Increased Growth of *Nicotiana glutinosa* as Partially Related to Accumulation of Ammonium-Nitrogen in Soil Fumigated with Methyl Bromide

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ABSTRACT

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Biological and chemical changes were measured in soil fumigated for various periods with methyl bromide (27,000 μl/L air) in a moving airstream. Height of *Nicotiana glutinosa* plants grown in fumigated soil increased as the period of fumigation increased above 16 hr. Increased growth was correlated with elimination of a coenocytic fungus from the roots of plants grown in soil fumigated for longer than 4 hr and with elimination of microorganisms other than recognized fungal pathogens. Increased growth was correlated with an increase in inorganic nitrogen (primarily ammonium) in soil fumigated for 16 hr or longer. Concentrations of various plant nutrients were determined in tissues of 35-day-old *N. glutinosa* plants grown in fumigated soil. Only nitrogen was closely correlated with increased growth, although high concentrations of copper, manganese, zinc, and phosphorus in plants grown in soil fumigated for 128 hr may have accounted for some increased growth.

The increased growth of plants in fumigated soil has been recognized since the late nineteenth century (14). Stover and Koch (13) suggested that factors other than elimination of pathogens might be involved, since tobacco grew better in soil treated with methyl bromide (MB), even though no pathogens were known to be present. In their work, the actual concentration of MB in the soil during the experiment was not determined. Therefore, data were not available to determine dosage responses needed for eliminating pathogens or to determine other changes occurring in the soil during fumigation.

In our experiments, continuous-flow fumigation methods (5) provided a system by which dosage response information was obtained for accumulation of ammonium, elimination of fungal pathogens, and growth of *Nicotiana glutinosa* L. in fumigated soil. Use of this information made it possible to separate the effects of pathogenic microorganisms and accumulation of ammonium on plant growth in fumigated soil.

MATERIALS AND METHODS

Fumigation of soil. Soil was fumigated with 27,000 μl MB/L air from a continuous-flow, controlled-concentration fumigation apparatus (5,10). Arlington loam soil (pH 7.0) containing 11–13% water was fumigated by placing 1 kg in 1-L glass cylinders and passing a constant concentration of MB:air through the column at the rate of 20 ml/min. The concentration of MB throughout the column equilibrated within 2.5 hr. This time was designated as E, and all times of exposures were based on E plus the added exposure period. Exposures were made at E, E+2, E+4, E+16, E+32, E+64, and E+128 hr, hereafter designated as the full series of exposures. Concentration of MB was monitored by gas chromatography (5). In experiments on the nitrogen cycle, approximately 150 g of soil was placed in 250-ml stoppered Erlenmeyer flasks through which MB was continuously circulated. E was approximately 30 min in this system. After fumigation, soil was immediately emptied from the flasks or humidified air was passed over it so that MB was dissipated rapidly.

Growth of *N. glutinosa* in fumigated soil. *N. glutinosa* was grown in autoclaved 10-cm diameter clay pots containing nonfumigated soil or soil fumigated for the full series of exposures. Pots were directly seeded with seed disinfested with 0.5% sodium hypochlorite for 3 min and rinsed with sterile distilled water. Seeds were planted within 1 day after the E+128 hr treatment was completed. The pots were subirrigated before germination and surface-irrigated after germination. Plants were thinned to one per pot, and pots were placed in a completely randomized block design in a greenhouse, using five replicates per treatment. Plant height and

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dry weight were determined after 90 days.

Colonization of roots by fungal pathogens from each treatment was determined by surface-sterilizing random segments of roots with 0.5% sodium hypochlorite for 3 min, rinsing with sterile distilled water, placing the segments on 2% water agar containing 150 μg of vancomycin per milliliter, and incubating at 25 C for five days. Hyphal tips of fungi that grew from the roots were transferred to Difco potato-dextrose agar.

A coenocytic fungus was routinely isolated from roots of plants grown in soil fumigated for short periods, and its pathogenicity on *N. glutinosa* was determined. Sterile vermiculite soaked with potato-dextrose broth (500 ml vermiculite/200 ml potato-dextrose broth) was infested with the fungus and added to steamed soil. Disinfested *N. glutinosa* seeds were added to pots of steamed soil. Noninfested autoclaved soil served as a control.

Total leaf area per plant was determined in a separate but identical experiment after plants had grown for 35 days. Leaves were compared with a set of templates representing leaves of known surface area (1).

Growth of *N. glutinosa* in soil fumigated for E+64 hr to which various soil suspensions were added. Nonautoclaved and autoclaved suspensions of soil were added to pots of fumigated soil to determine whether the factors involved in the growth responses were microbial in nature. Nonfumigated soil or soil fumigated for E+8 or E+64 hr was placed in autoclaved 10-cm diameter clay pots. Holes were made in the soil, and soil suspensions (0.5 g dry wt/ml sterile water) were added at the rate of 100 ml/pot as follows (Fig. 1): from nonfumigated soil to soil fumigated for E+8 or E+64 hr, from soil fumigated for E+8 hr to either nonfumigated soil or soil fumigated for E+64 hr, and from soil fumigated for E+64 hr to either nonfumigated soil or soil fumigated for E+8 hr. In a parallel series, the same suspensions were autoclaved 30 min before addition to the respective soils. Pots of nonfumigated soil or soil fumigated for E+8 or E+64 hr served as controls. Five replicate pots for each treatment, seeded as described, were placed in a completely randomized design and the plants were grown for 90 days.

Determination of inorganic nitrogen. Soil was fumigated, incubated at 23 C for 0, 32, or 144 hr, and stored at 0 C until all analyses were made. Soil was analyzed for nitrate and ammonium (total inorganic nitrogen) by steam distillation (2). Soil treated with humidified air for E+128 hr served as the nonfumigated control.

Since the soil used was low in nitrogen, a solution of ammonium

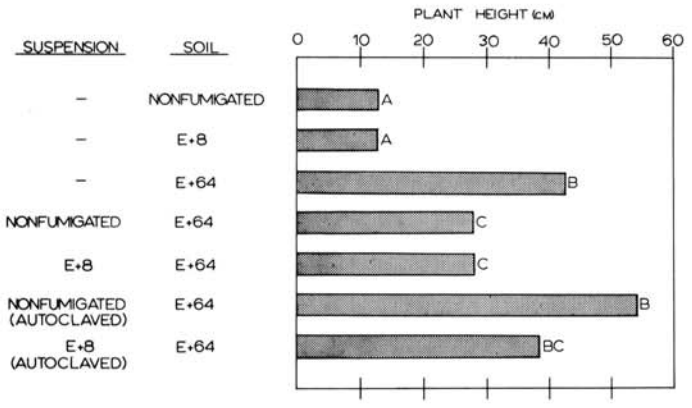

Fig. 1. Plant height of *Nicotiana glutinosa* grown for 90 days in pots of nonfumigated soil, soil fumigated for 8 or 64 hr, or soil fumigated for 64 hr to which soil suspensions of nonfumigated soil or soil fumigated for 8 hr had been added. Soil was fumigated with 27,000 μl methyl bromide/L air. E is the time required for the concentration of methyl bromide in reaction vessels to equilibrate. Values given are means of five replicates. Columns with the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.

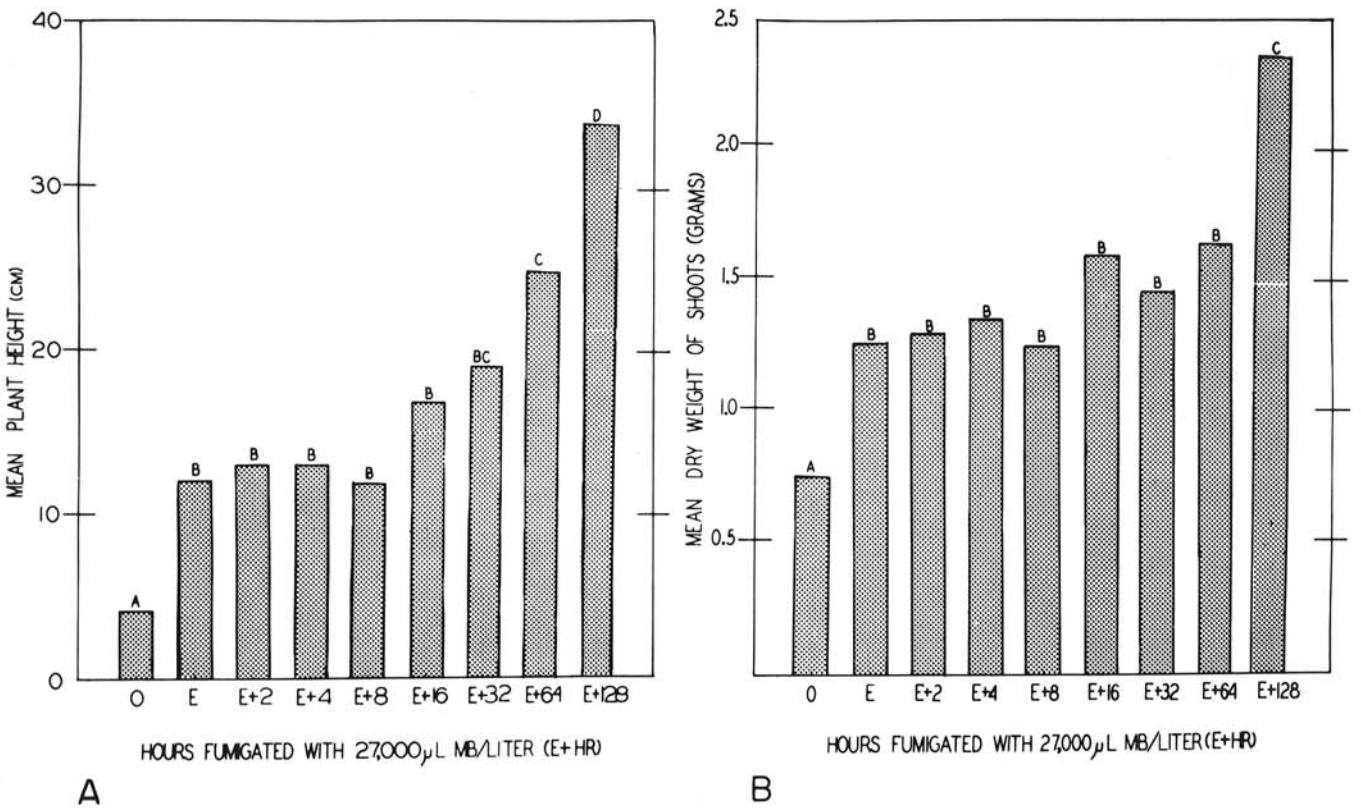

Fig. 2. **A,** Height and **B,** shoot weight of *Nicotiana glutinosa* plants grown in pots of nonfumigated soil and soil fumigated for 2, 4, 8, 16, 32, 64, or 128 hr with methyl bromide (MB). E is the time required for the concentration of MB in reaction vessels to equilibrate. Values given are means of five replicates. Columns with the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.

sulfate (1 g/25 ml distilled water) was added to 2 kg of moist soil (11% moisture) with an atomizer while the soil was being mixed in a cement mixer. Immediately after mixing, the soil was fumigated and incubated at 23 C. Ammonium and nitrate (total inorganic nitrogen) were determined in 25-g samples of soil from each flask 0, 7, 21, and 90 days after fumigation. Soil treated with humidified air for E+128 hr served as the nonfumigated control. Soil samples were handled as described.

In other experiments, ammonium sulfate (1 g/25 ml distilled water) and 10 g of cellulose were added to 2 kg of moist soil. Amended soil was stored at 23 C for 7 days to allow assimilation of the ammonium sulfate by microorganisms before fumigation. The soil was fumigated, incubated, and analyzed as described for the previous experiment.

Nitrogen plus other elements in *N. glutinosa* after 35 days of growth in fumigated soil. Nonfumigated soil and soil fumigated for the full series of exposures were placed in sterile clay pots and seeded with surface-disinfested *N. glutinosa* seed. Four replicate pots per treatment were arranged in a randomized block design in a greenhouse, and plants were grown for 35 days. Each pot contained approximately 100 seedlings, and plants were not fertilized. Dried plant tops from each pot were ground in a mortar and pestle, and the nitrogen content was determined by the micro-Kjeldahl method. Other elements were determined by atomic absorption and colorimetric procedures as described by Labanauskas and Bitters (6), except that a Perkin Elmer Model 460 atomic absorption spectrophotometer was used.

RESULTS

Growth of *N. glutinosa* in fumigated soil. Plants grown in fumigated soil were significantly ($P = 0.05$) taller (Fig. 2A) and heavier (Fig. 2B) than plants grown in nonfumigated soil. Although the increase was not always significant, plant height increased with each successive fumigation period after E+8 hr. The increased plant height was caused by the initiation of a flower stalk that emerged from the rosette sooner with each successive fumigation period after E+8 hr.

Leaf area per plant of plants grown in soil fumigated for E+128 hr was significantly ($P = 0.05$) greater than that of plants grown in soil fumigated for less than E+16 hr (Fig. 3). In contrast to plant height and weight, leaf areas of plants grown in soil fumigated for E, E+2, or E+4 hr were significantly less than those of plants grown in soil fumigated for E+8 hr. Also, the leaf area per plant of plants grown in nonfumigated soil was not significantly different from that of plants grown in soil fumigated for E, E+2, or E+4 hr.

A fungus with coenocytic mycelium was isolated from 70% of the roots of plants grown in nonfumigated soil and from 40 and 20%, respectively, of the roots of plants grown in soil fumigated for E and E+2 hr; it was also isolated infrequently from the roots of plants grown in soil fumigated for E+4 hr but not from roots of plants grown in soil fumigated for longer periods. The fungus grew on $P_{10}VP$ agar (15) and resembled *Pythium* in growth and morphology, but its identity is still obscure because it did not produce sporangia when grown on carnation leaf agar or when a mycelial plug was placed in pond water (16). Also, it did not produce fruiting structures when placed on the medium of Erwin and Katznelson (4) containing 20 mg of β-sitosterol per liter (7) or when subjected to various light regimens (11). The fungus caused damping-off of young *N. glutinosa* seedlings grown in autoclaved soil infested with it. Several other fungi isolated from the roots of plants grown in nonfumigated soil or soil fumigated for E or E+2 hr were species generally regarded as saprophytes and were not isolated regularly.

Growth of *N. glutinosa* in soil fumigated for E+64 hr to which various soil suspensions were added (Fig. 1). When nonautoclaved soil suspensions (either of soil fumigated for E+8 hr or of nonfumigated soil) were added to soil fumigated for E+64 hr, plant height was reduced significantly to a height intermediate between plants grown in soil fumigated for E+64 hr and those grown in nonfumigated soil or soil fumigated for E+8 hr. However, this effect was not produced (plant heights remained the same) if the suspensions were autoclaved before being added to the soil. The addition of autoclaved suspensions to nonfumigated soil or to soil fumigated for E+8 hr likewise did not affect plant growth. Other treatments that had no effect on plant growth were suspensions of nonfumigated soil added to soil fumigated for E+8 hr, suspensions of soil fumigated for E+8 hr added to nonfumigated soil, and suspensions of soil fumigated for E+64 hr added to either nonfumigated soil or soil fumigated for E+8 hr.

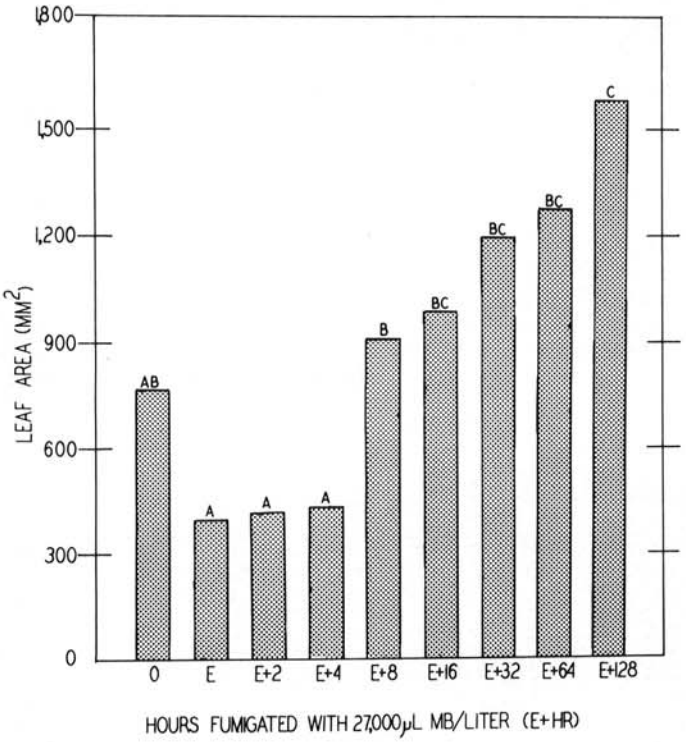

Fig. 3. Leaf area per plant of *Nicotiana glutinosa* after 35 days of growth in pots of nonfumigated soil and soil fumigated for 2, 4, 8, 16, 32, 64, or 128 hr with methyl bromide (MB). E is the time required for the concentration of MB in reaction vessels to equilibrate. Values given are means of five replicates. Columns with the same letter are not significantly different ($P = 0.05$) as determined by Duncan's multiple range test.

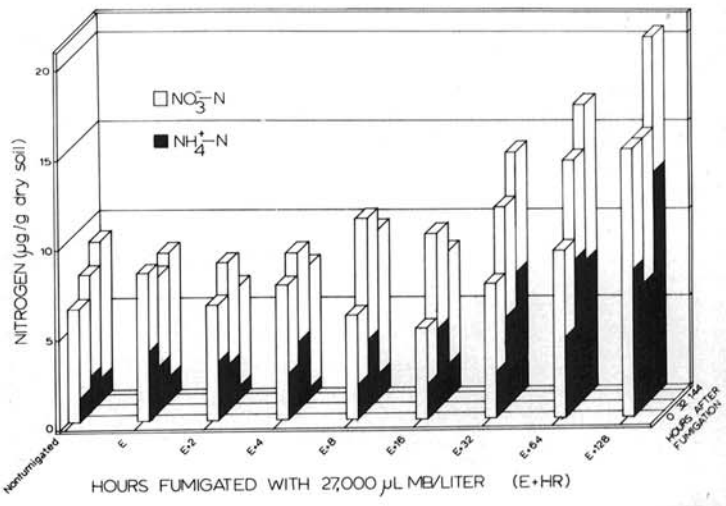

Fig. 4. Ammonium and nitrate (total inorganic nitrogen) in soil fumigated with methyl bromide (MB) for 2, 4, 8, 16, 32, 64, or 128 hr and incubated in closed flasks at 23 C for 0, 32, or 144 hr. E is the time required for the concentration of MB in reaction vessels to equilibrate. Inorganic nitrogen was determined by the steam distillation method of Bremner and Keeney (2).

Inorganic nitrogen in nonamended soil after MB fumigation (Fig. 4). The amount of nitrate and ammonium (total inorganic nitrogen) was very low in nonamended field soil but was relatively high in soil fumigated for E+32 hr and increased as the time of fumigation increased. The increase in inorganic nitrogen was due primarily to accumulation of ammonium.

Inorganic nitrogen in soil amended with ammonium sulfate before fumigation. Ammonium was present initially in amounts varying from 70–95 μg of nitrogen per gram of dry soil in soil fumigated for E, E+2, E+4, and E+8 hr. It disappeared rapidly from these soils, however, and by 21 days less than 1.5 μg of nitrogen per gram of dry soil was present as ammonium (Fig. 5A). This indicated that nitrification was not strongly inhibited when soil was fumigated for E to E+8 hr. Nitrification was inhibited in soils treated for longer periods; the inhibition lasted at least 90 days in soil fumigated for E+32, E+64, and E+128 hr, since amounts of ammonium were substantial (80–135 μg N/g dry soil) after 90 days. Generally, when the soil was incubated for longer than 7 days, the total amount of inorganic nitrogen increased as the length of time after fumigation increased. The total amount of inorganic nitrogen at any sampling period, however, was approximately the same for all treatments.

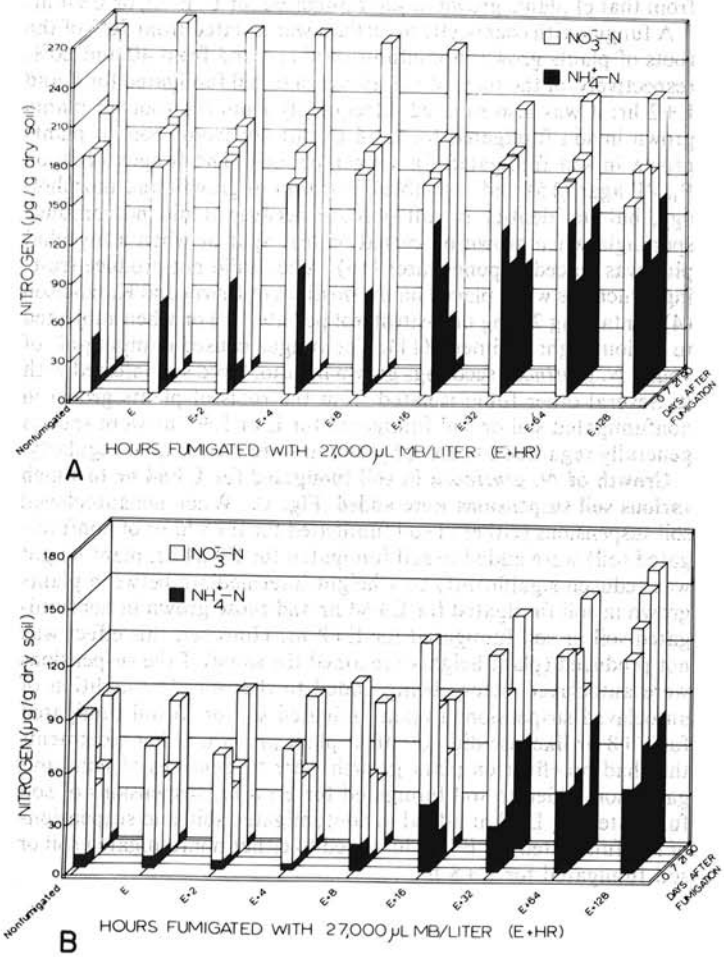

Fig. 5. Ammonium and nitrate in soil amended with **A,** ammonium sulfate or **B,** ammonium sulfate and cellulose and fumigated with methyl bromide (MB) for 2, 4, 8, 16, 32, 64, or 128 hr. Total inorganic nitrogen was determined after incubation in closed flasks at 23 C for 0, 7, 21, and 90 days. E is the time required for the concentration of MB in reaction vessels to equilibrate.

Inorganic nitrogen in soil amended with ammonium sulfate and cellulose before fumigation. The amount of total inorganic nitrogen in soil amended with ammonium sulfate and cellulose (Fig. 5B) was greater than in nonamended soil (Fig. 4) but less than in soil amended with only ammonium sulfate (Fig. 5A). The ammonium added to the soil presumably became incorporated into the protoplasm of soil organisms before fumigation because of the high carbon:nitrogen ratio due to the addition of cellulose. The amount of total inorganic nitrogen in soil amended with ammonium sulfate and cellulose was initially greater in soil fumigated for E+8 hr or longer (105–130 μg N/g dry soil) than in nonfumigated soil or soil fumigated for E, E+2, or E+4 hr (65–85 μg N/g dry soil). By the 7th day of incubation, however, the amount of nitrogen in soil fumigated for E+8 hr was similar to that in soil fumigated for shorter periods (40–50 μg N/g dry soil). Soil fumigated for E+16 hr or longer contained 90–140 μg of nitrogen per gram of dry soil. By the 90th day of incubation, the amount of inorganic nitrogen in soil fumigated for E+16 hr was similar to that found in soil fumigated for shorter periods (80–90 μg N/g dry soil). Soil fumigated for E+32 hr or longer contained 130 μg of nitrogen per gram of dry soil.

The amount of total inorganic nitrogen also varied with length of incubation after fumigation. In soil incubated for 7 days, the amount of inorganic nitrogen in each treatment (except E+64 and E+128 hr) was lower than the amount observed immediately after fumigation (0 day, Fig. 5A). The amount in soil fumigated for E+64 hr was the same as that inititally present, whereas the amount in soil fumigated for E+128 hr increased. In soil incubated for 21 days, the amounts in nonfumigated soil and soil fumigated for E, E+2, E+4, and E+8 hr were similar to those in soil after 7 days of incubation. Soil fumigated for E+128 hr contained more inorganic nitrogen after 21 days than after 7 days. After 90 days, all soils contained more inorganic nitrogen than after incubation for 7 or 21 days.

Concentrations of nitrogen and other nutrients in tops of *N. glutinosa* after 35 days of growth. Nitrogen in the tops of 35-day-old *N. glutinosa* grown in soil fumigated for E+8 hr or longer increased as the fumigation time increased. Concentration of nitrogen was maximum in seedlings grown in soil fumigated for E+128 hr (Table 1).

TABLE 1. Concentration of nutrients in tops of 35-day-old *Nicotiana glutinosa*[x] grown in soil fumigated with 27,000 μl methyl bromide/L air for various periods

Soil treatment (E[y] + hours)	Nitrogen (%)	Potassium (%)	Phosphorus (%)	Magnesium (%)	Sodium (%)	Zinc (ppm)	Manganese (ppm)	Copper (ppm)
E	2.73 a[z]	6.58 a	0.61 b	0.63 a	0.12 a	99.9 c	80.0 b	31.4 c
2	2.67 a	5.79 b	0.57 a	0.59 b	0.08 c	72.7 b	78.5 ab	27.7 ab
4	2.71 a	5.77 b	0.66 c	0.55 c	0.09 b	63.9 a	76.8 ab	27.1 a
8	2.88 b	5.41 c	0.63 bc	0.53 d	0.08 c	66.0 a	73.0 a	30.7 bc
16	2.84 b	5.01 d	0.65 c	0.49 e	0.08 c	62.3 a	81.0 ab	29.1 abc
32	3.77 c	4.63 f	0.66 c	0.39	0.08 c	86.9 c	78.6 ab	36.3 d
64	4.21 d	4.48 f	0.63 bc	0.35 h	0.08 c	92.9 c	75.3 ab	35.5 d
128	4.44 e	4.83 e	0.78 d	0.37 g	0.09 b	108.7 e	94.6 c	51.7 e

[x]Approximately 100 seedlings per replicate and six replicates per treatment.
[y]E is the time required for the concentration of methyl bromide in reaction vessels to equilibrate.
[z]Means in the same column with the same letter are not significantly different ($P = 0.01$) as determined by Duncan's multiple range test.

A trend toward increased concentration of phosphorus, zinc, manganese, and copper was noted in 35-day-old *N. glutinosa* but was not as pronounced as that for nitrogen in the same plants.

DISCUSSION

Increased growth of *N. glutinosa* plants in fumigated soil was not completely due to elimination of fungal pathogens, since plant height did not increase significantly until plants were grown in soil fumigated for periods far in excess (E+64 hr or longer) of those required for elimination of the coenocytic fungus isolated from roots of plants grown in soil fumigated for E+4 hr or less. Some pathogenic organisms may still have been present, but this is doubtful, since there was no difficulty in isolating fungi from the roots of plants grown in nonfumigated soil or soil fumigated for E, E+2, or E+4 hr. These results agree with those of Stover and Koch (13), although they did not attempt to isolate fungi from the roots of plants grown in either nonfumigated or MB-fumigated soil.

Increased growth of plants in MB-treated soil was due, in part, to elimination of microorganisms. At short exposures (E+4 hr or less) parasitic and nonparasitic microorganisms were responsible for the lack of increased growth seen in plants grown in soils exposed for longer times (E+64 and E+128 hr). Because a coenocytic pathogenic fungus was isolated from plants growing in soils fumigated for E+4 hr or less, assessment of the depressing roles on growth that other organisms may have had was difficult. This was resolved by using suspensions of autoclaved and nonautoclaved soil. Suspensions of soil treated for E+8 hr, which no longer contained the coenocytic fungus or other recognized pathogens, were added to soil (E+64 hr) known to be capable of supporting increased plant growth. The result was that subsequent growth in the amended soil was diminished. When the E+8-hr soil suspension was autoclaved before being added to the E+64-hr soil, subsequent plant growth was not diminished. When most of the organisms were eliminated from the soil by long fumigations (E+64 hr or longer) and autoclaved and nonautoclaved suspensions were added to E+64-hr soil, no effect on plant growth was noted. These experiments proved that decreased growth was the result of nonpathogenic as well as pathogenic organisms in the original soil.

Increased growth was due to the accumulation of nitrogen as well as to the elimination of microorganisms. In soils not associated with increased plant growth, populations of nitrifying microorganisms were sufficient to allow normal nitrification; ammonium was converted rapidly to nitrate, which, in turn, was leached from the soil during normal watering operations. In soils associated with increased growth (E+32 hr or longer), however, nitrification was inhibited, and nitrogen remained almost entirely in the unleachable ammonium form. Nitrifying bacteria were not completely eliminated because nitrification eventually resumed in fumigated soils; the longer the fumigation, the longer the delay before nitrification resumed. Also, organic nitrogen was converted to inorganic nitrogen because of increased ammonification. This was shown when cellulose plus ammonium sulfate were added to soil before fumigation. In soils fumigated for less than E+16 hr, the inorganic nitrogen was converted by the biomass (primarily fungi) into organic nitrogen because of a high carbon:nitrogen ratio. When soil was fumigated for longer than E+16 hr, however, the carbon:nitrogen ratio was reduced as fungi were killed and ammonification took place. In other studies, the general fungal flora of soil was drastically reduced by fumigation for E+16 hr or longer (8). Therefore, more nitrogen was available to plants growing in fumigated soil (E+16 hr or longer) because of inhibited nitrification and increased ammonification. This was confirmed by the close correlation between the concentration of nitrogen in 35-day-old *N. glutinosa* plants grown in soil fumigated with various doses of MB and the amount of inorganic nitrogen (ammonium) in the same soils.

In practical terms, the increase of inorganic nitrogen after soil was fumigated for E+128 hr was equivalent to the addition of approximately 74 kg/ha of nitrogen. In nitrogen-deficient soils, such as used here, increased growth would be expected to be pronounced. However, increased growth probably would not be so pronounced in soils containing adequate amounts of nitrogen. Any improvements of field fumigation techniques that would increase the effective concentration of fumigant in the soil (and therefore increase the accumulation of ammonium) could be of significant financial benefit because of the high cost of nitrogen fertilizers.

Concentrations of other plant nutrients in 35-day-old *N. glutinosa* grown in soil fumigated for the full series of exposures were not closely correlated with increased plant growth. Whereas concentrations of copper, manganese, zinc, and phosphorus were greater in plants grown in soil fumigated for E+128 hr, concentrations in plants grown in soil fumigated for E+64 hr were not different from those in plants grown in soil fumigated for E hr. Increased growth was noted, however, when plants were grown in soil fumigated for E+16 hr or longer. Therefore, nutrient status of these four elements in plants grown in soil fumigated for E+64 hr is not believed to be involved in increased growth of the plants but may play a role in the increased growth of plants grown in soil fumigated for E+128 hr or longer.

Previous workers (3,9,12) have reported that increased plant growth after soil fumigation is due to a reduction in pathogenic fungi and an increase in inorganic nitrogen; this study confirms their results. Previous workers, however, were unable to differentiate the effects of fungal pathogens from those of increased nitrogen content. In this study, the effects of increased nitrogen content and reduced fungal pathogens were separated.

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