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Effects of Different Fumigants on the Replanted Soil Environment and Growth of *Malus hupehensis* Rehd. Seedlings

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Abstract. **Apple replant disease (ARD) has been reported in all major fruit-growing regions of the world and is often caused by biotic factors (pathogen fungi) and abiotic factors (phenolic compounds). Soil chemical fumigation can kill soil pathogenic fungi; however, the traditionally used fumigant methyl bromide has been banned because of its ozone-depleting effects. There is thus a need to identify greener fumigant candidates. We characterized the effects of different fumigants on the replanted soil environment and the growth characteristics of *Malus hupehensis* Rehd. seedlings. All five experimental treatments [treatment 1 (T1), metham-sodium; treatment 2 (T2), dazomet; treatment 3 (T3), calcium cyanamide; treatment 4 (T4), 1,3-dichloropropene; and treatment 5 (T5), methyl bromide] promoted significantly the biomass, root growth, and root respiration rate of *M. hupehensis* seedlings and the ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) contents of replanted soil. Metham sodium (T1) and dazomet (T2) had stronger effects compared with 1,3-dichloropropene (T4) and calcium cyanamide (T3). At 172 days after T1, the height, root length, and root respiration rate of *Malus hupehensis* Rehd. seedlings, and the NH_4^+-N and NO_3^--N contents of replanted soil increased by 91.64%, 97.67%, 69.78%, 81.98%, and 27.44%, respectively, compared with the control. Thus, dazomet and metham sodium were determined to be the optimal fumigants for use in practical applications.**

Apple replant disease (ARD) is an inevitable problem associated with the development of the modern apple industry (Sheng et al., 2020; Wang et al., 2014) that seriously restricts the development of China's apple industry (Sheng et al., 2020). Several studies have shown that the increase of pathogenic microorganisms in soil under replanted conditions is the main cause of ARD (Yim et al., 2013). Therefore, controlling soil pathogenic microorganisms is key to preventing and controlling ARD. Current methods used to control ARD include soil fumigation, the application of large amounts of organic materials, and biological control (Nicola et al., 2017). Because organic material resources are often lacking and the effect of biological control is not substantial, soil fumigation has generally been the most effective method for preventing and controlling ARD (Ren et al., 2018).

Methyl bromide, a chemical fumigant that is also an effective disinfectant, has been banned globally because of its ozone-depleting effects (Hoffmann et al., 2020). Since then, several chemical fumigants have appeared on the market to replace methyl bromide, such as benzamide, sodium methylamine, and 1,3-dichloropropene (Yan et al., 2017). Metham sodium, a type of methylamine derivative that can decompose in moist soil to produce methyl isothiocyanate; block the division of biological cells as well as DNA, RNA, and protein synthesis; and has toxic effects (Bangarwa et al., 2009), is commonly used to control soil-borne diseases in fields of vegetables, peanuts, tobacco, and other crops (Frank et al., 1986). Pinkerton et al. (2000) found that metham sodium reduced significantly the incidence of *Phytophthora infestans* and verticillium wilt pathogens in eggplant. In addition, soil fumigation using metham sodium could also reduce root swelling and improve plant health (Hwang et al., 2017), control effectively corky ringspot of potato tuber and potato wilt disease caused by replanting, and improve tuber quality (Yellareddygari and Gudmestad, 2018). Dazomet, a broad-spectrum soil fungicide, is widely used to prevent and control soil-borne diseases such as ginger stem rot, *Phytophthora capsica*, and tomato root-end nematode, and protects crops from a series of pathogenic bacteria through the release of methyl isothiocyanate (Mao et al., 2014; Ślusarski and Pietr, 2009). Meng et al. (2019) found that dazomet fumigation affected primarily the characteristics of fungi, and could reduce the number of *Fusarium oxysporum*—a pathogenic bacterium—and improve the yield of watermelon. Chen et al. (2019) found that dazomet fumigation reduced significantly the number of bacteria and fungi in replanted soil and controlled chrysanthemum fusarium wilt effectively. 1,3-Dichloropropene, a soil fumigant and nematicide, is used primarily for strawberries, sweetpotatoes, melons, flowers, and other crops before planting, soil fumigation, and processing; it has been shown to be effective in controlling worms, plant pathogenic bacteria, and weeds (Qiao et al., 2010). Qiao et al. (2012) found that tomato root-knot nematode disease was reduced significantly, and the growth of tomatoes was promoted, after fumigating soil with 1,3-dichloropropene. Calcium cyanamide is an alkaline nitrogen fertilizer with insecticidal and herbicidal effects. Calcium cyanamide fumigation can optimize the replanted soil environment, improve disease resistance and yield, and promote the growth of cucumber (Bletsos, 2010). Fan et al. (2016) found that calcium cyanamide fumigation could alleviate strawberry replant disease effectively.

To date, most studies have focused on the control of nematodes and weeds by fumigants, especially in cash crops such as vegetables and flowers (Ibekwe, 2004). In contrast, few studies have focused on the prevention and reduction of ARD and compared the efficacy of different fumigants. In our study, apple rootstock *Malus hupehensis* seedlings were used in pot experiments to examine the effects of five fumigants on the biological characteristics of the soil microbial environment and *M. hupehensis* seedlings. In general, the goal of this study was to

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determine the most suitable fumigant for ARD control and to provide guiding information to aid in the selection of appropriate fumigants for treating soil for apple tree replanting.

Materials and Methods

Experimental materials and treatments

The experiment was conducted in the College of Horticulture Science and Engineering, Shandong Agricultural University, National Apple Engineering Technology Research Center in 2019 (lat. 36.16°N, long. 117.15°E). The soil was obtained from a 34-year-old apple orchard at Songjiezhuang village (lat. 36.09°N, long. 117.04°E) in Tai'an City, Shandong. The root stock was *Malus micromalus* Makino, and test soil was taken randomly from a 10- to 40-cm depth after removing the topsoil. Multipoint random sampling was used to sample the soil, which was then mixed evenly and air-dried before use. The soil type was brown loam, and its basic physical and chemical properties are summarized in Table 1. The collected soil was treated by five fumigants (Table 2).

Malus hupehensis Rehd. seedlings were used in experiments. The seeds of *M. hupehensis* Rehd. were stratified at 4 °C for ≈40 d. After the seeds became white, they were seeded in the seedling tray in Mar. 2019.

On 17 Apr. 2019, fumigants and soil at the recommended dosage were mixed and sealed for 15 d to fumigate the soil (Table 3). The plastic film was then opened so that the treated soil could be air-dried for 7 d (Li et al., 2016), after which the soil was turned regularly to remove the residual fumigant thoroughly. These experiments were conducted under natural conditions. On 9 May 2019, the seedlings grew five true leaves, and seedlings showing the same growth trend were transplanted to different treatments of clay tile pots (upper inner diameter, 25 cm; lower inner diameter, 17 cm; height, 18 cm), with 7 kg of soil in each basin. Thirty pots were used for each treatment, and two seedlings were planted in each pot for unified fertilizer and water management. Soil samples were collected and measured at 0 d (the day when the seedlings were exposed to fumigant), 9 d (2 d after seedlings were planted), 52 d (45 d after seedlings were planted), 112 d (105 d after seedlings were planted), and 172 d (165 d after seedlings were planted) after fumigation. Three pots were selected randomly for each treatment for use as three replicates. During sampling, the soil was removed around the basin and the surface layer, sifted through a 2-mm sieve, and placed into three sealed bags. One bag was stored in a refrigerator at 4 °C to measure the quantity of microbes and soil nutrient NO_3^--N and NH_4^+-N contents. Another bag was placed quickly into liquid nitrogen and stored at –80 °C for DNA extraction and real-time fluorescence quantitative polymerase chain reaction (PCR) analysis. The last bag was naturally air-dried to determine soil enzyme activity. At the same time, three seedlings of each treated *M. hupehensis* Rehd. were collected at 52 d, 112 d, and 172 d after fumigation. After being washed, samples were subjected to root scanning, and biomass and root respiration rate measurements were taken.

NO_3^--N and NH_4^+-N contents in soil

Samples were taken at 52 d (45 d after seedlings were planted), 112 d (105 d after seedlings were planted), and 172 d (165 d after seedlings were planted) after fumigation. At each sampling event, rhizosphere soil samples of three *M. hupehensis* Rehd. seedlings were collected from each treatment, placed immediately into zippered bags, and frozen at –20 °C. After leaching 6 g of soil with 50 mL potassium chloride (1 mol·L^{-1}) solution, the contents of NO_3^--N and NH_4^+-N in the soil were determined using an AA3 automatic flow analyzer (Xue et al., 2017).

Soil enzyme activity

Soil urease activity. Soil urease activity was determined by conducting a colorimetric assay using sodium phenate–sodium hypochlorite (Du et al., 2017). First, 5-g, air-dried soil samples were weighed in a 50-mL triangulated flask, and 1 mL toluene was added, followed by shaking until the contents were mixed evenly. After 15 min, 10 mL of 10% urea solution and citrate buffer solution were added, followed by shaking and incubation at 37 °C for 24 h. After culture, the filtrate was filtered, and 1 mL of filtrate was added to a 50-mL volumetric flask. Next, 4 mL sodium phenol solution and 3 mL sodium hypochlorite solution were added and shaken well. After 20 min, the mixture was diluted to the 50-mL mark, and the spectrophotometer was colorimetric at 578 nm (the blue of indophenol remained stable). Urease activity was calculated by subtracting the absorbance value of the sample from the difference in the absorbance value of the control sample, and the ammonia nitrogen (NH_4^+-N) content was calculated according to the standard curve.

The activity of urease was represented by the NH_3-N content (measured in milligrams) in 1 g of soil after 24 h. The formula for determining soil urease activity is as follows:

$$\text{Urease} = \frac{a \times V \times n}{m},$$

where a is the concentration of NH_4^+-N obtained from the standard curve (measured in milligrams per milliliter), V is the volume of the chromatic liquid (50 mL); n is the separation multiple; and m is the weight of the drying soil (measured in grams).

Soil phosphatase activity. Soil phosphatase activity was determined by conducting a colorimetric assay with disodium phenyl phosphate (Du et al., 2017). First, 5-g, air-dried soil samples were placed in a 200-mL triangulation flask, and 2.5 mL of toluene was added. After shaking for 15 min, 20 mL of 0.5% benzene–disodium phosphate was added. After shaking, the samples were placed in an incubator and cultured at 37 °C for 24 h. Next, 100 mL of 0.3% aluminum sulfate solution was added to the culture medium and filtered. Three milliliters of filtrate was then absorbed into 50-mL volumetric bottles, and 5 mL of buffer solution and four drops of chlorodibromo-p-benzoquinone imines reagent were added to each bottle. After color development, the solution was diluted to the scale, and the colorimetric determination was conducted 30 min later. The boric acid buffer was blue and colorimetric at 660 nm on the spectrophotometer. To draw the standard curves, 1, 3, 5, 7, 9, 11, and 13 mL of phenolic working fluids were taken for color development and volume determination. After color stability was achieved, the standard curve was drawn with the colorimetric method. Phosphatase activity was expressed in phenolic micrograms per gram of soil.

The activity of phosphatase was represented by the phenol content (measured in milligrams) in 1 g of soil after 24 h. The formula for determining soil phosphatase activity is as follows:

$$\text{Phosphatase} = \frac{a \times V \times n}{m},$$

where a is the concentration of phenol obtained from the standard curve (measured in milligrams per milliliter), V is the volume of the chromatic liquid (50 mL); n is the separation multiple, and m is the weight of the drying soil (measured in grams).

Soil sucrase activity. Soil sucrase activity was determined by conducting a colorimetric assay with 3,5-dinitrosalicylic acid (Du et al., 2017). First, 5-g, air-dried soil samples were placed in a 50-mL triangulated flask, and 10 mL of 1% starch solution was injected. This was followed by the addition of 10 mL of pH 5.6 phosphate buffer solution and five drops of toluene, shaking, and storage in an incubator. The samples were then cultured at 37 °C for 24 h. After culture, the suspension was filtered. Next, 1 mL of filtrate was poured into a 50-mL volumetric flask. Two milliliters of 3, 5-dinitrosalicylic acid solution was added and heated in a boiling water bath for 5 min; the solution was then moved to the volumetric flask and placed in running water

Table 1. Basic characteristics of the soil.

Soil texture	NO_3^--N (mg·kg^{-1})	NH_4^+-N (mg·kg^{-1})	Available K (mg·kg^{-1})	Available P (mg·kg^{-1})	Organic matter (g·kg^{-1})	pH
Brown loam	40.2	6.3	86.5	11.3	9.8	6.34

NH_4^+-N = ammonium nitrogen; NO_3^--N = nitrate nitrogen; K = potassium; P = phosphorus.

Table 2. Costs of different fumigant treatments.

Treatment	Name of fumigant	Fumigation agents ($/hm^2)	Plastic film ($/hm^2)	Total ($/hm^2)
T1	Metham sodium	183.7	459.3	643.0
T2	Dazomet	1,837.2	459.3	2,296.5
T3	Calcium cyanamide	344.5	459.3	803.8
T4	1,3-Dichloropropene	803.8	459.3	1,263.1
T5	Methyl bromide	2,755.8	459.3	3,215.1

to cool. After a constant volume of 50 mL was achieved, colorimetry was performed at 508 nm on a spectrophotometer. Glucose solution was used as the standard.

The activity of sucrase was represented by the glucose content (measured in milligrams) in 1 g of soil after 24 h. The formula for determining soil sucrase activity is as follows:

$$\text{Sucrase} = \frac{a \times V \times n}{m},$$

where a is the concentration of glucose obtained from the standard curve (measured in milligrams per milliliter), V is the volume of the chromatic liquid (50 mL), n is the separation multiple, and m is the weight of the drying soil (measured in grams).

Soil catalase activity. The soil catalase activity was determined by potassium permanganate ($KMnO_4$) titration (Du et al., 2017). First, 2-g, air-dried soil samples were placed in a 100-mL trigonometric bottle and injected with 40 mL of distilled water and 5 mL of 0.3% hydrogen peroxide (H_2O_2) solution. A control (40 mL of distilled water and 5 mL of 0.3% H_2O_2 solution) was injected into a triangular bottle without the addition of soil samples. The triangular bottle was shaken on a shaking machine for 20 min, and 5 mL of 3N sulfuric acid was added to stabilize the undecomposed H_2O_2. The suspension in the bottle was then filtered with a slow filter paper, followed by absorption of 25 mL of filtrate and titration with 0.1 N $KMnO_4$ to the light-pink terminal point.

The activity of catalase was represented by volume (measured in milliliters) of 0.1 N $KMnO_4$ in 1 g of soil after 20 min. The formula for determining soil catalase activity is as follows:

$$\text{Catalase} = (A - B) \times T,$$

where B is the amount of $KMnO_4$ (measured in milliliters) consumed for titrating the soil filtrate (measured in milliliters), A is the amount of $KMnO_4$ (measured in milliliters) consumed for titrating 25 mL of the original H_2O_2 mixture (measured in milliliters), and T is the correction value for $KMnO_4$ titration.

Soil culturable microorganisms

Soil microbial numbers were determined by a conventional plate count method. Bacteria were cultured in beef extract peptone medium (containing beef extract, peptone, and sodium chloride). The plates were inverted and cultured at 28 °C for 24 to 48 h before observing and counting colonies. Fungi were cultured in Martin medium (containing potassium dihydrogen phosphate, anhydrous magnesium sulfate, and peptone). The plates were inverted and cultured at 28 °C for 48 to 72 h before observing and counting colonies. Actinomycetes were cultured with Gauze's Medium No. 1 (containing potassium nitrate, dipotassium hydrogen phosphate, and anhydrous magnesium sulfate). The plates were inverted and cultured at 28 °C for 72 to 96 h before observing and counting colonies. The soil microbial number per gram of dry soil (colony-forming unit) = Average number of colonies × Dilution factor/dry weight of soil sample.

Soil microbial community structure

The extraction and purification of total DNA from the sample genome was performed per the instructions of the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA). The CFX96™ Connect real-time quantitative PCR system (BIO-RAD, Hercules, CA) was used to analyze the copy number of *Fusarium oxysporum* genes in the soil by real-time quantitative PCR. The system was implemented per the instructions of the SYBR Premix Ex TaqTM Kit (TaKaRa Biotech Co., Ltd., Dalian, China). Each reaction with the 25-μL PCR system included 1.5 μL DNA template, 12.5 μL SYBR Premix Ex TaqII (TaKaRa Biotech Co.), 1 μL of each primer; and 9 μL double distilled water. Reaction procedures involved predenaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s, for a total of 40 cycles.

Plant biomass

Samples were taken at 52 d (45 d after seedlings were planted), 112 d (105 d after seedlings were planted), and 172 d (165 d after seedlings were planted) after fumigation. At each sampling event, three *M. hupehensis* Rehd. seedlings were taken for each treatment and washed in clean water. Measurements of seedling height, ground diameter, and fresh weight were then acquired by using a ruler, vernier calipers, and electronic scale, respectively. After measurements, *M. hupehensis* Rehd. seedlings were wrapped tightly in paper bags and placed in a constant-temperature oven. After drying, seedlings were removed carefully and the dry mass was measured with an electronic scale.

Root configuration parameters

Samples were taken at 52 d (45 d after seedlings were planted), 112 d (105 d after seedlings were planted), and 172 d (165 d after seedlings were planted) after fumigation. At each sampling event, three *M. hupehensis* Rehd. seedlings were taken for each treatment. Roots were then washed in clean water, laid flat on a hard plastic container, and spread out in the water. The WinRHIZO root analysis system (version 2007; Seiko Epson Corporation, Nagano Prefecture, Japan) was then used to measure the root length, total volume, total surface area, and root tip number of seedlings from the sample images.

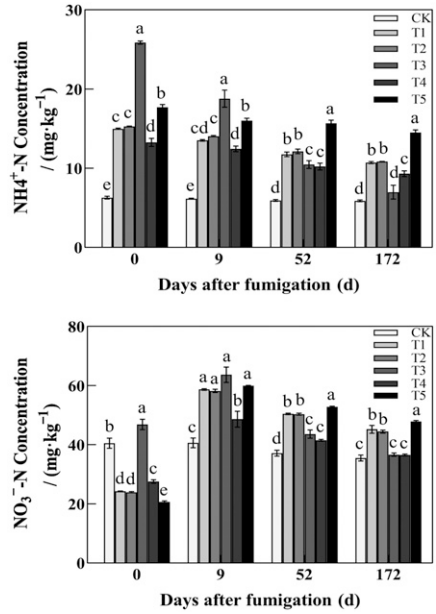

Fig. 1. Effects of ammonium nitrogen (**top**) and nitrate nitrogen (**bottom**) content in soil under different fumigant treatments at each sampling event. The number of days after fumigation starts the day when the plastic film was opened. Note that 0 d, 9 d, 52 d, and 172 d refer to 0 d, 9 d, 52 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 0 d, 2 d, 45 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted. Data are mean ± SE (n = 3). Values in the same column marked with the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple range test. NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; CK, control check; T1, metham sodium; T2, dazomet; T3, calcium cyanamide; T4, 1,3-dichloropropene; T5, methyl bromide.

Root respiration rate

Samples were taken at 52 d (45 d after seedlings were planted), 112 d (105 d after seedlings were planted), and 172 d (165 d after seedlings were planted) after fumigation. At each sampling event, three *M. hupehensis* Rehd. seedlings were taken for each treatment. Roots were then washed in clean water, and 0.5 g of fresh white roots was taken from the seedling roots and divided

evenly into 0.1-cm segments. An Oxytherm oxygen electrode (Hansatech Instruments Ltd, Pentney, UK) was used to determine the root respiration rate.

Statistical analysis

The experimental data were calculated and plotted using Microsoft Excel 2003. Analysis of variance was performed using SPSS19.0 software (version 19.0; SPSS Inc., Chicago, IL), and significant differences were detected by Duncan's new complex range method.

Results

NH_4^+-N and NO_3^--N content in soil

On the day after exposure, the NH_4^+-N content of each treatment differed significantly from the continuous control (Fig. 1). The NH_4^+-N content of T1, T2, T3, T4, and T5 was increased by 1.38%, 1.43%, 3.12%, 1.11%, and 1.82%, respectively, compared with the control. At 9 d, 52 d, and 172 d after fumigation, the NH_4^+-N content of each treatment was consistently greater than that of the control, and differences between the treatments and the control were significant. On the day after exposure, the content of NO_3^--N in T1, T2, T3, T4, and T5 was 0.60 times, 0.59 times, 1.16 times, 0.68 times, and 0.51 times greater, respectively, compared with the control. Nine days after fumigation, the NO_3^--N content of each treatment increased; specifically, the content of (bromomethane) NO_3^--N for T1, T2, T3, T4, and T5 was 1.45 times, 1.43 times, 1.57 times, 1.20 times, and 1.48 times greater, respectively, than that of the control. The NO_3^--N content of each treatment decreased over time, and the NO_3^--N content was always greater than the control.

Soil enzyme activity

The enzyme activity of soil treated with the five fumigants was significantly less than that of the control group (Fig. 2). The most obvious inhibitory effect on urease activity was observed in T5, followed by T2, T1, T4, and T3. On the first day after exposure, the urease activity of T1, T2, T3, T4, and T5 decreased by 58.66%, 80.24%, 13.10%, 52.02%, and 98.87%, respectively, compared with that of the control group, and differences between each treatment and the control were significant. The urease enzyme activity was restored gradually 52 d, 112 d, and 172 d after fumigation.

The day after the plastic film was removed, the H_2O_2 enzyme activity in T1, T2, T3, T4, and T5 was reduced by 37.45%, 28.54%, 17.45%, 23.82%, and 46.88%, respectively, compared with the control. The H_2O_2 enzyme activity was restored gradually 52 d, 112 d, and 172 d after fumigation.

On the day after exposure, the phosphatase activity of each treatment was reduced by 48.50%, 50.72%, 36.23%, 42.92%, and 77.48%, respectively, compared with the control group. At 172 d after fumigation, there was no significant difference in phosphatase activity between T3 and the control group, and the phosphatase activity of each treatment was still in a state of recovery and increasing. The pattern of change in the sucrase activity and phosphatase activity was nearly the same.

Number of microorganisms

The five fumigant treatments reduced significantly the number of soil fungi and bacteria (Table 4). The number of soil bacteria and fungi increased with time, and the growth rate of bacteria was more rapid than that of fungi. On the day after exposure, the number of bacteria, the number of fungi, and the ratio of bacteria to fungi between treatments differed significantly from the control. At 112 d after fumigation, the number of bacteria in T1, T2, T3, T4, and T5 was increased by 51.17%, 58.14%, 20.93%, 41.86%, and 102.33%, respectively, compared with the control. At 172 d after fumigation, the number of bacteria and fungi in each treatment continued to increase, and each treatment still differed significantly from the control.

Gene copies of *Fusarium oxysporum*

The copy number of *Fusarium oxysporum* genes in the soil at different periods was analyzed using real-time PCR. Each treatment reduced significantly the soil *Fusarium oxysporum* gene copy number, including sharp spores, 0 d after fumigation (Fig. 3). For example, for T1, T2, T3, T4, and T5, the gene copy number

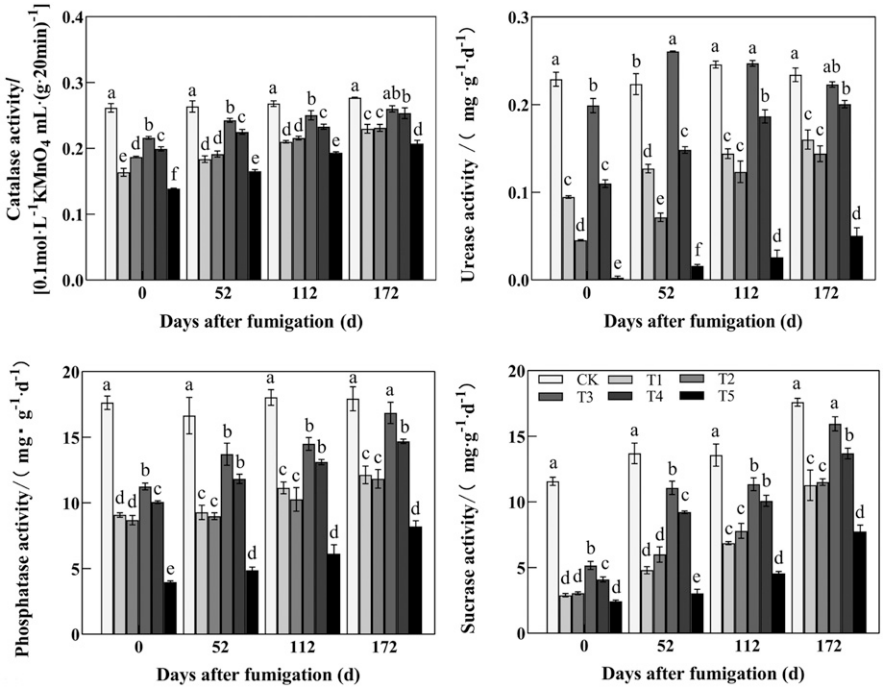

Fig. 2. Effects of different fumigant treatments on soil enzyme activities at each sampling event. (**Upper left**) Soil catalase activity. (**Upper right**) Soil urease activity. (**Lower left**) Soil phosphatase activity. (**Lower right**) Soil sucrase activity. The number of days after fumigation starts the day when the plastic film was opened. Note that 0 d, 52 d, 112 d, and 172 d refer to 0 d, 52 d, 112 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 0 d, 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted. Data are mean ± SE (n = 3). Values marked with the same letter within a sampling event are not significantly different at $P < 0.05$ according to Duncan's new multiple range test. $KMnO_4$, potassium permanganate; CK, control check; T1, metham sodium; T2, dazomet; T3, calcium cyanamide; T4, 1,3-dichloropropene; T5, methyl bromide.

Table 3. Name of fumigant, source, dosage form, and recommended concentration under different fumigant treatments.

Treatment	Name of fumigant	Dosage form	Recommended concn (kg·hm^{-2})	Source
T1	Metham sodium	Agent	90.0	Jiangsu LIMIN Chemical Co., Ltd.
T2	Dazomet	Particle agent	600.0	Jiangsu Nantong Shizhuang Chemical Co., Ltd.
T3	Calcium cyanamide	Granules	1500.0	Ningxia Jiafeng Chemical Co., Ltd.
T4	1,3-Dichloropropene	EC reagent	450.0	Hunan Levante Chemical Co., Ltd.
T5	Methyl bromide	Gas preparation	—	Jiangsu Lianyungang Dead Sea Bromide Co., Ltd.

Table 4. Effects of different fumigant treatments on the number of microorganisms at each sampling event.

Day	Treatment	Bacteria ($\times 10^5$ cfu/g)	Fungi ($\times 10^3$ cfu/g)	Bacteria/fungi
0[z] d	CK	12.33 ± 0.33[y] a[x]	33.00 ± 2.52 a	37.64 ± 1.75 c
	T1	2.67 ± 0.67 d	1.33 ± 0.33 d	200.00 ± 0.00 a
	T2	3.00 ± 0.58 d	1.00 ± 0.58 d	133.33 ± 66.67 ab
	T3	8.00 ± 0.58 b	12.33 ± 0.67 b	64.80 ± 2.29 bc
	T4	6.00 ± 0.58 c	7.00 ± 0.58 c	86.31 ± 8.27 bc
	T5	0.67 ± 0.33 e	0.33 ± 0.33 d	0.00 ± 0.00 c
52 d	CK	12.33 ± 0.33 bc	36.00 ± 2.65 a	34.64 ± 2.69 c
	T1	12.67 ± 0.33 b	5.33 ± 0.33 c	238.89 ± 12.52 b
	T2	11.67 ± 1.33 bc	4.33 ± 0.67 c	273.33 ± 13.33 b
	T3	10.33 ± 0.33 c	15.00 ± 0.58 b	69.09 ± 3.34 c
	T4	10.67 ± 0.33 bc	11.33 ± 1.33 b	96.19 ± 9.27 c
	T5	14.67 ± 0.33 a	2.33 ± 0.33 c	655.56 ± 94.44 a
112 d	CK	14.33 ± 0.33 d	45.33 ± 2.73 a	31.83 ± 1.86 c
	T1	21.67 ± 0.33 b	7.67 ± 0.33 d	283.93 ± 15.60 b
	T2	22.67 ± 0.88 b	7.33 ± 0.67 d	315.28 ± 35.71 b
	T3	17.33 ± 0.33 c	24.33 ± 0.88 b	71.32 ± 1.37 c
	T4	20.33 ± 1.33 b	17.67 ± 1.20 c	115.17 ± 2.02 c
	T5	29.00 ± 1.53 a	4.33 ± 0.33 d	681.67 ± 81.25 a
172 d	CK	14.00 ± 0.58 d	50.00 ± 0.58 a	27.99 ± 1.03 c
	T1	34.00 ± 0.58 b	11.67 ± 0.33 d	292.17 ± 13.23 b
	T2	35.00 ± 0.58 b	10.00 ± 0.58 d	352.73 ± 24.46 b
	T3	29.33 ± 0.33 c	29.67 ± 0.88 b	99.00 ± 2.28 c
	T4	31.33 ± 0.33 bc	12.33 ± 0.88 c	140.75 ± 5.82 c
	T5	46.00 ± 3.21 a	6.33 ± 0.33 e	734.92 ± 83.98 a

[z]The number of days after fumigation starts the day when the plastic film was opened. Note that 0 d, 52 d, 112 d, and 172 d refer to 0 d, 52 d, 112 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 0 d, 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted.
[y]Data are mean ± SE (n = 3).
[x]Values in the same column marked with the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple range test.
cfu = colony-forming unit; CK = control check; T1 = metham sodium; T2 = dazomet; T3 = calcium cyanamide; T4 = 1,3-dichloropropene; T5 = methyl bromide.

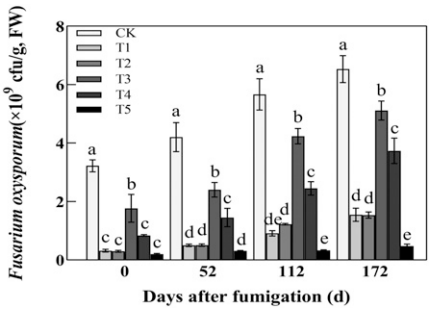

Fig. 3. Effects of different fumigant treatments on the gene copies of *Fusarium oxysporum* at each sampling event. The number of days after fumigation starts the day when the plastic film was opened. Note that 0 d, 52 d, 112 d, and 172 d refer to 0 d, 52 d, 112 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 0 d, 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted. Data are mean ± SE (n = 3). Values marked with the same letter within a sampling event are not significantly different at $P < 0.05$ according to Duncan's new multiple range test. cfu, colony-forming unit; FW, fresh weight; CK, control check; T1, metham sodium; T2, dazomet; T3, calcium cyanamide; T4, 1,3-dichloropropene; T5, methyl bromide.

of *Fusarium oxysporum*, was reduced by 90.11%, 90.82%, 45.17%, 74.11%, and 93.80%, respectively, compared with the control. Over time, the pointed spores all tended to restore the *Fusarium oxysporum* gradually. The pointed spore number of the *Fusarium* copy methyl bromide treatment (T5) was always the lowest. In addition, the recovery rate was the slowest for T5 and was significantly different from the control, followed by T2, T1, T4, and T3.

Biomass of *Malus hupehensis* Rehd. seedlings

Soil fumigation promoted the growth of *M. hupehensis* Rehd. seedlings, and different fumigants had different effects on the observed growth at different stages (Table 5). At 52 d after the end of fumigation, the growth of plants under each treatment was significantly different from that of the control. The height of the *M. hupehensis* Rehd. seedlings under T1, T2, T3, T4, and T5 increased by 42.83%, 41.57%, 19.97%, 26.26%, and 88.73%, respectively, compared with the control. At 112 d and 172 d after fumigation, the growth of plants under each treatment was still significantly different from that of the control. At 172 d after the end of fumigation, the height of the *M. hupehensis* Rehd. seedlings under T1, T2, T3, T4, and T5 increased by 91.64%, 85.34%, 39.00%, 28.88%, and 110.5%, respectively.

Root growth of *Malus hupehensis* Rehd. seedlings

Different fumigant treatments promoted the root growth of *M. hupehensis* Rehd. seedlings to different degrees (Table 6). After 52 d of fumigation in T1, T2, T3, T4, and T5, the root length of the seedlings increased by 82.37%, 81.72%, 62.00%, 69.03%, and 117.50%, respectively, compared with the control. The root surface, root volume, and root tip also increased significantly compared with the control. After 112 d and 172 d of fumigation, the root growth of *M. hupehensis* Rehd. seedlings under each treatment tended to be stable, but still differed significantly from the control.

Figure 4 shows that the five fumigation agents significantly improved the *M. hupehensis* Rehd. seedling root respiration rate 52 d after fumigation. The root respiration rate for T1 and T2 was significantly different from that of the control, but the root respiration rate for T1 and T2 did not differ significantly. At 112 d after fumigation, each of the five treatments differed significantly from the control with regard to root respiration rate; however, T1 and T2 did not differ significantly. The pattern in the root respiration rate observed at 172 d after fumigation was the same as that observed at 112 d after fumigation.

Discussion

Several years of agricultural production have shown that soil fumigation is the most effective measure for alleviating replant disease (Ren et al., 2018). On the one hand, soil fumigation can improve the soil environment by altering microbial community structure (Su et al., 2020); on the other hand, it can promote the growth of plants by improving soil physicochemical properties (Shao et al., 2015).

Soil physicochemical properties

Plant growth is related closely to the soil environment. Soil enzyme activity is an important factor for evaluating the level of

Table 5. Effects of different fumigants on the biomass of *Malus hupehensis* Rehd. seedlings at each sampling event.

Days	Treatment	Plant ht (cm)	Ground diam (mm)	Fresh wt (g)	Dry wt (g)
52[z] d	CK	15.88 ± 0.25[y] d[x]	2.32 ± 0.01 e	19.88 ± 0.23 d	9.87 ± 0.11 d
	T1	22.68 ± 0.56 b	2.74 ± 0.05 b	27.01 ± 0.55 b	13.25 ± 0.27 b
	T2	22.48 ± 0.39 b	2.63 ± 0.02 bc	26.81 ± 0.67 b	13.15 ± 0.08 b
	T3	19.05 ± 0.36 c	2.45 ± 0.08 d	24.21 ± 0.95 c	12.05 ± 0.48 c
	T4	20.05 ± 0.10 c	2.53 ± 0.04 cd	24.38 ± 0.40 c	12.06 ± 0.19 c
	T5	29.96 ± 1.05 a	3.23 ± 0.02 a	33.96 ± 1.06 a	16.79 ± 0.53 a
112 d	CK	35.42 ± 1.24 e	3.62 ± 0.01 e	39.42 ± 1.24 e	19.04 ± 0.77 e
	T1	62.72 ± 0.23 b	8.21 ± 0.01 b	68.46 ± 0.32 b	33.16 ± 0.52 b
	T2	60.19 ± 1.12 b	8.14 ± 0.06 b	65.05 ± 1.47 b	31.49 ± 0.57 b
	T3	41.17 ± 1.15 d	3.79 ± 0.01 d	44.84 ± 0.88 d	21.33 ± 0.37 d
	T4	46.85 ± 2.44 c	4.07 ± 0.02 c	50.85 ± 2.44 c	24.65 ± 0.85 c
	T5	68.12 ± 1.76 a	9.13 ± 0.02 a	74.65 ± 0.58 a	35.46 ± 0.24 a
172 d	CK	45.10 ± 3.06 e	3.95 ± 0.02 e	49.10 ± 3.05 e	24.30 ± 1.49 e
	T1	86.43 ± 0.52 b	11.32 ± 0.01 b	91.68 ± 1.45 b	45.09 ± 1.00 b
	T2	83.59 ± 0.57 b	11.21 ± 0.08 b	87.42 ± 1.33 b	43.06 ± 0.54 b
	T3	62.69 ± 4.06 d	8.44 ± 0.02 d	66.69 ± 4.07 d	32.86 ± 2.10 d
	T4	73.98 ± 4.13 c	10.05 ± 0.03 c	77.98 ± 4.15 c	38.51 ± 1.98 c
	T5	94.94 ± 0.39 a	12.05 ± 0.02 a	104.96 ± 2.06 a	51.92 ± 1.03 a

[z]The number of days after fumigation starts the day when the plastic film was opened. Note that 52 d, 112 d, and 172 d refer to 52 d, 105 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted.
[y]Data are mean ± SE (n = 3).
[x]Values in the same column marked with the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple range test.
CK = control check; T1 = metham sodium; T2 = dazomet; T3 = calcium cyanamide; T4 = 1,3-dichloropropene; T5 = methyl bromide.

Table 6. Effects of different fumigant treatments on the root growth of *Malus hupehensis* Rehd. seedlings at each sampling event.

Days	Treatment	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Tips per seedling
52[z] d	CK	1,040.20 ± 22.02[y] d[x]	635.63 ± 33.71 d	9.71 ± 0.33 d	1,502.97 ± 63.57 d
	T1	1,897.00 ± 16.37 b	822.67 ± 10.52 b	16.66 ± 1.09 b	2,367.50 ± 94.96 b
	T2	1,890.27 ± 46.03 b	817.86 ± 10.46 b	16.27 ± 0.82 b	2,320.88 ± 62.69 b
	T3	1,685.12 ± 24.12 c	716.18 ± 12.07 c	13.19 ± 0.35 c	1,963.44 ± 79.29 c
	T4	1,758.27 ± 30.16 c	720.14 ± 19.97 c	13.99 ± 0.06 c	2,039.08 ± 87.48 c
	T5	2,262.40 ± 75.48 a	897.21 ± 26.57 a	24.00 ± 1.84 a	2,961.78 ± 133.85 a
112 d	CK	2,596.49 ± 85.28 e	1,018.72 ± 16.16 e	26.57 ± 0.64 e	3,207.84 ± 120.52 e
	T1	4,784.35 ± 66.52 b	1,282.64 ± 10.32 b	61.65 ± 0.81 b	6,604.86 ± 103.93 b
	T2	4,649.96 ± 106.01 b	1,246.47 ± 15.92 b	59.36 ± 1.48 b	6,405.56 ± 199.92 b
	T3	3,458.50 ± 79.48 d	1,076.80 ± 14.95 d	29.50 ± 0.55 d	4,087.13 ± 139.45 d
	T4	3,751.01 ± 35.75 c	1,201.77 ± 10.45 c	32.44 ± 0.34 c	4,907.83 ± 142.12 c
	T5	5,351.54 ± 95.06 a	1,333.48 ± 16.85 a	64.75 ± 1.22 a	7,138.28 ± 219.55 a
172 d	CK	3,686.65 ± 55.48 e	1,133.97 ± 15.25 e	31.45 ± 1.74 e	4,556.90 ± 132.02 e
	T1	7,287.38 ± 115.97 b	1,646.83 ± 31.01 b	75.24 ± 1.36 b	8,341.04 ± 205.02 b
	T2	7,154.94 ± 101.06 b	1,627.06 ± 52.28 b	71.15 ± 1.16 bc	8,174.62 ± 185.29 b
	T3	4,775.10 ± 60.15 d	1,298.67 ± 19.65 d	61.37 ± 1.58 d	6,187.09 ± 136.95 d
	T4	5,787.90 ± 97.70 c	1,475.43 ± 16.55 c	66.61 ± 1.02 c	7,424.24 ± 251.65 c
	T5	7,953.03 ± 86.78 a	1,817.60 ± 33.69 a	83.31 ± 2.05 a	9,145.91 ± 418.39 a

[z]The number of days after fumigation starts the day when the plastic film was opened. Note that 52 d, 112 d, and 172 d refer to 52 d, 105 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted.
[y]Data are mean ± SE (n = 3).
[x]Values in the same column marked with the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple range test.
CK = control check; T1 = metham sodium; T2 = dazomet; T3 = calcium cyanamide; T4 = 1,3-dichloropropene; T5 = methyl bromide.

materials in soil, energy metabolism, and soil quality (Shao et al., 2015). Du et al. (2017) found that soil enzyme activity was altered to different degrees after fumigants were applied, and different fumigants had different effects on soil enzyme activity. Nannipieri et al. (2012) reported that soil fumigation led to a decline in soil microbial activity, which resulted in a decrease in soil enzyme activity. However, soil enzyme activity recovered gradually with time. In our study, T1, T2, T3, T4, and T5 reduced significantly replanted soil urease, sucrase, phosphatase, and catalase activity; but, as time progressed, the magnitude of the increases differed. This pattern may stem from the fact that fumigation can kill soil microorganisms to some extent, but as the beneficial microbes recover, so does the enzyme activity. Among treatments, the slowest recovery was observed for T5, followed by T1, T2, T4, and T3. The strength of the fumigant affected microbial recovery and thus enzyme activity. However, after the application of calcium cyanamide (T3), the lack of a significant difference in urease activity among the treatments and the control may stem from the fact that calcium cyanamide contains large amounts of nitrogen. Indeed, studies have found that applying nitrogen fertilizer not only provides the soil urease required for enzymatic reactions and enhances soil urease activity, but also improves nitrogen nutrition for soil microbial growth and thus contributes to the production of urease.

Number of microorganisms and the gene copies of *Fusarium oxysporum*

The transformation of substances in soil is related closely to microorganisms, and the structure of the soil microbial community can reflect the quality of soil to a certain extent (Su et al., 2020). Long-term replanting leads to imbalances in soil microbial community structure, increases in pathogenic bacteria, and the transformation of soil microorganisms from bacteria dominant to fungus dominant (Braun et al., 2010). Several studies have indicated that *Fusarium*, *Columbaria*, *Saproterus*, and *Trichinella* are the main causes of successive cropping obstacles in apple-producing areas in the United States and Italy (Manici et al., 2003; Schoor et al., 2009). The main pathogens of ARD in the Bohai Gulf region and the Loess Plateau region of northwest China are *Fusarium* fungi, which are highly pathogenic to apple rootstock *M. hupehensis* Rehd. seedlings (Wang et al., 2018). Previous studies have found that fumigants such as methyl bromide

and bitter chloride have significant inhibitory effects on *Fusarium oxysporum* (Dangi et al., 2015). Our study assessed the effects of five types of fumigant (metham-sodium, dazomet, 1,3-dichloropropene, calcium cyanamide, and methyl bromide) on replanted soil by measuring the number of soil bacteria, fungi, and *Fusarium oxysporum*. The five fumigants reduced significantly the number of soil bacteria, fungi, and gene copy number of *Fusarium oxysporum*. This finding may stem from the fact that different soil fumigants tend to kill replanted soil microorganisms, which is consistent with the findings of Kim et al. (2020). Among these fumigants, the greatest reductions were induced by methyl bromide, metham-sodium, and dazomet, followed by 1,3-dichloropropene and calcium cyanamide. The number of bacteria, fungi, and the copy number of *Fusarium oxysporum* genes in each treatment recovered gradually with time. The efficacy of fumigant might reduce gradually, and the structure of the soil microbial community might recover gradually; however, the recovery time and recovery effect require further study.

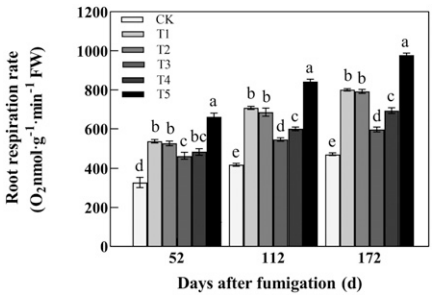

Fig. 4. Effects of different fumigant treatments on the root respiration rate of *Malus hupehensis* Rehd. The number of days after fumigation starts the day when the plastic film was opened. Note that 52 d, 112 d, and 172 d refer to 52 d, 112 d, and 172 d, respectively, since the opening of the plastic film, which corresponds to 45 d, 105 d, and 165 d, respectively, after *Malus hupehensis* Rehd. seedlings were planted. Data are mean ± SE (n = 3). Values marked with the same letter within a sampling event are not significantly different at $P < 0.05$ according to Duncan's new multiple range test. O_2, oxygen; CK, control check; T1, metham sodium; T2, dazomet; T3, calcium cyanamide; T4, 1,3-dichloropropene; T5, methyl bromide.

Plant morphological properties

After long-term replanting, the increase in soil pathogenic microorganisms, the accumulation of allelochemicals, and the imbalance of nutrients are all adverse factors restricting the growth of *M. hupehensis* Rehd. seedlings. Under these adverse conditions, the growth of plant roots is inhibited. As the center of metabolism of the underground part of plants, plant roots promote the growth of the aboveground part through the absorption of water and mineral elements (Suchoff et al., 2018). Wang et al. (2019) found that ARD inhibited root growth significantly, leading to significantly lower root length, root area, and root tip number relative to normal plants. However, all five fumigants in our study promoted the growth and root respiration rate of *M. hupehensis* seedlings. Thus, the five fumigants might have alleviated the adverse conditions of replanted soil, promoted the growth of plant roots, and improved aboveground growth. Methyl bromide was the most effective fumigant, followed by metam-sodium, dazomet, 1,3-dichloropropene, and calcium cyanamide.

NH_4^+-N and NO_3^--N content in soil

All types of mineral elements affect the growth of the root system (Miller et al., 2013). For example, the level of nitrogen is one of the most important factors affecting plant growth and development (Djidonou et al., 2019). Ayankojo et al. (2020) showed that NH_4^+-N and NO_3^--N are the main forms of nitrogen taken up by plant roots. As these forms of nitrogen can be transformed by nitrification and denitrification, nitrification is one of the most important processes in the process of soil nitrogen transformation. Apples are generally thought to be NO_3^--N plants, and the nitrogen uptake of both spring and summer plants is greater than that of NH_4^+-N (Gu et al., 1987). In our experiment, we found that the content of NH_4^+-N in the soil was increased after the fumigation treatment, whereas the content of NO_3^--N was decreased. This pattern might be explained by the fact that dead soil microorganisms decompose to produce a large amount of NH_4^+-N after fumigation (Yamamoto et al., 2008). In addition, soil fumigation might also depress microbiological nitrification and affect the conversion of NH_4^+-N to NO_3^--N, thus leading to the accumulation of NH_4^+-N and decreases in NO_3^--N (Yan et al., 2015). The contents of NH_4^+-N and NO_3^--N in each treatment generally showed a decreasing trend over time, which may stem from the fact that *M. hupehensis* Rehd. enters a flourishing growth period and its absorption capacity increases gradually. As a consequence, the contents of NH_4^+-N and NO_3^--N in the soil decrease; however, in our study, the concentrations of NH_4^+-N and NO_3^--N were always greater in the treatments than in the control.

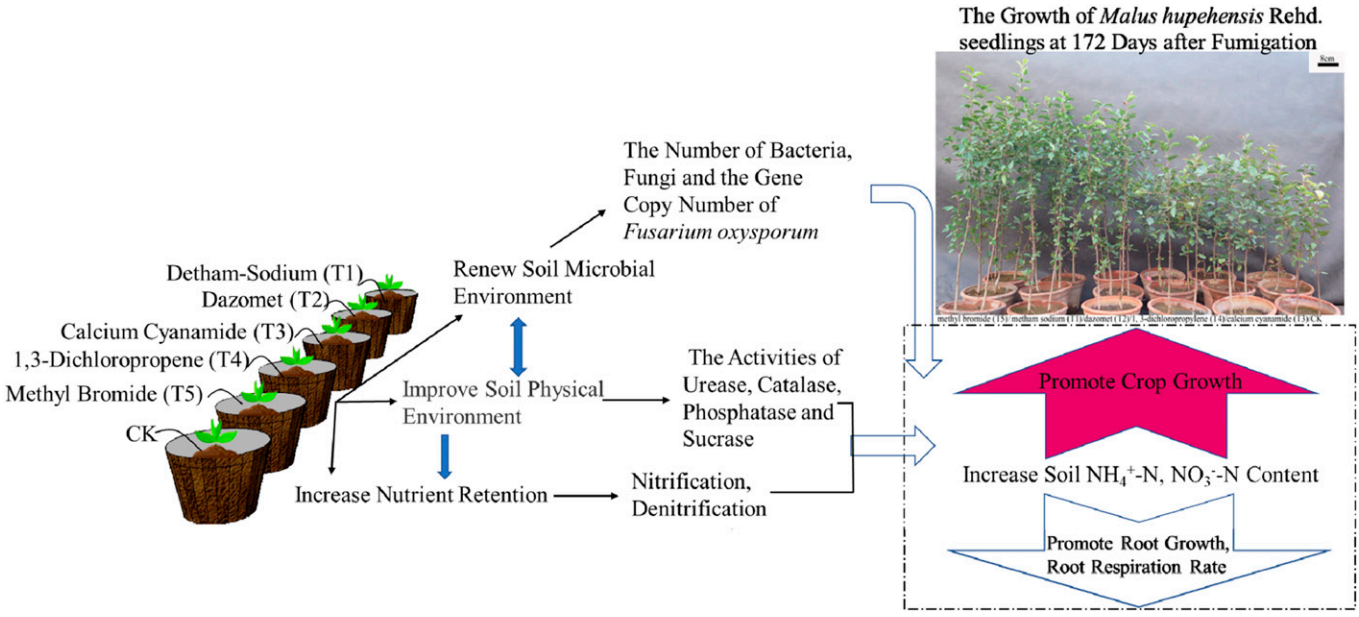

Fig. 5. Conceptual diagram depicting the effects of fumigants on the internal environment of the replanted soil of apple trees and the growth status of *Malus hupehensis* Rehd. seedlings 172 d after fumigation. NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; CK, control check; T1, metham sodium; T2, dazomet; T3, calcium cyanamide; T4, 1,3-dichloropropene; T5, methyl bromide.

Conclusions

We used five fumigants to treat the replanted soil of apple trees and investigated the effects of fumigants on the soil environment and the growth characteristics of *M. hupehensis* Rehd. seedlings. The results indicate that dazomet and metham-sodium had clear effects on the growth of *M. hupehensis* Rehd. and the soil environment, in addition to the banned fumigant methyl bromide. Furthermore, dazomet is more convenient to transport than metham-sodium, and metham-sodium is far cheaper than dazomet (Fig. 5). Thus, we recommend that metham-sodium or dazomet be used as needed to maximize production. In general, our experimental results provide guiding information that could be used to aid in the selection of suitable fumigants to treat the replanted soil of apple trees and thus control ARD. These findings also show promise in helping to promote the development of the apple industry.

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