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Nitrous Oxide Inhibits In Vitro Growth of Multiple Postharvest Fungi

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Abstract. **Nitrous oxide (N_2O) was tested as a potential fungicidal or fungistatic compound. Twelve postharvest fungi were exposed to 10 to 80 kPa with 20 kPa O_2 in a static system at 20 °C. These fungi were divided into N_2O high-, medium- and low-sensitive groups. Based on growth sensitivity, growth of high-sensitive fungi was completely inhibited, and that of medium-sensitive fungi up to 85%. With low-sensitive fungi, significant inhibition was achieved only when the fungi were exposed to N_2O continuously for 6 days. *Botrytis cinerea* Pers.: Fr., *Colletotrichum acutatum* Simmonds, *Monilinia fructicola* (Winter) Honey, *Penicillium expansum* Link, *Penicillium italicum* Wehmer, *Phytophthora citrophthora* (R.E. Smith and E.H. Smith) Leonian and *Rhizopus stolonifer* (Ehrens.: Fr.) Vuillemin, were high-sensitive; *Glomerella cingulata* (Stoneman) Spaulding was medium-sensitive, and *Alternaria alternata* (Fr.) Keissler, *Fusarium oxysporum* Schlechtend1: Fr. f. sp. *fragariae* Winks and Williams, *Fusarium oxysporum* Schlechtend1: Fr. f. sp. *lycopersici* (Saccardo) Snyder and Hansen., and *Geotrichum candidum* Link., were low-sensitive fungi. Addition of up to 100 $\mu L \cdot L^{-1}$ C_2H_4 did not reduce inhibition caused by N_2O. The inhibitory effect of N_2O was considered to be due to biophysical properties similar to CO_2, the competitive inhibition on C_2H_4 action, or the biosynthesis of methionine. These results indicate the potential of N_2O to control some postharvest decay fungi.**

Nitrous oxide (N_2O) is a chemically neutral, nontoxic, naturally occurring atmospheric gas (Leshem and Wills, 1998). The biophysical properties of N_2O, such as relative stability and high solubility, are similar to CO_2, which is known to suppress fungal growth. Various reports suggest that 3 to 80 kPa CO_2 partial pressures in the environment can suppress fungal growth (El-Goorani and Sommer, 1981). A competitive inhibitor of C_2H_4 production and action, 2,5-norbornodiene (NBD), has also been shown to suppress fungal growth (Képczynska, 1989). The inhibitory effect of NBD on germination and fungal growth could be partially reduced by the addition of C_2H_4 to the environment (Képczynska, 1989,1993), N_2O has also been demonstrated to be a competitive inhibitor of C_2H_4 production and action in fruits (Gouble et al., 1995).

The pyrimidinamine group of fungicides are known to inhibit methionine biosynthesis, leading to the inhibition of fungal growth (Masner et al., 1994). The addition of methionine to the growth medium partially reversed this inhibitory affect (Masner et al., 1994). N_2O also has the ability to interfere with methionine production in animals (Frontiera et al., 1994).

The potential of N_2O as a fungicidal or fungistatic compound is suggested because: 1) it has biophysical properties similar to CO_2; 2) it is a competitive inhibitor of C_2H_4 production and action; and 3) it has the ability to interfere in methionine biosynthesis. However, no research on the independent and direct effect of N_2O on pathogenic fungi has been published. The objective of our study was to determine the effects of N_2O on postharvest-decay causing fungi in vitro.

Materials and Methods

Fungal cultures. Twelve fungi which can cause postharvest-decay of fruits were tested viz., *Alternaria alternata* (Fr.) Keissler, *Botrytis cinerea* Pers.: Fr., *Colletotrichum acutatum* Simmonds, *Fusarium oxysporum* Schlechtend1: Fr. f. sp. *fragariae* Winks and Williams, *Fusarium oxysporum* Schlechtend1: Fr. f. sp. *lycopersici* (Saccardo) Snyder and Hansen., *Geotrichum candidum* Link., *Glomerella cingulata* (Stoneman) Spaulding, *Monilinia fructicola* (Winter) Honey, *Penicillium expansum* Link, *Penicillium italicum* Wehmer, *Phytophthora citrophthora* (R.E. Smith and E.H. Smith) Leonian and *Rhizopus stolonifer* (Ehrens.: Fr.) Vuillemin. These fungi were obtained from the Gene Bank, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, and the Plant Pathology Lab. Kagoshima Univ., Japan. All fungi were either fruit or vegetable strains, and during the course of the experiments, were maintained on malt agar medium (MA) at 20 °C. A 3-mm-diameter mycelial plug obtained from the edge of 3- to 14-d-old fungal cultures was centrally placed on MA plates. All experiments were carried out at 20 °C in the dark. Five replicated plates of each fungus were used. The experiments with each fungus were repeated at least three times.

N_2O dosing method. A static treatment system was used to expose the fungi to N_2O in 1.3-L or 3-L gas tight glass jars. A vacuum tube was used to direct the gas flow to and from the jar. N_2O and O_2 were metered into jars through a 1 mL syringe needle in a rubber bung in the lid of each jar. Separate compressed gas cylinders of N_2O and O_2 fitted with low-pressure gas valves and flow controllers (Hanshin Yodenki Kogyo Co., Tokyo) were used. Pressure was released from the jars through a second 1-mL syringe needle in a second rubber bung in each lid connected to a tube with its outlet submerged in water. Known amounts of gases were flushed into the jars in a stepwise procedure. The process was repeated several times to obtain higher N_2O concentrations in the jars.

Oxygen partial pressure was maintained at 20 kPa. Therefore, the volume of O_2 added (VO_2) was in proportion to the volume of N_2O added (VN_2O). For each addition of N_2O and O_2 and release of pressure from the jar, the fraction of N_2O ($FracN_2O$) was calculated using the equation:

$$FracN_2O\{new\} = (FracN_2O\{old\} \times Vol + VN_2O) / (Vol + VN_2O + VO_2)$$

where; Vol = volume of jar (m^3). In the case of multiple additions, this equation was used recursively.

To avoid the negative effects of a CO_2 free environment (Hartman et al., 1972), after flushing the N_2O and O_2, the jars were also flushed with CO_2 to maintain 0.03 kPa.

The N_2O and O_2 concentrations were verified using a thermal conductivity detector (Qadir and Hashinaga, 2001).

Experiments. In the first experiment, the inoculated plates were exposed to 10, 20, 30, 40, 50, 60, 70, and 80 kPa N_2O. The mycelial growth of different fungi was determined every other day for 6 d by measuring mycelial diameter. After recording the data, plates were replaces and required partial pressure of gases were reestablished. In the second experiment, fungi were exposed to 30, 50, and 80 kPa N_2O continuously for 6 d. The mycelial growth rate was measured after 6 d of incubation. The gas inside the jar was stirred every other day by drawing and flushing back the same gas with a 60-mL gas tight syringe. In the third experiment, the effect of methionione, aminooxyacetic acid (AOA), or both on the mycelial growth of *B. cinerea* was tested. The protocol followed was that of Qadir et al. (1997), briefly 0.5 mL spore suspension (1×10^7 spores/mL) 10 mL modified Pratt's liquid medium containing (in mM): glucose 111, KH_2PO_4 28.6, NH_4NO_3 50, $MgSO_4 \cdot 7H_2O$ 4.9, $FeCl_3 \cdot 6H_2O$ 0.07, $ZnSO_4 \cdot 7H_2O$ 1.7, citric acid 31.2, H_3BO_3 28.6 and diethylbarbituric acid 28.2. Basal medium was amended with 35 mM methionine, AOA, or both. Cultures were placed on a rotovator shaker (200 rpm). The dry weight of the mycelium, was measured after 6 d of incubation.

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Statistical analysis was done by calculating a least significant distance (LSD) for the arcsine-transformed data (ignoring 0% and 100%), and data transforming this back to get a conservative LSD for the mean percentages. The 0% and 100% means are obviously significantly different from the other means since they have no variance.

Results

Preliminary experiments indicated that mycelial growth rates were not affected by either the potential gradient in N_2O or the change in atmospheric pressure in jars resulting from the N_2O and O_2 dosing methods (data not shown).

To determine the effect of N_2O on mycelial growth, fungi were exposed to 10 to 80 kPa N_2O. In high-sensitive group complete inhibition was obtained, while in medium-sensitive group up to 85% inhibition was achieved. In the low-sensitive group of fungi there was a slight enhancement in growth rate at the lower N_2O levels and a small inhibition in growth rate at 80 kPa N_2O. Of the twelve fungi tested, *B. cinerea, C. acutatum, M. fructicola, P. expansum, P. italicum, P. citrophthora,* and *R. stolonifer* were high-sensitive, only *G. cingulata* was medium and *A. alternata, F. oxysporum* f. sp. *fragariae, F. oxysporum* f. sp. *lycopersici,* and *G. candidum* were low-sensitive fungi to N_2O (Table 1). The addition of 1, 10 and 100 $\mu L \cdot L^{-1}$ C_2H_4 to the 30, 50, and 80 kPa N_2O treatments did not reduce the inhibitory effect on the mycelial growth rate or increase growth rate of controls (data not shown). In general, greater inhibition was achieved when the fungi were exposed to 30, 50 and 80 kPa N_2O concentrations continuously for 6 d (compare Table 1 and 2).

The addition of 35 mM methionine to modified Pratt's liquid medium in shake culture of *B. cinerea* increased the dry weight of the mycelium by about 50%, compared with control (Table 3). Addition of 35 mM AOA, an inhibitor of C_2H_4 production in *B. cinerea* (Qadir, 1994), to the basal or methionine-amended medium decreased the mycelial dry weight by ≈25% and 40% respectively, compared with controls (Table 3).

The addition of 35 mM methionine to MA medium did not reduce the N_2O inhibitory effect on *B. cinerea.* After the treatment, however, when the cultures were exposed to air, the colony growth rate was faster on methionine amended MA plates (data not shown).

Table 1. Percentage of inhibition of fungal colony growth rate in various N_2O levels at 20 °C.[z]

	N_2O partial pressure (kPa)								
Fungi	0	10	20	30	40	50	60	70	80
High sensitive									
Botrytis cinera	0	46	81	92	94	97	100	100	100
Colletotrichum acutatum	0	24	20	20	48	44	76	92	100
Monilinia fructicola	0	37	52	41	77	100	100	100	100
Penicillium expansum	0	6	25	31	56	75	81	100	100
Penicillium italicum	0	50	50	50	85	79	79	100	100
Phytophthora citrophthora	0	18	54	43	57	89	89	100	100
Rhizopus stolonifer	0	45	89	96	97	98	100	100	100
Medium sensitive									
Glomerella cingulata	0	0	10	20	25	40	60	65	85
Low sensitive									
Alternaria alternata	0	–23	–23	9	–20	14	–11	–14	26
Fusarium oxysporum f.sp. *fragariae*	0	–8.3	17	5	3	17	12.5	33	25
Fusarium oxysporum f.sp. *lycopersici*	0	–18	–3	–3	0	3	21	9	9
Geotrichum candidum	0	---	---	37	---	5	---	---	19

[z]Data are mean of three experiments, each consisting of five plates. Mean colony diameter (mm) was recorded every other day for 6 d. The LSD for comparing means at 5% probability is 5.1.

Table 2. Percentage of inhibition of fungal colony growth rate in various N_2O levels during 6 d continuous exposure to 20 °C.[z]

	N_2O partial pressure (kPa)			
Fungi	0	30	50	80
High sensitive				
Botrytis cinera	0	96	98	100
Colletotrichum acutatum	0	40	60	100
Monilinia fructicola	0	71	100	100
Penicillium expansum	0	65	87	100
Penicillium italicum	0	87	93	100
Phytophthora citrophthora	0	45	76	100
Rhizopus stolonifer	0	100	100	100
Medium sensitive				
Glomerella cingulata	0	41	59	73
Low sensitive				
Alternaria alternata	0	9	23	57
Fusarium oxysporum f.sp. *fragariae*	0	29	35	68
Fusarium oxysporum f.sp. *lycopersici*	0	29	35	68
Geotrichum candidum	0	4	15	55

[z]Data are mean of three experiments, each consisting of five plates. Mean colony diameter (mm) was recorded after 6 d. The LSD for comparing means at 5% probability is 2.4.

Discussion

Nitrous oxide, a competitive inhibitor of C_2H_4 production and action (Gouble et al., 1995), suppressed the growth rate of most of the fungi tested. Elevated CO_2 also suppresses fungal growth (El-Goorani and Sommer, 1981). The biophysical similarity between N_2O and CO_2 may be the relevant factor in fungal growth suppression. In this study, complete inhibition was achieved with the high-sensitive group of fungi at 50 to 80 kPa N_2O. However, in the low-sensitive group, this inhibitory effect was minimal (Table 1). This variation in inhibition suggests that N_2O may have suppressed the fungal metabolic process at multiple locations.

In animal and plant systems, N_2O has shown two C_2H_4 related responses: 1) interference in the methionine pathway (Frontiera et al., 1994), which can lead to the growth inhibition of some fungi (Masner et al., 1994); and 2) inhibition of C_2H_4 action (Gouble et al., 1995), that may be essential for the growth of some fungi (Kepczynska, 1989, 1993). In the present study, the addition up to 100 $mL \cdot L^{-1}$ C_2H_4 to N_2O did not reduce the inhibitory effect or increase growth rate in the control. However, after the treatment, when plates were exposed to air, regardless of the treatment, the N_2O inhibitory effect disappeared and the mycelial growth rate on methionine amended MA plates was more rapid. This indicate that N_2O may have an C_2H_4 related fungistatic effect.

Overall a greater inhibition in growth rate was achieved in the high- and low-sensitive groups of fungi when they were exposed to N_2O continuously (Table 2). The intermittent exposure to air of ≈2 h every other day in Expt. 1, may have diminished the N_2O inhibitory effect in low-sensitive group of fungi.

Table 3. Mycelial dry weight of *B. cinera* grown on basal medium with methionine, aminooxyacetic acid (AOA), or both in shake culture at 20 °C for 6 d.[z]

Treatments	Mycelial dry wt (g)
Control	0.21 ± 0.02
Methionine	0.43 ± 0.03
Methionine + AOA	0.18 ± 0.01
AOA	0.16 ± 0.01

[z]Means ± SE (10 replicated flasks).

In summary, 80 kPa N_2O inhibited the in vitro growth of most fungi tested, particularly during continuous exposure for 6 d at 20 °C. While the current study has shown the direct effect of N_2O on the growth rate of fungi, the mechanisms of this growth inhibition are as yet unclear and cannot be inferred from these results. The role of N_2O in postharvest decay and the mechanisms of in vitro inhibition warrant further investigation.

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