtesy of Dr. A. Hasegawa. These tester strains formed a havily powdery thallus with abundant sporulation on 1/10 Sabouraud dextrose agar with salts.

One hundred and twenty-three isolates of *M. canis* used for crosses were from the culture collection in our laboratory. They had been isolated from human and animal ringworms for the 12 years from 1967 to 1978 in 9 cities of Japan.

All the strains were maintained on soil and hair and on 1/10 Sabouraud dextrose agar with salts at 5 °C and also at −36 °C, with infrequent transfers.

Media used for mating

The following 8 (1 keratinous and 7 non-keratinous) agar media were used: 1. diluted Sabouraud dextrose agar with salts (6, 7, 8), 2. diluted Sabouraud dextrose agar with both salts and yeast extract, 3. oatmeal salts agar (9), 4 & 5. variants of oatmeal salts agar enriched with yeast extract, 6. oatmeal salts agar with both yeast extract and horsehair powder, 7. another variant of oatmeal salts agar added with neopeptone, dextrose and yeast extract, 8. niger seed salts agar (1) with yeast extract. The composition of each of these eight media is given in the Table 1. Media were autclaved and distributed in 20 ml amount into sterilized plastic Petri-dishes of 9 cm diameter and 2 cm depth.

Mating experiments

A pair of tester strains of *N. otae*, VUT 77054'+' and VUT 77055'−', were crossed with each other on all of the 8 media above-mentioned. After that, ascocarp production on each of the 8 media was compared with regard to the

Table 1. One keratinous and 7 non-keratinous agar media used in the present study for crosses of *Nannizzia otae* and 123 isolates of *Microsporum canis*.

Medium No. / Contents (g/l liter)	1.	2.	3.	4.	5.	6.	7.	8.
Neopeptone	1	0.5	-	-	-	-	0.5	-
Oatmeal*	-	-	50	20	10	20	20	-
Niger seed**	-	-	-	-	-	-	-	50
Dextrose	2	1	-	-	-	-	0.5	1
Yeast extract	-	1	-	1	1	1	1	1
NaNO3	-	-	-	1	1	1	1	-
MgSO4·7H2O	1	1	1	1	1	1	1	0.5
KH2PO4	1	0.5	1	0.5	0.5	0.5	0.5	1
Agar agar	20	20	10	10	20	10	10	20
Horsehair powder	-	-	-	-	-	added	-	-

*Oatmeal(Baby Heinz oatmeal) and **niger seed(seed of *Guizotia abyssinica*) in blender were soaked with a portion of water at 60-70°C for 1.5 hours, then filtrated through a sheet of velvet, respectively. The filtrate was added to other ingredients in the remainder.

number of gymnothecia produced per plate, the germination rate of ascospores, and the degree of suppression of sporulation. In addition, 123 Japanese isolates of *M. canis* were crossed with the tester strains of *N. otae* on selected 3 media 4, 7 and 8, to determine their mating type.

Each of the strains maintained was transferred onto a 1/10 Sabouraud dextrose salts agar in slant and incubated for 2-3 weeks at 24 °C before they were used for crosses. Small pieces of young, vigorously growing cultures were cut out of the colonies and placed approximately 5 mm apart from each other on the center of agar plates in Petri-dishes. All mating experiments were carried out in triplicate.

All crosses were incubated in the dark at 23–24 °C for 4-6 weeks after which they were examined periodically for the presence of gymnothecia, pseudogymnothecia, or the absence of these structures. Whenever gymnothecia were observed, they were examined for asci and ascospores.

A number of ascospores from the cross VUT 77054'+' × VUT 77055'−' that yielded fertile gymnothecia on each of the 8 media were randomly isolated with a micromanipulator, after that they were examined for germination. Especially, the monoascospore F_1 progeny from the cross that produced abundant fertile gymnothecia on niger seed salts agar with yeast extract were back crossed to the parentals to determine their mating type.

Results

A comparison of ascocarp production and sporulation by *N. otae* (VUT 77054'+' x VUT 77055'−') on 8 different agar media are summarized in the Table 2.

Diluted Sabouraud dextrose agar with salts (medium 1) and diluted Sabouraud dextrose salts agar with yeast extract (medim 2) did not sufficiently support ascocarp production; that is, the cross formed a small number of gymnothecia or pseudogymnothecia (ranging from 40 to 70 per plate in total), while the size of which being generally small and the germination rate of ascospores from the cross being considerably low (20–21 %). Oatmeal salts agar (medium 3) was also insufficient in this regard; the cross produced 180-200 gymnothecia per plate (Figure 1), while their maturity was regularly poor and only 44 % of ascospores isolated germinated to make mature colonies.

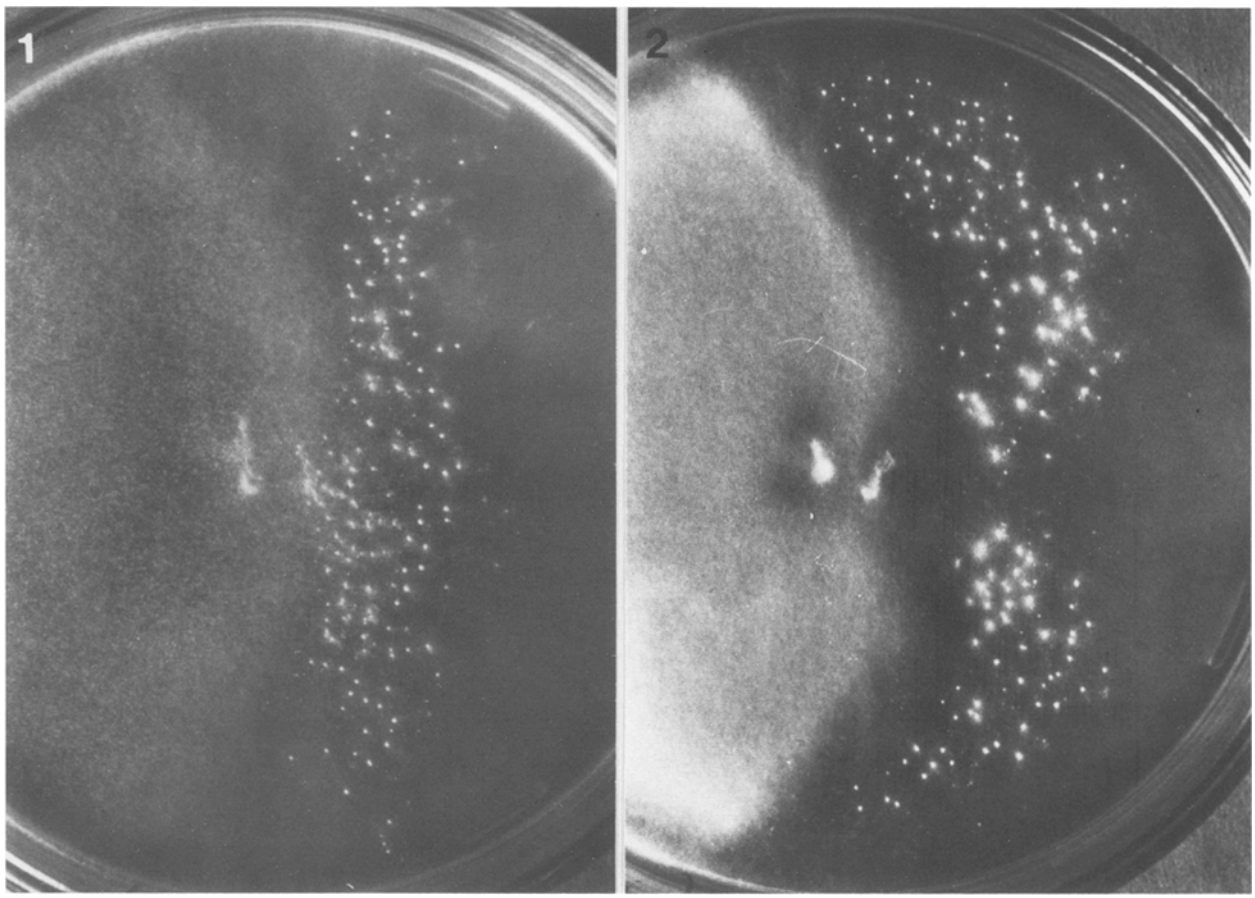
In contrast, three variants of oatmeal salts agar enriched with yeast extract (media 4, 5 and 7) satisfactorily supported ascocarp production; 370–740 gymnothecia were produced per plate in the crosses (Figure 2 and 3), and the fertility of which was fairly good with a high germination rate of ascospores (82–92 %). Against our anticipation, modified oatmeal salts agar enriched with both yeast extract and horsehair powder (medium 6) was not necessarily superior to the above three media with regard to the number of gymnothecia per plate.

Above all, niger seed salts agar with yeast extract (medium 8) was the most superior to any other seven media tested in the following respects; i.e., the excellent production of fertile gymnothecia that ranged from 670 to 710 per plate in number (Figure 4), the high germination rate of ascospores (92 %), and the appropriate suppression of asexual growth (sporulation). When 47 monoascospore

Table 2. Sexual reproduction in *Nannizzia otae* (VUT 77054'+' x VUT 77055'−') on 8 (1 keratinous and 7 non-keratinous) agar media.

Medium No.	1.	2.	3.	4.	5.	6.	7.	8.
No. gymnothecia per plate	~40	~70	180 ~200	450 ~470	370 ~390	330 ~350	720 ~740	680 ~710
Relative size of gymnothecia	small	small	small	large	large	large	small	large
Germination rate of random ascospores*	12/62 (20%)	12/58 (21%)	20/46 (44%)	39/42 (92%)	43/53 (82%)	36/48 (73%)	57/66 (86%)	47/51 (92%)
Asexual sporulation	+++	+++	++	+	+	+	++	+

+++ = luxriant, ++ = moderate, + = suppressed
*Germination rate = No. ascospores germinating/No. ascospores isolated



Figures 1-4. Pictures of ascocarp production by *Nannizzia otae*, VUT 77054'+' x VUT 77055'−', on 4 different agar media: (1) oatmeal salts agar (medium 3), (2) modified oatmeal salts agar with yeast extract (medium 4), (3) modified oatmeal salts agar added with dextrose, peptone and yeast extract (medium 7), and (4) niger seed salts agar with yeast extract (medium 8).

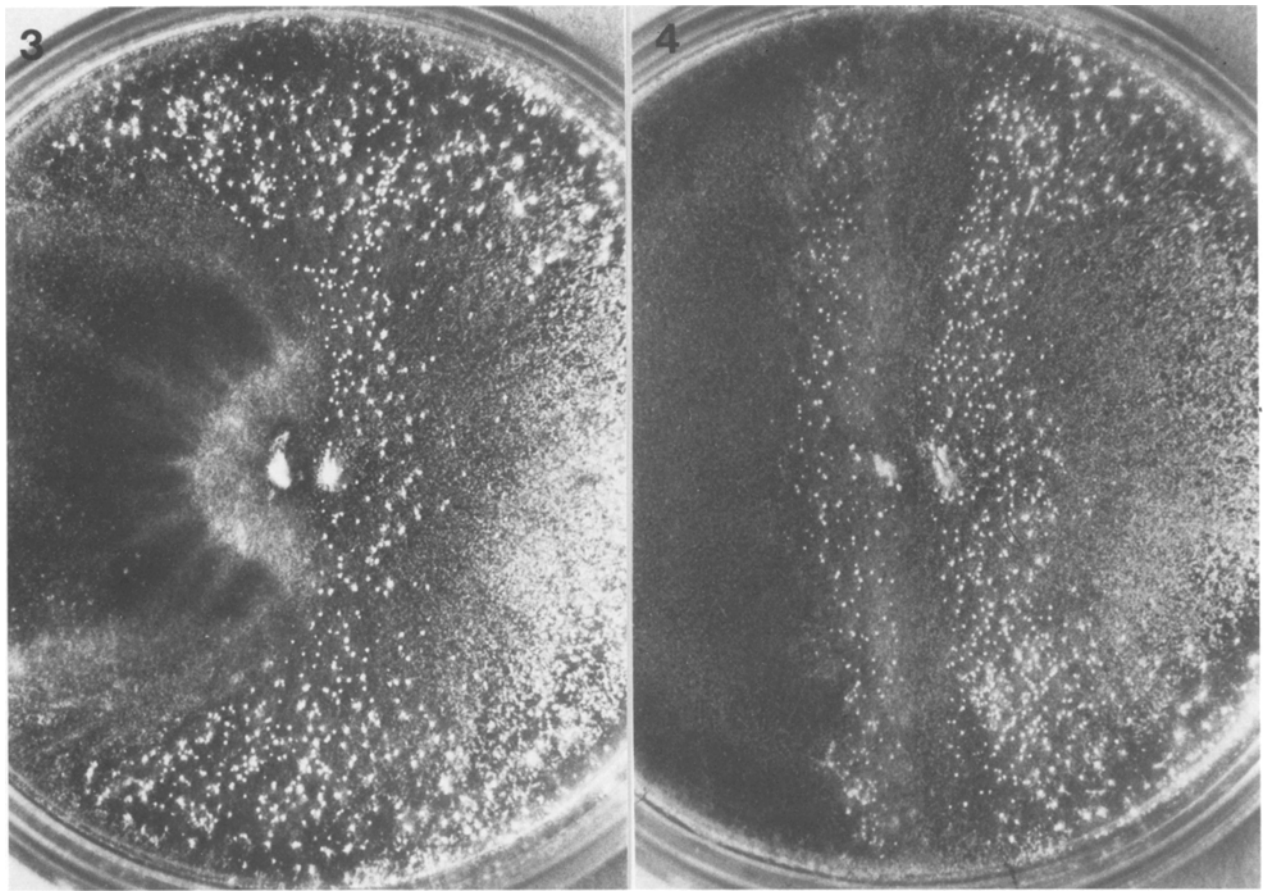
F_1 progeny from this cross were back crossed to the parentals, 21 (45 %) of them mated or reacted with the '+' mating type, 18 (38 %) did with the '−' mating type, while the remaining 8 (17 %) were non-reactive.

On the other hand, mating behavior of the 123 Japanese isolates of *M. canis* paired with the tester strains of *N. otae* (VUT 77054'+' and VUT 77055'−') is summarized in the Table 3. Out of the 123 isolates, 113 (92 %) produced fertile gymnothecia in crosses with the '+' mating type, 9 (7 %) were sexually non-reactive, and the only one strain isolated from human in Osaka city formed fertile gymnothecia in crosses with the '−' mating type. Most of the such non-reactive strains had been downy and had lost their characteristic sporulation and pigmentation.

Discussion

As the results of their mating study of *N. otae*, Weitzman & Padheye (11) pointed out that F_1 generations from the strongly reactive three crosses were all characterized by poor ascospore germination and most of the monoascospore progeny were non-reactive in crosses to determine their mating type. They suggested several possible factors to account for the poor ascospore germination and the non-reactors, such as an associated lethal factor, incompatibility factors in the strains, or the spontaneous deterioration of sexual power. Their further genetic analysis of the F_1 progeny precluded the likelihood of the first possibility, but the latter two remained to be elucidated.

Of course, many other factors besides these, such as the media used for crosses or maintenance of the strains, the



temperature, the age of culture, and the technique for crosses, also may affect ascocarp production in the species. Indeed during our experiments we observed that maintaining of cultures on media containing high content of sugars and peptones at room temperature caused a rapid loss of sexual power in the previously strongly reactive strains.

Medium used for crosses, however, is one of the most critical such factors because of variation in nutritional requirement for sexual reproduction depending on species. It is thus important to use the most reliable medium for crosses of *N. otae* when mating reactions were used as one of the criteria for identification or genetic analyses of this species.

Several non-keratinous agar media, such as niger seed agar (1), different variants of oatmeal salts agar (9, 10), and diluted Sabouraud dextrose agar with salts (6, 7, 8), have been reported to support ascocarp production in many dermatophytes or other Gymnoascaceae. Our present study, however, demonstrated that these above media were all insufficient to support ascocarp production in *N. otae*, thereby suggesting a possibility that the media that Weitzman & Padhye used for crosses in their study might partially account for the poor ascospore germination and the non-reactors.

A small amount of yeast extract promoted ascocarp production by *N. otae* when it was added to each of oatmeal salts agars and niger seed salts agar. Niger seed salts agar enriched with yeast extract was the most superior to any other seven media in all of the following points; i.e. the number of gymnothecia formed per plate, the germination rate of ascospores, and the degree of suppression of sporulation.

In fact, 92 % of ascospores from the cross VUT 77054'+' x VUT 77055'−' that produced abundant gymnothecia on this medium germinated to make mature colonies. Of the 47 monoascospore F_1 progeny, 21 (45 %) reacted as '+' mating type, and 18 (38 %) as '−' mating type. The

Table 3. Summary of mating reactions of 123 Japanese isolates of *M. canis* paired with the tester strains of *N. otae* (VUT 77054'+' and VUT 77055'−').

Source		Isolates producing fertile gymnothecia with "+" mating type	Isolates producing fertile gymnothecia with "-" mating type	Non-reactive isolates
Prefecture	City			
Shizuoka	Hamamatsu	11	0	1
Kyoto	Kyoto	33	0	3
Osaka	Moriguchi	10	0	0
	Osaka	7	1	0
	Kishiwada	12	0	0
	Sayama	3	0	0
Wakayama	Wakayama	16	0	2
Kagawa	Takamatsu	10	0	2
Oita	Beppu	11	0	1
Total		113(92%)	1 (1%)	9 (7%)

segregation pattern of mating types in this cross was a 21 : 18 ratio for '+' and '−' mating type, thereby precluding the likelihood of an associated lethal factors in the strains or the tetrapolar compatibility system. Further precise genetic study of the F_1 generations from many crosses are under investigation.

On the other hand, of 114 Japanese isolates of *M. canis* compatible with our tester strains of *N. otae*, 113 (92 %) belonged to the '−' mating type, and only one belonged to the '+' mating type. The results well correspond to those reported by Hasegawa & Usui (3), and by Weitzman & Padhye (12) with regard to the distribution of mating type. These data suggest that *N. otae (M. canis)* exists predominantly as one mating type. The other mating type is possibly restricted within certain narrow geographic areas, so far within Japan.

Similar unequal distribution of mating type, however, has been recognized also in other zoophilic species, such as *A. benhamiae*, *A. vanbreuseghemii* and *Trichophyton mentagrophytes* (4, 8, 9), thus this phenomenon can be regarded as a common characteristic feature of these animal-dependent fungi.

Weitzman & Padhye (11) suggested three possible explanations for the unequal distribution of mating type in *M. canis*, i.e., 1) selective factor, 2) pathogenicity and 3) geographic distribution. Of these three possibilities, the former two appear to be less promissing, but the last one is likely. In this connection, the varying epidemiologic factors, that continually acted on the fungus during its propagation from the original habitat to all the world as an animal-dependent parasite, presumably have caused and promoted such unequal distribution of mating type in *N. otae*, and also in other zoophilic species. For example, we can suggest such as the speed, quantity and geographic spread of moving animal populations carrying the fungus, and also the contact frequencies among the animal populations and the survival or fall of them.

Acknowledgement

We thank Dr. Hasegawa, A., Department of Medicine, School of Veterinary Medicine, Faculty of Agriculture, University of Tokyo, for the cultures of the tester strains of *N. otae*.

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