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GROWTH OF FUNGI IN HIGH CARBON DIOXIDE AND LOW-OXYGEN ATMOSPHERES

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ABSTRACT

Food and grain spoilage fungi are traditionally regarded as aerobic organisms, but some species are efficient oxygen (O_2) scavengers capable of near normal growth in very low concentrations of O_2. The extent to which fungi are able to develop in low-O_2 atmospheres often depends on the concentration of carbon dioxide (CO_2) that is present as atmospheres high in CO_2 are often more effective in controlling fungal growth. Although atmospheres of 20% CO_2 inhibit the growth of many fungi, >80% CO_2 may be required to prevent fungal deterioration in high-moisture commodities. Growth and mycotoxin production by seven species of spoilage fungi was studied in atmospheres where residual O_2 was controlled at less than 0.5%, and the CO_2 concentrations were 20, 40 or 60%, with the balance being the inert filler gas, nitrogen. The potential for mycotoxin production was also assessed. Residual O_2 was a much more critical factor than CO_2 concentrations in reducing growth of *Penicillium* and *Aspergillus* species. *Mucor plumbeus, Fusarium oxysporum* and two *Byssochlamys* species were able to grow in all atmospheres tested although growth was reduced as CO_2 concentration increased. All species tested were able to grow in an atmosphere of 80% CO_2 with 20% O_2, but growth was slower than in air, particularly for the *Penicillium* species and *Aspergillus flavus*. Gas composition had a more pronounced effect on mycotoxin production than on growth.

INTRODUCTION

The important parameters controlling fungal growth in modified or controlled atmospheres are the minimum amount of oxygen (O_2) needed for growth to occur, the inhibitory effects of carbon dioxide (CO_2) and any interactions or synergism between these two gases in mixture. Nitrogen (N_2) has no inherent inhibitory effects; atmospheres high in N_2 may be inhibitory simply because of the lack of available O_2.

Fungi are normally considered to be obligate aerobes, requiring some molecular O_2 for germination, sporulation and growth. However, many species are able to grow in the presence of very small amounts of O_2 (Gunner and Alexander, 1964; Curtis, 1969; King *et al.*, 1969; Wells and Uota, 1970; Gibb and Walsh, 1980; Hesseltine *et al.*, 1985; Hocking, 1988; Taniwaki, 1995), and many fungi have been demonstrated to be efficient O_2 scavengers; thus the total amount of O_2 available, rather than O_2 tension, may determine the extent of growth (Pitt and Hocking, 1985).

Many field and storage fungi important in the spoilage of grains and processed foods are able to grow quite well in atmospheres containing 1% O_2 or less. Many *Fusarium* species, which can be classed as field fungi, are well adapted to growth in low-O_2 environments: *Fusarium moniliforme, F. oxysporum, F. culmorum* and *F. solani* all grow strongly in atmospheres containing 1.0–0.1% O_2 or even less (Gunner and Alexander, 1964; Tabak and Cooke, 1968; Walsh, 1972; Gibb and Walsh, 1980; Magan and Lacey, 1984) if other growth conditions (e.g. temperature and water activity) are favourable. Many *Mucor* and some *Rhizopus* species grow well at low-O_2 tensions (Wells and Uota, 1970; Gibb and Walsh, 1980; Yanai *et al.*, 1980) or even anaerobically (Hesseltine *et al.*, 1985) and can proliferate in high-moisture commodities even when stored under low-O_2 atmospheres (Bottomley *et al.*, 1950; Wilson *et al.*, 1975). Other field fungi such as *Alternaria* species and *Cladosporium herbarum* are more sensitive to reduced O_2 tensions (Magan and Lacey, 1984), and gradually die off during storage.

Storage and food spoilage fungi such as *Penicillium* and *Aspergillus* species generally are more sensitive to low levels of O_2 than the more tolerant field fungi. With the exception of *P. roqueforti*, the growth rates of many *Penicillium* species are reduced by more than 50% in atmospheres of 1% O_2 or less (Yanai *et al.*, 1980; Magan and Lacey, 1984). Of the Aspergilli, *A. candidus* is the most tolerant of reduced O_2 conditions (Magan and Lacey, 1984) and thus can proliferate in controlled-atmosphere stored grains (Di Maggio *et al.*, 1976). Some *Eurotium* species are also reasonably tolerant of low O_2 (Peterson *et al.*, 1956; Yanai *et al.*, 1980).

While CO_2 is known to be inhibitory to growth of many microorganisms, including fungi (Jones and Greenfield, 1982; Daniels *et al.*, 1985), the inhibitory concentration varies markedly among fungal species. Levels of CO_2 from 4 to 20% can be stimulatory to growth of many fungi in atmospheres containing low levels of O_2 (Wells and Uota, 1970; Gibb and Walsh, 1980), but atmospheres containing >50% CO_2 will substantially inhibit growth of most spoilage fungi (Petersen *et al.*, 1956; Wells and Uota, 1970). Some fungi, e.g. *Rhizopus, Mucor* and *Fusarium* species, are very tolerant of high levels of CO_2 and can grow in atmospheres containing 95–100% CO_2 (Stotzky and Goos, 1965).

The interactions between low-O_2 and high-CO_2 atmospheres and their effects on fungal growth are not well understood. The work described here investigated the effects on the growth of several food spoilage fungi of low-O_2 (≤0.5%) atmospheres in combination with elevated (20, 40 and 60%) CO_2 compared with both air and an atmosphere containing 80% CO_2 and 20% O_2.

METHODS

Fungi

Seven species of fungi were chosen for this study primarily because of their recognised ability to grow at low O_2 tension. Most were isolated from spoiled packaged processed foods containing low levels of O_2.

The species studied were *Mucor plumbeus* FRR 2414, isolated from spoiled, fermenting apple juice, Sydney, Australia; *Fusarium oxysporum* FRR 3414, from spoiled, fermenting orange juice, Sydney, Australia; *Byssochlamys fulva* FRR 3792, from fermenting strawberry puree, Sydney, Australia; *B. nivea* FRR 4421 from strawberries, Brazil, capable of producing patulin; *Penicillium roqueforti* FRR 2162, from spoiled Cheddar cheese, Lincoln, Nebraska, USA, capable of producing roquefortine C and reported to be tolerant of low O_2 and high CO_2; *P. commune* FRR 3932, from spoilage of Cheddar cheese packed in low-O_2 atmospheres and capable of producing cyclopiazonic acid; and *Aspergillus flavus* FRR 2757, from peanuts, Kingaroy, Queensland, Australia, a producer of aflatoxin B_1. *A. flavus* is not recognised as being particularly tolerant to modified atmospheres but was included because it is an important mycotoxin producer in many stored products.

Gas systems

The fungi were grown in anaerobe jars (HP11, Oxoid, Basingstoke, UK), evacuated and flushed several times with the appropriate gas mixture. O_2 scavengers ("Ageless"; Mitsubishi Gas Chemical Company, Japan, supplied by W.R. Grace, Melbourne, Australia) were added to jars to maintain residual O_2 at less than 0.5%. Gas concentrations were measured using a gas chromatograph (Model 8AIT, Shimadzu Corporation, Japan) as described by Taniwaki (1995). Atmospheres were checked daily, and the jars were flushed again when CO_2 varied by more than 2% or O_2 exceeded 0.5%.

Media

Cultures were grown on Czapek Yeast extract Agar (CYA; Pitt and Hocking, 1985), a mineral salts medium containing sucrose (3%) and yeast extract (0.5%), and Potato Dextrose Agar (PDA), as being more representative of a natural substrate.

Incubation

The anaerobe jars were incubated at 25°C for up to 30 d. At each sampling time, one jar containing plates of each fungus was opened.

Estimation of fungal growth

The extent of growth was measured using several methods. The simplest of these was measuring colony diameters as an indication of the extent of growth. Fungal biomass was estimated by hyphal length, following the method of Schnürer (1993), or, where growth was not filamentous, by using mycelia dry weight determined by a method based on Paster *et al.* (1983) and Zill *et al.* (1988). Ergosterol content of colonies was also

measured, using a method based on Zill *et al.* (1988). These methods are described in detail in Taniwaki (1995).

Mycotoxin analyses

Mycotoxins were analysed by HPLC. Aflatoxins, after derivatisation with trifluoracetic acid, were analysed according to the method of Beebe (1978). Cyclopiazonic acid was analysed using the method of Urano *et al.* (1992), roquefortine C using the method of Ware *et al.* (1980) and patulin using the method of Burda *et al.* (1992). These assay methods are described in detail in Taniwaki (1995).

RESULTS

Growth at low O_2 in various concentrations of CO_2

P. commune failed to grow in any of the low-O_2 atmospheres tested. *P. roqueforti* grew quite well in 20% CO_2, developing colonies of 25–30 mm on both media, but failed to grow at the higher concentrations of CO_2.

A. flavus showed very weak growth after 30 d in an atmosphere of 20% CO_2 with <0.5% O_2, with colony diameters of 11.0 and 9.5 mm on CYA and PDA. It did not grow at the higher CO_2 concentrations and consequently was used as a biological indicator of O_2 content in these experiments; if the "control" plate of *A. flavus* showed growth when a jar was opened, the plates of other fungi from that jar were not analysed as it was assumed that the O_2 had risen above 0.5%.

The other four fungi, *M. plumbeus, F. oxysporum, B. fulva* and *B. nivea*, were able to grow in all three concentrations (20, 40 and 60%) of CO_2 with <0.5% O_2.

M. plumbeus grew more strongly on CYA than on PDA, and growth responses for this species are shown in Fig. 1. Very thin colonies were produced, and at the higher CO_2

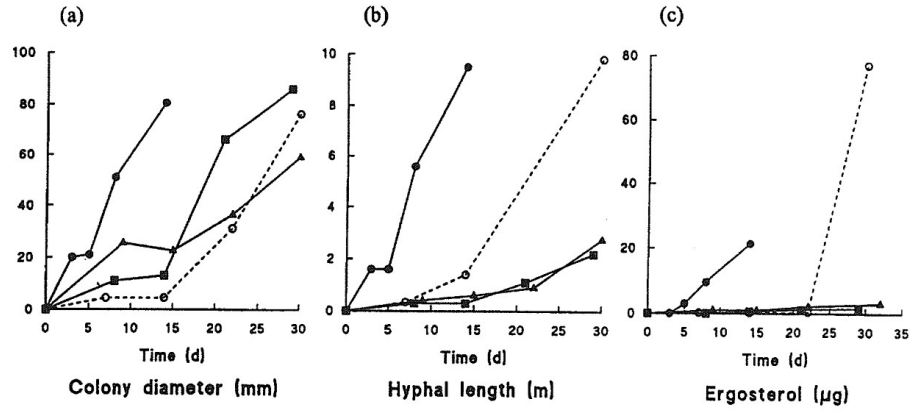

Fig. 1. Growth of *Mucor plumbeus* on CYA in four concentrations of CO_2 as measured by (a) colony diameter, (b) hyphal length and (c) ergosterol content (on the ordinates). full circles = 20% CO_2/<0.5% O_2; squares = 40% CO_2/<0.5 O_2; triangles = 60% CO_2/<0.5% O_2; empty circles = 80% CO_2/20% O_2.

concentrations individual hyphae could be seen at the colony margins. Ergosterol production in 40 and 60% CO_2 was very low compared with colony diameter, as was hyphal length, reflecting the sparseness of the colonies in these atmospheres.

F. oxysporum exhibited similar growth responses on CYA and PDA. Data for growth on CYA are shown in Fig. 2. A distinct difference was observed in response between 20% CO_2 and the two higher concentrations at which much greater (but similar) inhibition occurred. This difference was more obvious in the hyphal length and ergosterol data than in the colony diameter data.

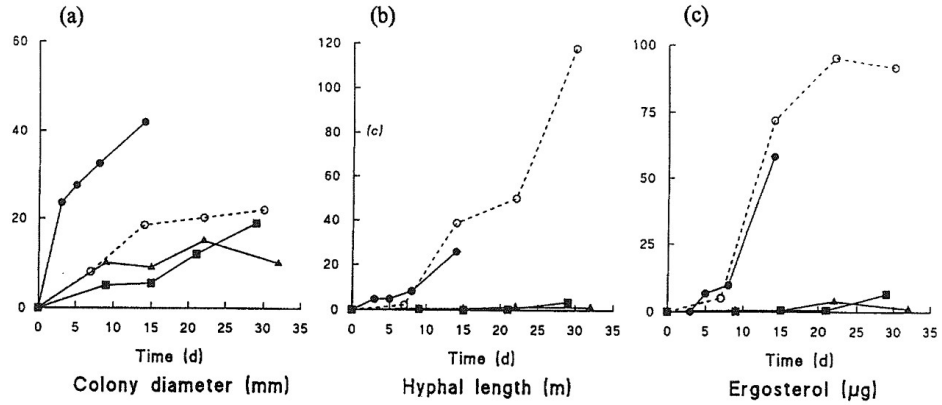

Fig 2. Growth of *Fusarium oxysporum* on CYA in four concentrations of CO_2 as measured by (a) colony diameter, (b) hyphal length and (c) ergosterol content (on the ordinates). full circles = 20% CO_2/<0.5% O_2; squares = 40% CO_2/<0.5% O_2; triangles = 60% CO_2/<0.5% O_2; empty circles = 80% CO_2/20% O_2.

B. fulva and *B. nivea* grew better on PDA than CYA. Both were capable of growth in the three concentrations of CO_2, but inhibition increased with increasing CO_2 concentration. Growth responses for *B. fulva* on PDA are shown in Fig. 3. *B. nivea* reacted similarly but produced lower concentrations of ergosterol than did *B. fulva* at 40 and 60% CO_2.

Growth in 80% CO_2, 20% O_2

P. commune was able to grow in this atmosphere, producing compact wrinkled colonies (approx 15 mm after 30 d) with some sporulation (data not shown). *P. roqueforti* grew well with abundant sporulation in this atmosphere (but better on PDA than on CYA), producing 20–30 mm sporulating colonies in 2–3 weeks. However, ergosterol production was very low on both media. *A. flavus* grew very slowly on both media, with white, non-sporulation colonies only reaching 10 mm after 30 d. As with the *Penicillium* species, ergosterol production was very low.

M. plumbeus produced colonies that were smaller, but more dense, than those produced in 40% and 60% CO_2 with <0.5% O_2 (Fig. 1). This was reflected in the hyphal length and ergosterol figures.

F. oxysporum showed little inhibition in the 80% CO_2/20% O_2 atmosphere, and although its growth rate as measured by colony diameter was less, the hyphal length and ergosterol production were both much greater than in any of the low-O_2 atmospheres (Fig. 2).

B. fulva produced smaller colonies in the high-CO_2 atmosphere, but, as with *F. oxysporum,* hyphal length and ergosterol concentration both exceeded those produced in any of the CO_2 atmospheres with <0.5% O_2 (Fig. 3).

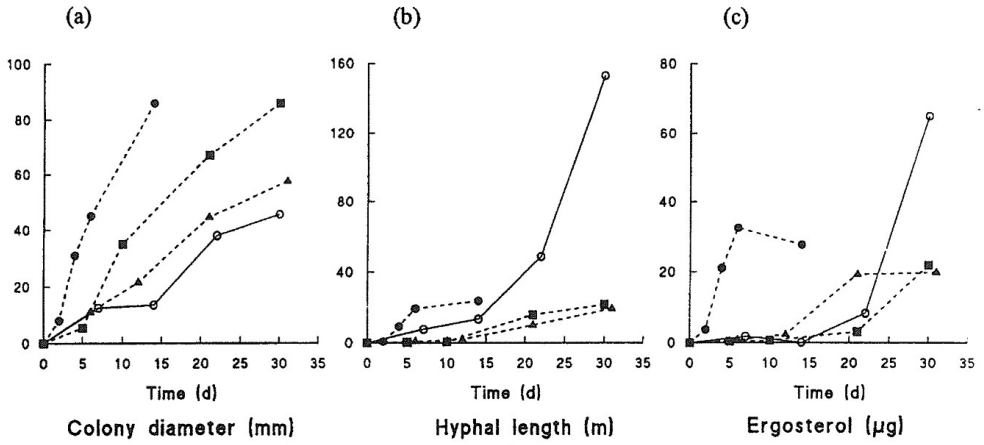

Fig 3. Growth of *Byssochlamys fulva* on PDA in four concentrations of CO_2 as measured by (a) colony diameter, (b) hyphal length and (c) ergosterol content (on the ordinates). full circles = 20% CO_2/<0.5% O_2; squares = 40% CO_2/<0.5% O_2; triangles = 60% CO_2/<0.5% O_2; empty circles = 80% CO_2/20% O_2.

Mycotoxin production

Mycotoxin production was generally inhibited by the atmospheres tested. Aflatoxins were not produced by *A. flavus* in either of the atmospheres in which growth was observed, i.e. 20% CO_2/<0.5% O_2 or 80% CO_2/20% O_2. This is in agreement with other reports in the literature which observed that increased CO_2 levels inhibited aflatoxin production to a much greater degree than it did the growth of *A. flavus* in natural substrates (Landers *et al.*, 1967; Sanders *et al.*, 1968; Wilson and Jay, 1975).

Patulin was produced by *B. nivea* in 20, 40 and 60% CO_2 on PDA, but at very low levels. Paster and Lisker (1985) observed similar inhibition of patulin production by *P. griseofulvum* (synonym *P. patulum*) in low-O_2 atmospheres (1% and 5% O_2) or in 20% CO_2 with 20% O_2.

Roquefortine C was produced by *P. roqueforti* in 20% CO_2, peaking in 10–14 d (0.2–0.3 µg per agar plate) but not at higher CO_2 concentrations in the absence of O_2. Roquefortine C production was also observed in 80% CO_2/20% O_2 (0.9 µg/plate) after 30 d. *P. commune* produced low concentrations of cyclopiazonic acid (0.5 µg/plate) in 80% CO_2/20% O_2 after 30 d.

CONCLUSIONS

The work described here shows that there are important interactive effects between low-O_2 and high-CO_2 atmospheres with respect to their inhibitory effects on fungal growth and metabolism. Some fungi, for example *Fusarium* species and mucoraceous fungi (represented in this study by *M. plumbeus*) are well adapted to growth in atmospheres low in O_2 and rich in CO_2, whereas the species more likely to be found in stored grains (*Aspergillus* and *Penicillium*) are substantially inhibited under these conditions. Mycotoxin production is almost totally suppressed under all the conditions studied.

There is no single satisfactory method for estimating fungal growth under modified atmospheres. Extension of colony radius or diameter has been used to measure fungal growth in many physiological studies (Pitt and Hocking, 1977; Hocking and Pitt, 1979; Wheeler and Hocking, 1988, and others) but this does not take colony density into account and thus is not a good measure of fungal biomass. Hyphal length and colony dry weight are better measures of fungal biomass, but these measurements, together with colony diameter, are unsuitable for estimating fungal growth in foods and stored commodities. Ergosterol content has been used to estimate the extent of fungal invasion in grains and grain products (Seitz *et al.*, 1977; Schnürer, 1991), but since the formation of sterols usually proceeds via aerobic biochemical pathways, the amount of ergosterol in the membranes of fungi growing under O_2 stress may not reflect the true extent of fungal growth.

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