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Water Relations of Xerophilic Fungi Isolated from Prunes

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The predominant spoilage fungi of dried and high-moisture prunes were members of the *Aspergillus glaucus* group and *Xeromyces bisporus*. *Chrysosporium* spp. were also important. At the mean *p*H of prune flesh (3.8) and at 25 C, *X. bisporus* grew at water activities (a_w) down to 0.605, and *Chrysosporium fastidium* grew to 0.686. Germination was always followed by growth, but within the 120-day incubation period, the minimum a_w permitting asexual sporulation was usually higher than that permitting germination. Sexual sporulation often required an even higher a_w. The water requirements of aspergilli were appreciably greater at this *p*H than near neutrality, no species germinating below 0.738 a_w. This was probably a consequence of a high spore-death rate during incubation at low a_w and *p*H.

Dried prunes, which may have water contents in the range of 15 to 22%, are generally stable against microbial attack unless substantial rehydration is permitted. In contrast, prunes containing about 35% water ("high-moisture" prunes) and distributed in plastic pouches require chemical or heat treatment to protect the product against fungal spoilage (5). Such a product is relatively concentrated, with an intrinsic water activity (a_w) permitting growth of only xerophilic fungi.

There appear to be no published descriptions of the spoilage flora of high-moisture prunes. However, Tanaka and Miller (17) made 124 isolations of molds from 48 spoiled samples of dried California prunes. Of these isolates, 45% were of the *Aspergillus glaucus* group, 15% were *A. niger*, and 4% were other aspergilli. *Penicillium* spp. comprised 33% of the total; two strains of *Alternaria* and one each of *Monilia*, *Mucor*, and *Chaetomella* were the only other genera represented. Tanaka and Miller (18) also reported that the strains of *A. glaucus* had pronounced osmophilic properties, *A. niger* and the penicillia were less osmophilic, and the remaining isolates had no such properties.

The object of the present work was to identify the fungi responsible for the spoilage of high-moisture prunes, to compare the Australian prune-spoilage flora with that described for California, and to determine the water relations of germination and of sexual and asexual sporulation of these isolates at *p*H 3.8 and 25 C.

MATERIALS AND METHODS

Isolations were attempted from 56 spoiled samples of which 47 were high-moisture prunes in plastic pouches and 9 were dried prunes. The primary isolation medium, a prune decoction, was prepared by boiling 100 g of split dried prunes in 1,000 ml of water for 1 hr. Agar and the required amount of sucrose were added to the extract. For culturing high-moisture prunes, 40% (w/w) sucrose was added to give a final a_w of 0.94. For dried prunes, 0.86 a_w was achieved by adding 65% (w/w) sucrose. After growth at 25 C, the isolates were examined microscopically and identified, if necessary, after transfer to secondary media. The latter were all based on Czapek agar as modified by Smith (13) and are described in Table 1.

Whole prunes would appear to be ideal substrates on which to study the water relations of these fungi. However, it was apparent from the data of Miller and Tanaka (6) that equilibration to the desired levels of a_w was very slow. In addition, the opacity of prunes made microscopic examination difficult. To overcome these disadvantages, Czapek invert malic agar (CIMA) was devised, based on Czapek agar with *p*H adjusted with malic acid to 3.8. This was the mean *p*H of 216 prunes tested, the range being 3.3 to 4.3 (J. I. Pitt, M.Sc. Thesis, University of New South Wales, Kensington, 1965).

In early experiments, CIMA was adjusted to desired levels of a_w by varying the concentration of invert sugar in accordance with the empirical formula of Money and Born (7). In later work, thin layers of the basal medium containing invert sugar were equilibrated under vacuum over various aqueous solutions for 2 weeks prior to inoculation. This proved the more accurate method for obtaining the required a_w levels. Small portions (0.2 to 0.5 ml) of molten CIMA

TABLE 1. *Characteristics and applications of media employed*

Medium	Sucrose concn (per cent, w/w)[a]	*p*H	Water activity	Application
Czapek agar	3	6.8	0.999	Growth and identification of most molds
Czapek agar	20	6.8	0.98	Growth and identification of *A. glaucus* group
Czapek agar	40	6.8	0.95	Increased growth of some of *A. glaucus* group
Czapek agar with peptone	20	7.0	0.98	Growth of *A. restrictus*. Increased production of conidia by *A. glaucus*
Acid Czapek agar with peptone	40	5.0	0.95	Growth of *S. sebi* and *E. fertilis*
Czapek agar with peptone, *p*H 4	40	4.0	0.95	Growth of *Chrysosporium* spp.
Czapek agar with peptone, *p*H 4	55	4.0	0.91	Growth of *X. bisporus*

[a] Sucrose concentration in osmophilic media is frequently reported as per cent (w/v) or simply, per cent. Particularly in very concentrated media, it is more convenient as well as more precise to use per cent (w/w): 40% (w/w) = 67% (w/v); 55% (w/w) = 122% (w/v).

medium were poured onto sterile 1.5-inch (3.81 cm) watchglasses. These were supported in metal frames on glass pedestals set in 1-lb (454 g) screw-cap glass jars containing appropriate controlling solutions. Saturated aqueous salt solutions, as listed by Robinson and Stokes (10), were employed to give a_w levels of 0.902, 0.843, 0.800, 0.771, 0.753, 0.738, and 0.708. Data of Stokes and Robinson (16) were used to prepare sulfuric acid solutions of 0.720, 0.697, 0.686, 0.672, 0.656, 0.644, 0.633, 0.623, 0.615, and 0.605 a_w.

Heintzeler (4) reported that, at reduced water activities, spores from young cultures germinated more rapidly than those from old cultures. Care was therefore taken to use mature young spores, the state of maturity being judged by their resistance to staining.

Raper and Fennell (9) indicated that ascospores of most members of the *A. glaucus* group, when grown at 25 C on 20% sucrose Czapek agar, produced mature ascospores in 2 to 4 weeks. However, ascospores of some of these species, grown under these conditions and used in a preliminary experiment at an age of 4 weeks, germinated erratically, and the resulting mycelia grew very slowly in comparison with those from the corresponding conidia. More consistent results, reported here, were obtained by using spores from the same cultures when 12 weeks old.

Judged by their resistance to staining, *Xeromyces bisporus* ascospores matured in 6 to 10 weeks when cultured on 55% (w/w) sucrose Czapek agar with peptone, *p*H 4, at 25 C. Latent periods obtained in the first trial, when the inocula were 3.5 months old, were longer than those obtained when the culture was 5.5 months old. The latter results were recorded.

After inoculating each watchglass with an estimated 5,000 to 10,000 spores, the jars were closed and incubated at 25 C for 120 days. Microscopic examination, at 100 × and 400 × to detect germination and at 40 × for sporulation, was made regularly throughout this period. When a species produced more than one type of spore, the water relations of each were studied separately. In some cases, separation of different spore types on a single strain was achieved by growth on a medium favoring production of one type of spore. For some other species, two strains, each exhibiting a different predominant spore type, were used. The times recorded for germination and sporulation refer to the *first* clear signs of the change occurring. In most cases, these were unequivocal, germination or sporulation occurring simultaneously at a number of places in the culture.

RESULTS

Isolations. From the 56 samples of prunes, 70 isolations were made. The species isolated and the frequency of their occurrence are shown in Table 2.

The most unexpected feature of these results is that *X. bisporus* was the most frequently isolated species. This is regarded (12) as a very rare organism whose isolation has been reported only twice previously. Clearly, *X. bisporous* is not so rare if appropriate media are employed for isolation. This organism does not grow at a_w levels above 0.97 (12), and experiment showed that profuse growth of this species can be obtained on Czapek agar with 0.5% peptone added, acidified to *p*H 4, and containing 55% (w/w) sucrose.

About two thirds of the spoilage isolates were aspergilli, with species of the *A. glaucus* group predominating. Three of the four "small-spored" species of this group regarded by Raper and Fennell (9) as cosmopolitan were isolated fre-

quently (*A. chevalieri*, *A. repens*, *A. ruber*), but the fourth (*A. amstelodami*) was not found. The relatively frequent isolation of *A. mangini*, regarded as a rare species, was also notable. Altogether, six species from the *A. glaucus* group were found.

Aspergillus spp. outside the *A. glaucus* group were infrequently isolated, as were *Penicillium* spp. *A. niger* was not found.

Two species (five strains) assignable to the genus *Chrysosporium* Corda were isolated. These did not correspond to any of the species accepted by Carmichael (1) in the latest taxonomic study of the genus and have been described as new species by Pitt (8).

The only other molds isolated were *Eremascus fertilis* Stoppel, a rare organism (3), and *Sporendonema sebi* Fries, known as the "dun mold," which has frequently caused spoilage of salt fish (2).

TABLE 2. *Mold species isolated from prunes*

Species	No. of isolations		Total	Per cent
	High-moisture prunes	Dried prunes		
Xeromyces bisporus Fraser	14	2	16	19
Aspergillus chevalieri (Mangin) Thom and Church[a]	12	1	13	15
A. repens De Bary[a]	10	3	13	15
A. ruber (K., S., and B.) Thom and Church[a]	11	2	13	15
A. mangini Thom and Raper[a]	7	1	8	10
Chrysosporium fastidium Pitt		4	4	6
C. xerophilum Pitt	1		1	6
Penicillium spp.	4		4	5
A. versicolor (Vuill.) Tiraboschi	3		3	4
A. tonophilus Ohtsuki[a]	2		2	2
A. carnoyi Thom and Raper[a]	1		1	9
A. echinulatus (Delacr.) Thom and Church[a]	1		1	9
A. ochraceous Wilhelm	1		1	9
A. restrictus Smith		1	1	9
A. wentii Wehmer	1		1	9
Eremascus fertilis Stoppel	1		1	9
Sporendonema sebi Fries	1		1	9
Total	70	14	84	100
Total *A. glaucus* group	44	7	51	61

[a] Member of the *A. glaucus* group.

Water relations of germination. One or two strains of each species isolated from prunes (Table 2) were examined. To make the study more comprehensive, three species of xerophilic molds from the Commonwealth Mycological Institute, Kew, England, and seven species isolated in the laboratory from various relatively low-moisture foodstuffs were included. A second strain of *A. echinulatus*, isolated from stale bread, was also studied. Designated type "L," it possesses ascospores averaging 9 μm in long axis; the length of ascospores of the prune isolate, type S, averaged 7 μm.

The minimum a_w levels permitting germination of various spore types of a range of species within 120 days at *p*H 3.8 and 25 C are shown in Table 3.

Under these conditions, germination of the two spore types of *X. bisporus* showed an outstanding tolerance of low water activity, the aleuriospores germinating at 0.605 a_w. The two species of *Chrysosporium* isolated here are also exceptionally xerophilic, being more drought tolerant under these conditions than even the *A. glaucus* group.

The members of the *A. glaucus* group tested were all capable of germination at a lower a_w or earlier at the same a_w, than any of the remaining organisms except *S. sebi*. *A. carnoyi* and *A. mangini* were particularly xerophilic members of the group.

E. fertilis and *A. ochraceous* were the only other molds to germinate below 0.80 a_w. Only one species examined, *E. albus*, failed to germinate at 0.902 a_w. It has since been found that CIMA medium does not support growth of this species without additional nutrients.

Microscopic observations showed that germination was always followed by growth, and there was no evidence of death or inhibition of a mold while the mycelium was still of microscopic

TABLE 3. *Minimum a_w and time for germination and sporulation of fungi*

Species and spore type	Minimum for germination		Minimum for asexual sporulation		Minimum for sexual sporulation	
	a_w	Days	a_w	Days	a_w	Days
Xeromyces bisporus (aleurio-spores)	0.605	120	0.663	80	0.672	116
X. bisporus[a] A.	0.644	80	0.656	90	0.697	83
Chrysosporium fastidium	0.686	48	0.697	64		
C. xerophilum	0.708	37	0.708	80		
Aspergillus carnoyi[b] A.	0.738	14	0.753	59	0.771	41
A. carnoyi[b] C.	0.738	19	0.753	68	0.771	41
A. mangini[b] A.	0.738	19	0.771	55	0.771	68
A. echinulatus[b] S.A.	0.738	50	0.771	42	0.800	86
A. amstelodami[b, c] A.	0.738	63	0.753	63	0.843	27
A. chevalieri[b] A.	0.738	75	0.753	87	0.843	28
A. repens[b] A.	0.738	78	0.753	67	0.843	20
A. tonophilus[b] A.	0.738	89	0.771	42	0.843	29
A. mangini[b] C.	0.753	14	0.753	99	0.771	51
A. amstelodami[b, c] C.	0.753	23	0.753	75	0.843	25
A. repens[b] C.	0.753	24	0.753	62	0.843	25
A. tonophilus[b] C.	0.753	84	0.771	46	0.843	29
A. chevalieri[b] C.	0.753	91	0.771	24	0.843	40
A. ruber[b] A.	0.753	98	0.771	60	0.800	41
A. ruber[b] C.	0.771	7	0.771	53	0.771	92
A. echinulatus[b] L.C.	0.771	8	0.771	37	0.800	63
A. restrictus	0.771	8	0.800	43		
Sporendonema sebi	0.771	8	0.771	14		
A. echinulatus[b] S.C.	0.771	11	0.771	47	0.800	51
A. echinulatus[b] L.A.	0.771	17	0.771	46	0.800	75
Eremascus fertilis	0.771	38			0.771	80
A. ochraceous	0.771	57	0.800	47		
A. candidus	0.800	23	0.843	19		
Penicillium fellutanum[c]	0.800	28	0.843	13		
A. sydowi[c]	0.800	30	0.800	64		
A. versicolor	0.800	63	0.800	80		
P. citrinum[c]	0.843	5	0.843	14		
A. wentii	0.843	8	0.843	21		
Paecilomyces varioti[c]	0.843	9	0.843	55		
P. brevi-compactum[c]	0.843	9	0.843	24		
A. niger[c]	0.843	10	0.843	31		
A. flavus[c]	0.902	10	0.902	15		

[a] A = ascospores, C = conidia, L = large, S = small.
[b] Member of the *A. glaucus* group.
[c] Not isolated from prunes.

size. Thus, the ability of a spore to germinate appears to be, at least under the conditions tested, an indication of the ability of the organism to form macroscopic colonies and hence, in time, to cause visible food spoilage at that a_w level.

For some spore types studied, plots of water activity against log latent period (15) showed abrupt termination at lower a_w levels. For example, *A. restrictus* germinated at 0.771 a_w in 8 days but not within 120 days at 0.753 a_w; similar results were obtained with a number of other species. At the end of the experiment, ungerminated inocula were subcultured on media of 0.94 or 0.98 a_w. Almost all were nonviable. Since Snow (14) found that spores of many such molds can remain viable for periods of years, it appears probable that the *p*H of CIMA medium was rapidly lethal to some spore types.

Subsequently, large numbers of spores from several species were inoculated into CIMA medium at an a_w level sufficiently low to prevent germination, and were subcultured weekly onto Czapek agar with 20% (w/w) sucrose (0.98 a_w). Some spore types showed increasing latent periods after only 2 weeks of incubation. After 7 weeks at

low a_w, conidia of *A. wentii* failed to germinate at 0.98 a_w within 2 weeks. *A. flavus*, *A. ochraceous*, and *A. restrictus* were unable to germinate after 10 weeks of incubation. Thus, the ability of spores to germinate at low a_w levels will sometimes depend upon their ability to remain viable during the latent period.

At 0.902 and 0.843 a_w, conidia of the *A. glaucus* group almost always germinated more rapidly than ascospores and produced more vigorous mycelia. However, the ascospores were consistently capable of germination at a lower a_w level than were the conidia (Table 3). During this experiment, microscopic examination showed that, at lower a_w levels, inocula of ascospores germinated by producing germ tubes through the walls of intact cleistothecia. Presumably, such enclosed ascospores were able to remain viable for longer periods than individual ascospores or conidia, and hence could germinate at lower levels of a_w.

Water relations of sporulation. The minimal water activities at which the species examined could sporulate asexually and sexually are also shown in Table 3.

Only *X. bisporus* and the two *Chrysosporium* spp. sporulated asexually below 0.753 a_w, whereas only *X. bisporus* formed cleistothecia below 0.771 a_w. The ability of *X. bisporus* to form aleuriospores at 0.656 a_w and cleistothecia at 0.672 a_w, water activities which prevent the growth of almost all microorganisms, is remarkable.

It was observed that, for a given species, asexual sporulation occurred when the mycelium reached a critical size, irrespective of the a_w level. The time required for asexual sporulation to commence was thus an indication of the relative rate of mycelial growth at various a_w levels. However, the variations between species were very large. Whereas *S. sebi* sporulated as soon as aerial hyphae were formed, *A. carnoyi* and *A. mangini* delayed asexual sporulation until mycelia were 1 to 1.5 cm in diameter, and sexual sporulation was commencing.

For some species, Table 3 shows appreciable differences between minimal a_w levels for germination and asexual sporulation. There is no evidence of an intrinsic inability to sporulate at the lower a_w, and the difference may be due to premature termination of the experiment.

The ability of the species with perfect stages to sporulate sexually at low a_w levels, however, appeared to depend on water requirements rather than time, except for *X. bisporus*. Several species which sporulated readily at 0.843 a_w showed no sign of producing any sexual spore-bearing apparatus at 0.800 a_w, despite vigorous mycelial growth and profuse asexual sporulation within the period of the experiment.

The times required for sexual sporulation from conidia and ascospores showed remarkable grouping within species. In some cases conidia and ascospores came from strains of widely differing appearance; this provides an unexpected type of evidence for the validity of species concepts in the *A. glaucus* group as arranged by Thom and Raper (19). In contrast, asexual sporulation times frequently differed widely for inocula of conidia and ascospores from the same species.

Several species of aspergilli formed atypical spore-bearing apparatus at low water activities, producing branched conidiophores or abortive vesicles before typical forms appeared. In some members of the *A. glaucus* group there appeared structures similar to "primitive" spore types such as aleuriospores and chlamydospores, which are not normally found in the aspergilli. However, no observations were made to show whether these were, in fact, spores. Under very adverse environmental conditions, the most drought tolerant species in the *A. glaucus* group produced coiled ascogonia without subsequent formation of cleistothecia.

DISCUSSION

As might be expected in general from their known xerophilic character (12) and in particular from data for California prunes (17), the *A. glaucus* group predominated amongst the spoilage molds of Australian high-moisture prunes. The unexpected occurrence of *X. bisporus* as the most common single species was less surprising in view of its water relations. Scott (12) reported its growth down to 0.62 a_w, and his data clearly extrapolate to a lower value. The present finding of germination and growth at 0.605 a_w appears to be the lowest authentic value reported for any microorganism. Frank and Hess (2) claimed germination of *S. sebi* at 0.60 a_w, but it is clear that the a_w level of their medium when inoculated was substantially higher than this figure.

None of the aspergilli tested in this trial germinated at a_w levels as low as those reported previously. The probable reason was the unfavorable *p*H, causing both extension of latent periods (11) and loss of spore viability. Germination studies of *A. carnoyi* and *A. mangini* have not been reported previously. Both *S. sebi* and *Paecilomyces varioti* showed a lower drought tolerance than had been reported previously. Again, the probable cause was the unfavorable *p*H.

The lowest minimum water activities for asexual sporulation reported previously appear to be those of Snow (14), who observed asexual sporulation of *A. chevalieri* and *A. repens* at 0.78 a_w

during a 56-day experiment. During the present investigation, all members of the *A. glaucus* group sporulated at 0.771 a_w within 60 days, and the mycelia produced by nine spore types sporulated at 0.753 a_w after periods varying from 53 to 99 days. The aleuriosporic species were capable of sporulation at very low a_w levels, *X. bisporus* producing clearly defined aleuriospores at 0.66 a_w, and the least xerophilic of the three species of this type, *C. xerophilum*, at 0.708 a_w.

Sexual sporulation of *A. chevalieri* was reported by Snow (14) at 0.84 a_w in 19 days. In the present experiment, sexual sporulation of *A. chevalieri* at 0.843 a_w required 28 days. However, *A. carnoyi* and *A. mangini* produced cleistothecia at 0.771 a_w, and *A. ruber* and *A. echinulatus* produced cleistothecia at 0.800 a_w. The most outstanding organism was *X. bisporus*, which produced cleistothecia at 0.771 a_w within 17 days after germination. At 0.672 a_w, fully developed perithecia were apparent 106 days after germination. As maturation of ascospores of this species normally requires several weeks after cleistothecial formation, even under favorable growth conditions, it is doubtful whether mature ascospores would ever be produced at these very low a_w levels.

The study of the water relations of these mold species over the range 0.9 to 0.6 a_w on a medium of *p*H 3.8 has shown that this *p*H is somewhat inhibitory to most aspergilli, but that *X. bisporus* and, to a lesser extent, the *Chrysosporium* spp. isolated in this work are particularly well adapted to growth under acid conditions. Further data of the effect of *p*H upon the water relations of xerophiles are clearly desirable.

Although dried prunes of 22% moisture content (0.68 a_w) are unlikely to be spoiled by any of the aspergilli, even slightly higher moisture contents may render them susceptible to *A. mangini*, which was isolated fairly frequently in this work. However, the results indicate that contamination of dried prunes at 22% moisture with spores of *X. bisporus* will almost inevitably cause spoilage. That only occasional outbreaks of this type have been reported in Australian prunes presumably indicates a relatively low incidence of contamination by such spores.

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