Navarro, S. and Donahaye, E. [Eds.](1993) Proc. Int. Conf. Controlled Atmosphere and Fumigation in Grain Storages, Winnipeg, Canada, June 1992, Caspit Press Ltd., Jerusalem, pp. 163-171.

USE OF SULPHUR DIOXIDE TO CONTROL FUNGI IN STORED GRAIN

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ABSTRACT

The effect of different concentrations of sulphur dioxide (SO_2) in solution on the growth of field fungi, *Penicillium* and *Aspergillus* spp., was determined in laboratory experiments on 1% malt extract agar at pH 3.5. Field fungi such as *Cladosporium herbarum* and *Epicoccum nigrum* were tolerant of up to 200 ppm SO_2, while *Aureobasidium pullulans* and *Botrytis cinerea* were inhibited by this concentration. *Penicillium* spp. were able to tolerate up to 250 ppm SO_2, at 25°C, with growth being stimulated by intermediate concentrations of 100 ppm SO_2. However, this stimulation did not occur at 0.95 water activity. For *Penicillium* spp., at both 15 and 25°C, there was an increase in the lag time (in days) for initiation of growth. In contrast, with the exception of *Aspergillus niger*, other *Aspergillus* spp. were more sensitive than *Penicillium* spp. and inhibited by 50 ppm SO_2. Treatment of wheat grain (cv Avalon) with 500-2,000 ppm SO_2 in solution at two moisture contents initially resulted in a decrease in the total fungal populations on the grain. However, over a 28-day storage period, there was little difference between the rates of moulding of treated and untreated wheat grain. *Penicillium* spp. were responsible predominantly for the moulding of treated grain. This suggests that the threshold treatment levels necessary need to be chosen with care so that such tolerant fungi can be inhibited effectively.

INTRODUCTION

Sulphur dioxide (SO_2) is one of the oldest food additives known to man and was employed by the ancient Romans, Greeks, and Egyptians to preserve wine. Historically, it was used for centuries as a disinfectant. With the development of inorganic chemistry, SO_2 and its salts commonly became used as preservatives, particularly of food and beverages. Sulphur dioxide is a colourless gas, with a characteristic pungent odour and smell, that is readily soluble in water. It is very reactive, but also very versatile, hence its extensive use for the prevention of growth of food-borne spoilage microorganisms, prevention of enzymic and non enzymic-browning, use as a

bleaching agent of flour, silk, and straw, for stabilisation of vitamin C, and inhibition of fungal growth in preserved fruit, fruit juices, and cereal grains (Green, 1978).

Sulphur dioxide and other fumigants, such as ethylene oxide and methyl bromide, were reported by Majumder *et al.* (1973) to inhibit both fungal growth and mycotoxin production on grain. Raghunathan *et al.* (1969) found that treatment of 13% moisture content (m.c.) sorghum with sodium metabisulphite (64 mgl^{-3}; 48hr at 25°C) inhibited 95% of the internal seed fungi, but also significantly reduced seed viability. More recently, treatment of moist maize (24% m.c.) with 0.3% SO_2 resulted in a significant decrease in microbial colonisation and practically no deleterious effect on grain quality (Eckhoff *et al.*, 1979). In laboratory studies, Vidal and Jayaraman (1979) suggested that combinations of ammonia (NH_3) and SO_2 (3:1) had a synergistic effect on control of contaminant microorganisms. Treatment of moist maize (26.5% m.c.) with 0.066% SO_2 alone or combined with 0.018% NH_3 (Eckhoff *et al.*, 1983) showed that after 60 mould-free days, *Penicillium* spp. were found to have colonised the top third of the bin.

There is, however, surprisingly little detailed information on the actual tolerance and sensitivity of grain fungi, particularly *Penicillium* and *Aspergillus* spp., to different concentrations of SO_2. This paper presents information on the effect of SO_2 in solution on growth of grain fungi, and the effect of treatment on fungal colonisation of moist wheat grain of different water activities.

MATERIALS AND METHODS

Fungal isolates: The grain fungi studied included field fungi such as *Aureobasidium pullulans* (de Bary) Arnoud, *Botrytis cinerea* Pers., *Cladosporium herbarum* (Pers) Link, *Epicoccum nigrum* Link; and storage fungi such as *Aspergillus flavus* Link, *A. niger* van Tiegham, *A. terreus* Thom, *A. ochraceus* Wilhelm, *A. versicolor* (Vuill.) Tiraboschi, *Penicillium aurantiogriseum* Dierckx, *P. chrysogenum* Thom, *P. expansum* Link ex Gray, *P. griseofulvum* Dierckx, and *P. hordei* Stolk. All fungi were maintained on 2% malt extract agar (MEA).

Sulphur dioxide media: Filter-sterilised sulphur dioxide (in solution) was incorporated into molten 1% MEA (pH 3.5; modified with 1M HCL) at a concentration ranging from 10-250 ppm ($\mu g\ l^{-1}$), and poured into 90 mm plastic Petri plates. The water activity of the medium was also modified to 0.95 a_w by the addition of appropriate glycerol/water mixtures instead of water using the molal concentrations recommended by Dallyn and Fox (1981). The accuracy of the final concentrations was measured using a sodium thiosulphate/iodine back titration.

Inoculation and measurement: Spores were obtained from 10-4 day-old cultures of each species and suspended in small vials containing 0.5 ml of 0.1% sterile water agar (plus 0.01% Tween 20). A small loop (0.5 mm diameter) was used to centrally inoculate Petri plates containing media with different SO_2 concentrations. Experiments were carried out with 3 - 5 replicates per treatment. Plates inoculated with field fungi and *Penicillium* spp. were incubated at 15 and 25°C, and the *Aspergillus* spp. at 25°C only. The plates were examined regularly and the lag time prior to initiation of growth was monitored with a dissecting microscope. The growth of each colony was measured in two directions at right angles to each other and the data used to determine the radial growth rate in mm day^{-1}.

Effect of SO_2 on fungal growth in grain: Citric acid/disodium hydrogen phosphate buffer (pH 3.4) was made up, and SO_2 in solution added to provide concentrations of 500, 1,000, and 2,000 ppm. Appropriate amounts of these concentrations or the buffer alone (control) were added to 250 g quantities of winter wheat grain (cv Avalon) to provide three replicates of each treatment at 0.92 a_w (22% m.c.) and 0.96 a_w (26% m.c.). The grain was agitated regularly while being stored for 24 hr at 5°C to allow equilibration to occur. Afterwards, samples were taken and the populations of fungi present on all treatments assessed using the serial dilution method and spread plating 0.1 or 0.2 ml of grain washings onto three replicate 2% MEA and 2% MEA + 10% NaCl plates. These plates were incubated at 25°C for 7-10 days and the fungal populations enumerated. Wheat grain was stored at 25°C and samples taken every 7 days for a period of up to 28 days.

RESULTS

In vitro effect of SO_2 on growth of grain fungi

Fig. 1 shows the effect of up to 200 ppm SO_2 on the growth rate of four common grain fungi found on freshly harvested cereal grain. As the SO_2 concentration was increased there was a decrease in the growth of the these species. However, at 200 ppm SO_2, some growth of *C. herbarum* and *E. nigrum* still occurred.

Table 1 shows the effect of different concentrations of SO_2 on the growth rate (mm day^{-1}), and the lag time for growth initiation of a range of *Penicillium* spp. at 15 and 25°C. *P. expansum* and *P. aurantiogriseum* were the most tolerant species, showing a capability for growth with 250 ppm SO_2 at both temperatures. In almost all cases, intermediate concentrations of 100 ppm SO_2 resulted in a stimulation of growth. However, the lag time prior to growth initiation increased with concentration of SO_2. At reduced a_w, this stimulation of growth by intermediate SO_2 concentrations was not evident (Table 2). Up to 100 ppm SO_2 had little effect on growth of *A. niger*,

although some other *Aspergillus* spp. were inhibited by 50 ppm SO_2 (Table 3).

Effect of SO_2 treatment of moist wheat grain on fungal populations

Table 4 shows the effect of treatment of wheat grain with up to 2,000 ppm SO_2 in solution at 0.96 and 0.92 a_w. Immediately after treatment, there was a significant decrease in total populations of filamentous fungi and yeasts. In the controls, particularly at 0.96 a_w, significant moulding occurred after seven days due to the growth of *Penicillium* and *Aspergillus* spp. At 0.92 a_w, the increase in total fungal colony forming units (CFUs) was slower than at 0.96 a_w. Generally, treatments of up to 2,000 ppm SO_2 had very little inhibitory effect on rate of moulding on moist wheat grain.

DISCUSSION

The *in vitro* tests of the efficacy of SO_2 in solution on growth of grain fungi demonstrated that *C. herbarum*, *E. nigrum*, and a range of *Penicillium* spp. were tolerant of concentrations of up to 200 ppm in solution. Indeed, growth of some species such as *P. expansum* and *P. aurantiogriseum* were

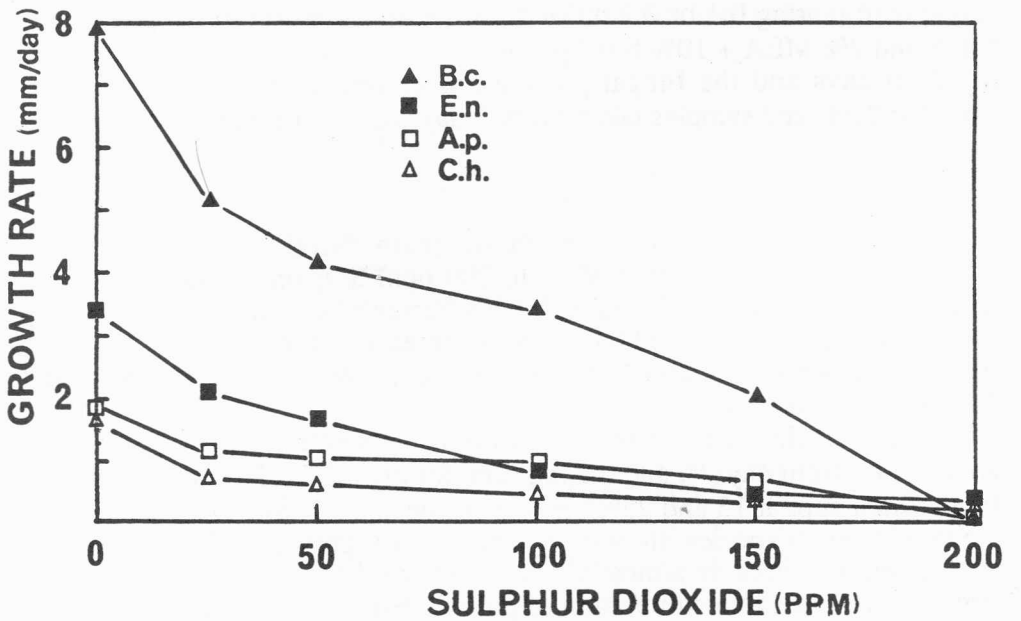

Fig. 1: The effect of different concentrations (ppm) of sulphur dioxide (SO_2) on the growth of four field fungi. Key to fungi: B.c. = *Botrytis cinerea*; E.n. = *Epicoccum nigrum*, A.p. = *Aureobasidium pullulans*, and C.h. = *Cladosporium herbarum*.

Table 1: Effect of different sulphur dioxide concentrations (ppm) on (a) the growth of *Penicillium* species and (b) the lag time for growth initiation on 1% malt extract agar, pH 3.5, at 15° and 25°C.

Sulphur dioxide (ppm)	0	100	200	250	0	100	200	250
Temperature: 15°C **Species**	Growth rate (mm day^{-1})				Lag time (days)			
P. aurantiogriseum	1.1	1.3	1.2	1.0	1	2	4	13
P. chrysogenum	0.4	0.7	0.2	< 0.2	1	2	11	>28
P. expansum	0.8	1.3	0.9	0.5	1	2	4	10
P. griseofulvum	0.2	0.3	0.2	NG	2	3	12	>28
P. hordei	0.3	0.4	0.3	0.3	1	2	11	14
Temperature: 25°C **Species**								
P. aurantiogriseum	1.1	1.2	1.1	1.1	1	1	3	12
P. chrysogenum	0.6	0.8	0.6	NG	1	2	9	>28
P. expansum	1.0	1.2	1.1	< 0.2	1	2	3	13
P. griseofulvum	0.4	0.6	0.5	NG	1	3	4	>28
P. hordei	0.4	0.6	0.5	0.3	1	2	3	14

NG = no growth

Table 2: Effect of different sulphur dioxide concentrations (ppm) on (a) the growth *Penicillium* spp. at 0.95 a_w and (b) lag time for growth initiation on 1% malt extract agar, pH 3.5 and 15° and 25°C.

Sulphur dioxide (ppm	0	50	100	200	0	50	100	200
Temperature: 15°C **Species**	Growth rate (mm day^{-1})				Lag time (days)			
P. aurantiogriseum	0.3	0.3	0.2	< 0.2	1	2	3	6
P. chrysogenum	0.3	0.3	0.1	NG	1	2	6	> 28
P. expansum	0.4	0.5	0.4	NG	1	2	4	> 28
P. griseofulvum	0.3	0.2	NG	NG	4	5	> 28	> 28
P. hordei	0.4	0.3	0.3	NG	2	3	12	> 28
Temperature: 25°C **Species**								
P. aurantiogriseum	0.8	0.7	0.7	0.3	1	1	3	8
P. chrysogenum	0.5	0.4	0.4	NG	1	2	3	> 28
P. expansum	0.8	0.9	0.7	0.4	1	1	3	9
P. griseofulvum	0.2	0.2	NG	NG	2	2	5	> 28
P. hordei	0.5	0.6	0.5	NG	1	2	3	9

NG = no growth

Table 3: Effect of sulphur dioxide (ppm) in solution on growth of five *Aspergillus* spp. on 1% malt extract agar, pH 3.5, at 25°C.

Concentration (ppm)	0	10	20	30	40	50	100
Species	Growth rate (mm/day)						
Aspergillus flavus	4.1	3.9	3.4	3.9	2.5	NG	NG
A. niger	4.6	4.8	4.4	4.3	3.9	3.3	1.9
A. ochraceus	1.7	1.8	1.7	1.6	1.4	NG	NG
A. terreus	1.8	1.2	0.9	0.5	0.2	NG	NG
A. versicolor	1.2	0.9	0.5	0.5	0.4	0.4	0.2

NG = no growth

Table 4: The total fungal colony forming units (CFU, g/grain) present on wheat grain of two water activities, treated with 500, 1,000, and 2,000 ppm sulphur dioxide in solution and stored for up to 28 days at 25°C.

Storage time (days)	0	7	14	21	28
Water activity: 0.96 SO_2 treatment (ppm)	Mean Log_{10} CFU (g/grain)				
Control	4.03	5.88	6.50	6.85	7.75
500	3.94	5.80	6.30	6.72	7.30
1,000	2.96	5.89	6.45	6.64	7.41
2,000	2.96	4.67	5.85	6.21	6.93
Water activity: 0.92 SO_2 treatment (ppm)					
Control	3.82	4.55	5.58	6.32	7.65
500	3.42	4.21	5.51	5.87	6.82
1,000	3.44	3.98	5.53	6.03	6.90
2,000	2.33	3.63	4.79	5.78	6.95

stimulated by 100 ppm SO_2. However, for all *Penicillium* species tested, there was an increase in lag times prior to germination and growth as the SO_2 was increased. Under conditions of water stress, the tolerance to SO_2 was reduced, but growth still occurred over a range of concentrations.

Previously, Couey and Uota (1961) found that reduction in spore germination of fungi such as *B. cinerea* was quantitatively proportional to the SO_2 concentration (up to 340 ppm). Germination also decreased linearly with increasing time of exposure to gaseous SO_2. Interactions have also been found previously between moisture, temperature, and gaseous SO_2. For example, germination of wet spores of a *Alternaria* sp. (unknown) decreased by 60% after exposure to 50 ppm, while 100 ppm SO_2 was required for dry spores, even at 98% relative humidity. There was an inverse relationship

between SO_2 concentration required and relative humidity (Uota, 1965). Other studies with much lower concentrations of SO_2 (< 200 ppb) showed that while germination of fungi such as *Fusarium culmorum* (W.G.Sm.) Sacc., *E. nigrum*, and *Cladosporium* spp. were unaffected, germ tube extension was significantly decreased (Magan and McLeod, 1986). Hibben (1966) found that up to 100 ppm gaseous SO_2 had little influence on germination of spores of *A. terreus* and *Penicillium egyptiacum* v. Beyma. The stimulation of growth and tolerance of the *Penicillium* spp. and *A. niger* to 100 ppm SO_2 was also found previously in liquid culture studies (Saunders, 1966). The thermotolerant spoilage fungus, *Byssochlamys nivea* Westling, grew with up to 50-75 ppm SO_2, depending on temperature, accompanied by the production of the mycotoxin patulin with up to 50 ppm SO_2, particularly at 21 and 30°C (Roland and Beuchat, 1984). This information suggests that the threshold SO_2 concentration may vary considerably for different spoilage fungi. It has been suggested that tolerance of filamentous fungi, particularly *Penicillium* spp., may be due to their ability to actively transport the SO_2 into the mycelia (Tweedie and Segel, 1970; Benitez *et al.*, 1983). The primary method of subsequent inactivation may be by binding or oxidation of the SO_2 which inactivates its antimicrobial properties. King *et al.*, (1981) found that SO_2-binding substances, mainly acetaldehyde, enabled spoilage yeasts to grow with much higher concentrations of SO_2. However, pH, contact time, and concentration were found to be important determinants of efficacy.

The treatment of moist wheat grain with up to 2,000 ppm SO_2 was ineffective in controlling the rate of moulding. Furthermore, *Penicillium* spp. predominated in the treated samples. This suggests that two factors may be interacting. Firstly, that a percentage of the SO_2 is being absorbed and bound to the grain components reducing its antifungal activity, and secondly that this reduction in concentration enables the growth of tolerant grain fungi, particularly *Penicillium* spp. Both Maleque (1989) and Serre (1991) have shown that, depending on the wheat grain m.c., between 20-30% of gaseous SO_2 became bound to the grain during treatment. Grain of intermediate m.c.'s may thus be more effectively treated than moist grain (> 20%) where a greater percentage of the SO_2 can be inactivated.

Studies by Eckhoff *et al.*, (1979; 1983) suggested that very effective control of mould growth could be achieved in moist maize with between 0.066-0.3% SO_2. More recently, Katangaza (1990) fumigated wheat grain of 18-24% m.c. with a range of concentrations of between 0.88 - 7% (g/kg grain). In these studies, there was correlation between decrease in both internal fungal colonisation of grain and germination and an increase in SO_2 concentration. However, for medium-term storage (5 months) at least 4.4% SO_2 (g/kg grain) was required to preserve wheat grain. Serre (1991) carried out similar experiments with wheat grain and found that high concentrations of gaseous SO_2 were necessary to control moulding. This contrasts with the

results obtained with moist maize in the USA (Eckhoff *et al.* 1979, 1983). As mentioned earlier, the binding of a percentage of SO_2 to components of maize and wheat may differ and influence the inhibitory effect of the treatment used. Furthermore, the threshold exposure concentrations necessary may also vary with the type of cereal under consideration. This may be critical when dealing with contaminant mycotoxigenic fungi that could be stimulated to grow and produce mycotoxins under sub-optimal treatment conditions.

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