Influence of Oxygen Concentrations on Chinese Chestnuts and Their Spoilage Organisms

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ABSTRACT

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Members of the genera *Phoma, Fusarium, Paecilomyces, Candida, Diplodia, Penicillium, Pestalotia, Phomopsis, Alternaria, Aspergillus, Gloeosporium, Trichoderma, Rhizopus, Curvularia, Gliocladium,* and *Trichothecium* and bacteria (mainly *Bacillus* spp.) were isolated from chestnuts at harvest or during storage. *Aspergillus* spp. were isolated infrequently, but some isolates produced appreciable amounts of aflatoxin. Nuts stored in a 2% O_2 atmosphere deteriorated less rapidly and yielded fewer fungi when plated than those stored at 21% O_2. Nuts that were not fully mature at harvest deteriorated more rapidly than mature nuts. Four *Fusarium* spp., *Phoma* sp., and *Pestalotia* sp. grew 22–68% slower in vitro at 2% than at 21% O_2. Sporulation was also markedly reduced at low O_2. *Diplodia* sp. was not affected greatly by O_2 level.

Interest in the culture of Chinese chestnut (*Castanea mollissima* Blume) in Georgia and certain other states has occurred primarily because of the loss of the American chestnut (*C. dentata* (Marsh.) Borkle) to Endothia blight. Low quality of nuts resulting in part from fungal deterioration and insect attack has limited commercial production of Chinese chestnuts, particularly in the southeastern United States (3,4,6,8). Initial fungal invasion may occur while nuts are still on the tree (3,12). Deterioration may occur while nuts are on the ground before gathering (3) and is especially severe during conventional storage (6).

Methods that have been used commercially or evaluated experimentally to minimize spoilage losses include daily harvesting, rapid marketing, storage at low temperatures above freezing, kernel removal and freezing, irradiation, application of chemicals to the trees or harvested nuts, and selection of trees with low rot incidence (2–4,6,12). Mechanical harvest is considered essential for the expanded production of chestnuts in the United States, and storage requirements may be affected as nuts of different maturity stages result from once-over harvest (8).

We found little information on the use of controlled atmosphere storage to extend the storage life and minimize spoilage losses of Chinese chestnuts, although the method is commonly used for certain other perishable commodities (5,9). In the present study we determined the microorganisms associated with Chinese chestnuts at harvest and studied fungal development and nut deterioration at different oxygen concentrations in storage.

MATERIALS AND METHODS

Chestnuts at three stages of maturity were mechanically harvested from an orchard near Byron, GA. One group of nuts, the most mature used in the test, had dark pigmentation and fell to the ground free of the burrs when the tree was shaken. A second group, considered to be intermediate in maturity, had developed most of their coat pigmentation but remained in partially open burrs at harvest. A third group, considered to be immature, had developed little pigmentation and remained in closed or partially open burrs. These groups were selected to represent stages of nut maturity common to once-over mechanical harvest.

Two experiments were conducted. In the first, nuts of the three maturity stages were stored in the laboratory at 21 C in 10-L desiccator jars adjusted to 2, 5, 10.5, and 21% internal O_2. The environment in each chamber was adjusted with a vacuum pump fitted with a mercury manometer to provide the appropriate oxygen partial pressure, followed by the restoration to 760 mm Hg with nitrogen gas (research purity 99.9%). The internal environment of the flasks was recharged daily to prevent significant change in the gas concentrations. The oxygen and carbon dioxide levels were monitored periodically by removing 1-ml gas samples from the jars and chromatographing them with a Fisher-Hamilton gas partitioner (thermal conductivity detector; dual columns: 1.8 m × 6.4 mm column of 30% di-2-ethyloxysebacate on column-pak 60-80 mesh and 2.0 m × 4.8 mm column of molecular sieve 13X, 42-60 mesh; helium carrier gas at 80

Table 1. Marketable Chinese chestnuts after storage at four oxygen levels[a]

Maturity stage[b]	Storage (% O_2)	Percent marketable		
		7 days	18 days	35 days
Mature	2.0	100 x[c]	100 w	87 w
	5.0	96 x	100 w	93 w
	10.5	99 x	94 wx	52 x
	21.0	99 x	86 x	50 x
Intermediate	2.0	100 x	98 w	88 w
	5.0	81 x	59 y	54 x
	10.5	96 x	55 y	7 y
	21.0	83 x	58 y	11 y
Immature	2.0	97 x	51 y	7 y
	5.0	...[d]	...[d]	...[d]
	10.5	12 y	0 z	0 z
	21.0	0 z	0 z	0 z

[a] All nuts were marketable when placed in storage.
[b] Mature nuts had dark pigmentation and fell free of the burrs when the tree was shaken. Intermediate nuts had most of their coat pigmentation and remained in partially open burrs at harvest. Immature nuts were from closed or slightly open burrs and had little pigmentation.
[c] Each value is a mean of three replications of 10 nuts. Values in column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[d] Treatment not included because of shortage of nuts.

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ml/min). Changes in the internal environment were maintained within 5–6% of the desired gas levels. Three replications of each treatment were used. After 7, 18, and 35 day storage, samples of 10 nuts were removed from each replication and classified as cull or marketable, based on external appearance.

As the study progressed, we observed that fungal deterioration was more severe at 21% than at lower O_2 levels, particularly in comparison with the 2% level. Consequently, isolations were made to determine the organisms associated with the deteriorating nuts. Seventy-five nuts were selected at random from the three replications at 21%, aseptically separated into pericarp and kernel portions, plated on water agar, and incubated on a laboratory bench. The genera of fungal colonies growing from the pericarp and kernel sections were identified directly on the plates if possible; colonies were transferred to plates of potato-dextrose agar if additional study was necessary for identification.

The second study was similar, except that only two oxygen concentrations (2 and 21%) were used and the nuts were examined after 33 and 70 day storage. Also, additional isolations were made to determine microorganisms associated with chestnuts at harvest and during storage at the two oxygen concentrations. Ten nuts from each maturity stage at harvest and 10 from each of three replications after 33 and 70 days of storage were plated and organisms identified as described.

Aspergillus flavus Link ex Fries was isolated infrequently (<2%) from both the pericarps and kernels but was of particular interest because of its toxigenic nature. Thirteen isolates selected at random were assayed for production of aflatoxins B_1, B_2, G_1, and G_2, as described by Lillard et al (7).

Because the oxygen concentration influenced the deterioration of nuts in storage, tests were also done to determine whether oxygen concentration also influenced the growth of selected fungi in pure culture. The test organisms were those commonly isolated from chestnuts at high O_2 levels and included *Phoma, Pestalotia, Diplodia,* and *Fusarium* spp. (*F. solani, F. lateritium, F. moniliforme, F. semitectum*).

Each organism was grown in 1-L vacuum flasks containing 150 ml of potato-dextrose agar. Each flask was fitted with a rubber stopper and a clamped rubber hose on the side arm to seal the system. Desired O_2 levels were established in the flasks as described above. To prevent contamination, the nitrogen gas was filtered through a sterile packed cotton filter before it was transferred to the vacuum flask. To eliminate the possible effect of brief exposure to low atmospheric pressure on the fungi, the 21% O_2 treatment was lowered to 90 mm Hg and returned to 760 mm Hg with filtered air rather than N_2. The flasks (three replications per treatment) were arranged in a randomized complete block design on a laboratory bench at 24–27 C under continuous fluorescent lighting. During the experiment, the gas concentrations were monitored and the flasks recharged as needed to maintain the 2 and 21% O_2 levels and to prevent CO_2 buildup. The temperature in the flasks ranged from 24 to 28 C.

Growth rates of the fungi were determined by measuring radial expansion daily for 10 days or until the agar surface in the flasks was completely covered with mycelium. Any differences in profuseness of growth were also recorded. At the end of the study, spore counts were made on representative fungi (*F. moniliforme* and *Pestalotia* sp.) grown at the two oxygen levels. Spore production was estimated by pouring 100 ml of sterile distilled water over the agar surface of each flask, scraping the surface gently with a rubber policeman to free the spores, and counting the spores in suspension with a Levy Improved Neubauer counting cell.

RESULTS

Effect of nut maturity and O_2 level. The stage of maturity of chestnuts when harvested and the oxygen level during storage significantly influenced the amount of deterioration that occurred, particularly as the storage period increased (Tables 1 and 2). In the first study the least mature nuts deteriorated rapidly, and none was marketable after 18 days of storage at 10.5 and 21% O_2 (Table 1). Deterioration of these nuts was severe even in the 2% O_2 environment, and only 7% were marketable after 35 days. Mature nuts deteriorated less rapidly than those of intermediate maturity. The stage of maturity had less effect on deterioration in the second study (Table 2). In both studies the nuts deteriorated more rapidly and severely at 21% than at 2% O_2, regardless of the maturity stage. Nuts stored at 21% had internal deterioration and also often had fungal growth visible on their external surfaces.

Organisms at different O_2 levels. Isolations made from deteriorating nuts at 21 C in the first study yielded members of 16 genera including (in order of frequency of occurrence) *Fusarium, Phoma, Diplodia, Paecilomyces, Phomopsis, Gloeosporium, Penicillium, Aspergillus, Rhizopus, Pestalotia, Alternaria, Curvularia, Mucor,*

Table 2. Marketable Chinese chestnuts after storage at two oxygen levels[a]

Maturity stage[b]	Storage (% O_2)	Percent marketable	
		33 days	70 days
Mature	2	98 w[c]	92 x
	21	80 x	20 z
Intermediate	2	95 w	64 y
	21	72 y	27 z
Immature	2	93 w	...[d]
	21	55 z	...[d]

[a] All nuts were marketable when placed in storage.
[b] Mature nuts had dark pigmentation and fell free of the burr when the tree was shaken. Intermediate nuts had developed most of their pigmentation but remained in partially open burrs at harvest. Immature nuts had little pigmentation and remained in closed or slightly open burrs.
[c] Each value is a mean of three replications of 16 nuts. Values in column followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).
[d] Not included because of shortage of nuts.

Table 3. Microorganisms in kernels of Chinese chestnuts plated at harvest and after storage at two oxygen levels[a]

Organism	At harvest	Frequency of isolation after storage at: 21% O_2		2% O_2	
		33 days	70 days	33 days	70 days
Alternaria	6.3	3.6	0.0	4.4	0.0
Aspergillus	2.1	8.5	3.6	1.5	2.1
Candida	2.1	2.6	30.1	2.2	47.9
Diplodia	2.1	5.6	9.7	2.9	8.3
Fusarium	20.8	8.9	2.1	4.4	3.1
Gloeosporium	2.1	1.3	1.5	0.7	0.0
Paecilomyces	0.0	11.2	11.8	13.9	3.1
Penicillium	4.2	28.5	6.7	6.6	6.3
Pestalotia	2.1	2.0	8.2	0.7	0.0
Phoma	43.7	24.3	20.0	51.1	9.4
Phomopsis	2.1	1.3	2.1	5.1	0.0
Trichoderma	6.3	0.0	0.0	0.0	2.1
Bacteria (mostly *Bacillus* spp.)	2.1	1.3	1.5	5.8	14.6
Miscellaneous and unknown	4.2	1.0	2.6	0.7	3.1

[a] Data from the three maturity categories were combined to determine organism frequency.

Gliocladium, *Trichothecium*, and *Trichoderma*, as well as several unknown genera. The first three genera accounted for about 70% of the isolations. The predominant *Fusarium* spp. were *F. solani*, *F. moniliforme*, *F. lateritium*, and *F. semitectum*. *Trichoderma* sp. was isolated mainly from nuts collected from the ground. Bacterial colonies (mainly *Bacillus* spp.) grew occasionally (<5%) from the plated kernel sections. In a few cases specific organisms such as *Diplodia* and *Gloeosporium* were associated with a characterisitic internal rot. In most cases, however, more general deterioration occurred, involving a complex of the weakly parasitic fungi.

Many of the same fungi that occurred in nuts during the first storage experiment were isolated from kernels of freshly harvested chestnuts in the second experiment (Table 3). *Phoma* sp. and *Fusarium* spp. were isolated most commonly and together accounted for about 65% of the isolates at harvest. *Fusarium* spp. and often *Phoma* sp. accounted for a lower percentage of the isolates in storage than at harvest. The genera *Penicillium*, *Paecilomyces*, and *Candida*, all relatively unimportant in freshly harvested nuts, made up a significant percentage of isolates from nuts in storage. *Candida* was a significant component of the microflora after 70 days at both 21 and 2% O_2. *Phoma* sp. accounted for about half of all isolates from nuts stored for 33 days at 2% but accounted for only about 10% of isolates after 70 days. Bacteria were more frequent in nuts stored at 2% than at 21% O_2.

Pericarp tissue sections were heavily infected (80–95% for the three maturity stages) with microorganisms at harvest and did not change significantly at the two oxygen levels during storage. Kernels from mature nuts collected from the ground yielded more microorganisms at harvest than those from intermediate and immature nuts that did not contact the soil (Table 4). However, the organisms developed rapidly in nuts stored at 21% O_2, and sections of kernels from the immature and intermediately mature nuts were 83 and 76% infected, respectively, after 33-day storage. A significantly higher percentage of kernel sections of all maturity categories were infected at 21% than at 2% O_2 after 33 and 70 days in storage.

Production of aflatoxin by *Aspergillus*. Seven of the 13 *A. flavus* isolates selected at random produced no aflatoxins, two produced a low level (mean of 41 μg/g of mycelium, dry wt) of B_1, and four produced fairly high levels of B_1 (mean of 4,818 μg/g), G_1 (9,723 μg/g), and G_2 (2,430 μg/g).

Effect of O_2 level on growth of fungi. Six of the seven fungi tested grew significantly slower at 2% than at 21% O_2 (Figs. 1 and 2). Growth reduction ranged from 22% (*Phoma* sp.) to 68% (*Fusarium lateritium*). Radial expansion of *Diplodia* sp. was not reduced at the 2% O_2 concentration, but aerial mycelium grew less profusely. Sporulation of most of the test organisms was also markedly reduced at the low oxygen concentration. Spore counts showed that *Pestalotia* sp. (Fig. 2B) produced 540 times more spores per flask at 21% than at 2% O_2, and *F. moniliforme* produced 11 times more. Although the reduced spore counts reflected in part differences in the size of the mycelial mats, microscopic observation indicated that existing mycelium at 2% O_2 had only a few poorly developed spores.

DISCUSSION

These results indicate that Chinese chestnuts grown in the southeastern United States are heavily infected with an array of weakly parasitic organisms that may cause serious nut deterioration, particularly under unfavorable storage conditions. Wright (12) reported that 62% of kernels from chestnuts were visibly infected with microorganisms. A fungus that he identified as *Phoma castanea* accounted for 34% of the isolations. A *Phoma* sp., presumably the same as that isolated by Wright, as well as many other organisms, were also commonly present on chestnuts at harvest and in our storage trials. Wells and Payne (10) found that *Aspergillus* spp. made up 16.8% and *Penicillium* spp. 40.7% of fungi isolated from weevil-damaged chestnuts plated on malt-salt agar. We found a much lower frequency of these organisms in undamaged nuts plated on water agar, but some of our isolates of *Aspergillus* spp. produced

Table 4. Percent of kernel sections of Chinese chestnuts yielding microorganisms at harvest and after storage at the two oxygen levels[a]

Stage	At harvest	% O_2 in storage	Percent kernels with organisms	
			33 days	70 days
Mature	65	21	86 y[b]	87 y
		2	53 z	48 z
Intermediate	35	21	76 y	85 y
		2	40 z	38 z
Immature	20	21	83 y	90 y
		2	19 z	48 z

[a] Kernel sections from 10 nuts from each maturity stage at harvest and from 10 nuts from each of three replications in storage were plated.

[b] Oxygen level comparisons at each maturity stage followed by a different letter are significantly different ($P = 0.05$).

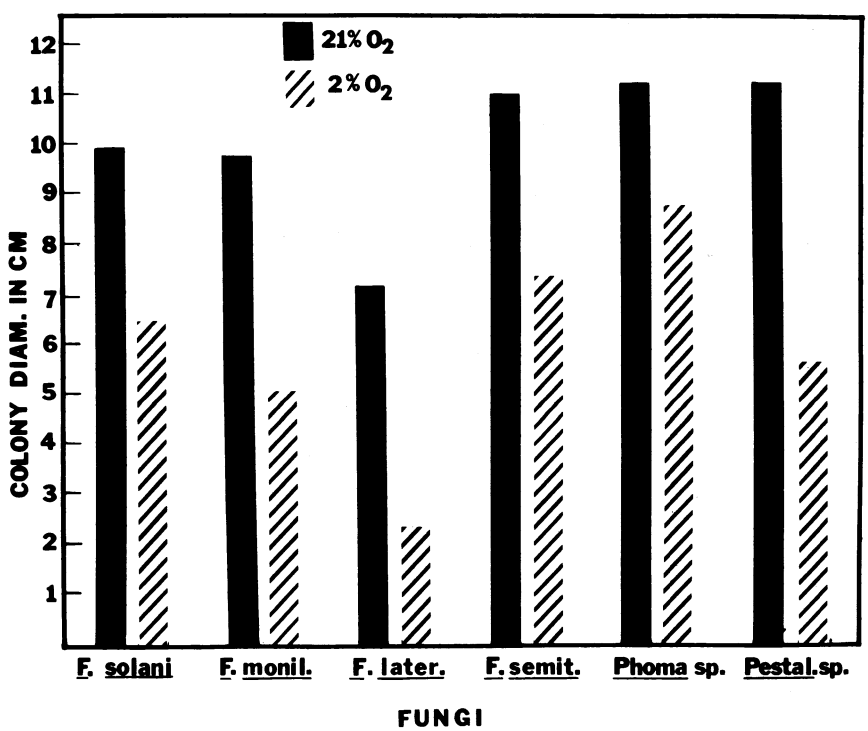

Fig. 1. Radial growth of selected fungi after 8 days (*Fusarium moniliforme*) or 10 days (all others) on potato-dextrose agar in sealed flasks at 2 and 21% O_2 at 24–28 C. All oxygen concentration comparisons for each organism are significantly different ($P = 0.05$).

appreciable amounts of toxins, as did theirs. *Penicillium* isolates were not tested for toxin production.

Storage of chestnuts in the laboratory in a 2% O_2 atmosphere reduced fungal proliferation, as indicated by the absence of external fungal growth and lower recovery rate of fungi from plated kernel sections. It was not uncommon for two or more organisms to grow from a single plated kernel section, particularly after storage at 21% O_2. Competition between resident organisms before and during plating may explain some of the apparent irregularities in the isolation data presented in Table 3. A larger sample size for isolation would also reduce the variation.

A 2% O_2 atmosphere also reduced mycelial growth of six of seven fungi tested in vitro. Follstad (1) found that the mycelial growth rate of all isolates of *Alternaria tenuis* (= *A. alternata*), *Botrytis cinerea, Cladosporium herbarum,* and *Rhizopus stolonifer* decreased significantly at 1% compared with 21% O_2 and with each decrease of O_2 concentration below 1%. *R. stolonifer* grew at a lower O_2 concentration than the other three fungi. Sporulation of *B. cinerea* and *R. stolonifer* was retarded greatly below 1% O_2. Follstad (1) concluded that O_2 concentration below 1% would be required to control common postharvest decay organisms since higher O_2 concentrations had little effect on their growth. However, Wells and Uota (11) worked with liquid cultures of the four fungi studied by Follstad plus *Fusarium roseum* and concluded that mycelial growth was more sensitive to low O_2 concentrations than previously suggested. In liquid culture, mycelial growth decreased linearly with decreasing O_2 concentrations below 4%. Mycelial growth of *A. tenuis, B. cinerea, C. herbarum,* and *F. roseum* was inhibited more than 50% in 4% O_2 compared with normal O_2. They also found that *R. stolonifer* grew at lower oxygen levels than other fungi. The fungi in our tests also responded differently at the different O_2 concentrations. Our findings agree with those of Wells and Uota (11) that mycelial growth is markedly inhibited at low O_2 concentrations above 1%, although cultural techniques and measurements differed. A closed system for modified atmospheric studies has limitations since growth differences could conceivably be caused by accumulation of gases such as ethylene, ammonia, and CO_2. We monitored our system frequently to rule out that possibility.

The stage of nut maturity at harvest was a critical factor in the storage life of the crop. At present, typical existing orchards are composed mostly of heterogenous seedling trees with a wide range in nut maturity dates among trees and on individual trees. Our results suggest that nuts of varying maturity harvested by a once-over mechanized process will not store as well as uniformly mature nuts. Although immature chestnuts would not be harvested intentionally, they would be a component of crops harvested mechanically (8).

The results of our studies suggest that storage in a controlled atmosphere may retard fungal deterioration of Chinese chestnuts. The economics of such storage must, however, be weighed against the quality retention.

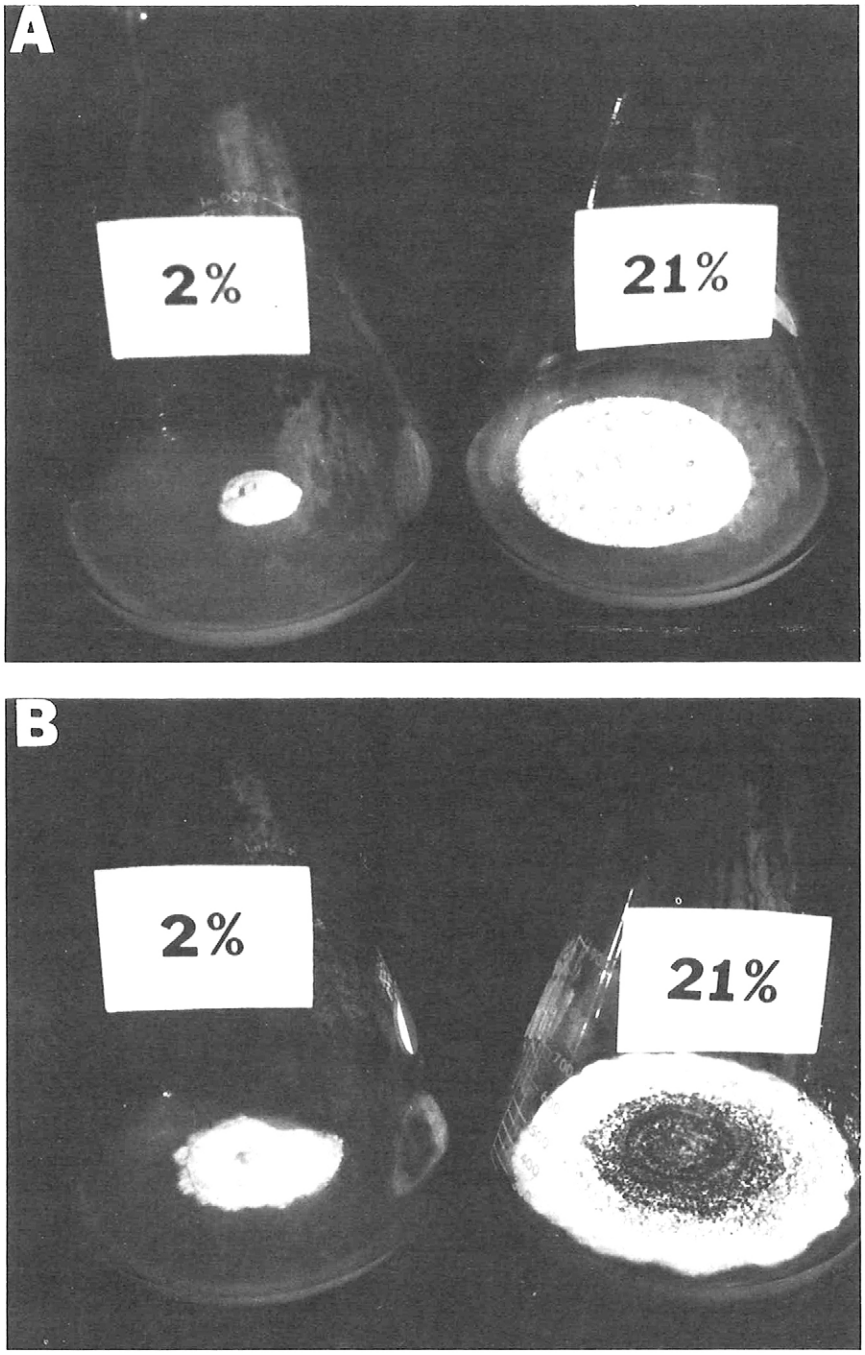

Fig. 2. Cultures of *Fusarium lateritium* **(A)** and *Pestalotia* sp. **(B)** after 8 days of growth on potato-dextrose agar in sealed flasks at 2 and 21% oxygen at 24–28 C.

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