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Integrated Management of Clubroot in Zhejiang Province, China

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Abstract: Clubroot, caused by *Plasmodiophora brassicae*, is a destructive soil-borne disease significantly harming global *Brassica* crop production. This study employed the Williams and European Clubroot Differential (ECD) and Williams systems to identify the pathotypes of *P. brassicae* collected from Hangzhou City, Yuhang District and Quzhou City, Kaihua County in Zhejiang Province. Greenhouse and field trials were conducted to evaluate the effects of plastic film covering and four chemical agents on the growth parameters and clubroot severity of the Chinese cabbage cultivar 'Granaat'. Potential treatment mechanisms on clubroot were explored through a qPCR analysis of the resting spore density and pH measurement of the soil. Furthermore, treatment with 1-napthaleneacetic acid (NAA), a synthetic auxin, was also evaluated for its potential role in suppressing clubroot. The results indicate that the pathotypes of *P. brassicae* in the two districts were P1, ECD20/31/12, and P3, ECD20/15/4. While an individual application of plastic film covering could not effectively control clubroot, calcium cyanamid, dazomet and ammonium bicarbonate demonstrated significant efficacy in its management. These three agents significantly reduced the resting spore density in the soil, with calcium cyanamid and ammonium bicarbonate also increasing soil alkalinity. Additionally, ammonium bicarbonate promotes lateral root development in 'Granaat,' helping infected plants access adequate water and nutrients. However, NAA exhibited no efficacy in clubroot control. Therefore, sustained lateral root development is crucial for effectively resisting *P. brassicae* invasion. Considering application costs and environmental friendliness, we propose the field application of ammonium bicarbonate as the optimal method for clubroot disease management in Zhejiang Province.

Keywords: *Plasmodiphora brassicae*; pathotype; integrated control; plastic film covering; chemical agents

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1. Introduction

Clubroot, caused by the soil-borne obligatory eparasite *Plasmodiophora brassicae* Woronin, is the most destructive disease of *Brassica* crops in many regions [1]. *P. brassicae* primarily exists as protoplast, and studies have indicated that its resting spores can survive in field soil for over 17 years under specific conditions [2]. Resting spores germinate in suitable conditions, releasing primary zoospores. These primary zoospores undergo a series of structural and physical changes, commencing primary infection in both the root hairs and epidermal cells of the host plant. Subsequently, the product of the primary infection, the primary plasmodium, undergoes condensation, primary infection, and conjugation within the root epidermal cells. This process establishes secondary infection and eventually leads to the appearance of root gall symptoms [3,4].

Brassica is one of the most economically important and nutritionally essential crops worldwide. Infection by *P. brassicae* induces root swelling, thereby impeding nutrients

and water transport, hindering plant growth, and increasing susceptibility to wilting. Consequently, clubroot outbreaks pose substantial threats to agricultural production in many countries. China has a total of 95 genera and 425 species of the Brassicaceae and stands as the world's leading producer of brassicas vegetables [5]. The first report of clubroot disease in China emerged from Jiangxi Province, by Huang et al. [6]. Presently, clubroot is spreading rapidly across China, with the most severe outbreaks in the southwest, northeast, and middle regions. Zhejiang Province, identified as one of the most heavily infested areas in China, has successively reported clubroot occurrences in various cities and counties, including Dongyang, Huangyan, Yongjia, Rui'an, Lanxi, and Hangzhou [7,8]. Notably, the cultivation area of *Brassica* crops has undergone a large expansion in Zhejiang Province, driven by agricultural landscape restructuring. This expansion underscores the urgency for coordinated research on clubroot disease.

Early researchers in field studies noticed and validated the pathotype differentiation in *P. brassicae* [9]. Currently, bioassays with various hosts are used to differentiate pathotypes based on pathogen virulence patterns, focusing on the development of root galls as the measure of the host response. These assays capture both the occurrence and level of pathotype specialization within the pathogen population. Several differential systems have been proposed to study the pathotype differentiation of *P. brassicae* [10]. Among these, the Williams and European Clubroot Differential (ECD) system has gained the highest acceptance and widest application. Williams [11] aimed to theoretically differentiate 16 distinct pathotypes using two *Brassica napus* materials (Laurentian and Wilhelmsburger) and two *B. oleracea* materials (Jersey Queen and Badger Shipper). However, only 9 different pathotypes were identified among the 124 pathogen isolates employed in the study. Buczacki et al. [12] established the ECD system, which consists of three groups of *Brassica* crops (*B. campestris*, *B. napus*, and *B. oleracea*), each comprising five hosts. This system uses a modified binary naming system to encode pathotypes of *P. brassicae*. Pang et al. [13] developed a Sinitic clubroot differential set (SCD system) using eight differential inbred lines of Chinese cabbage with known or novel clubroot resistant (CR) genes, validating the significant genetic diversity with P4. However, since the plant materials in the SCD system are core parents in current breeding programs, challenges such as breeder's right protection and material acquisition restrict the broad application of this system in research and production. The diverse abilities of distinct pathotypes, including the infection of a variety of *Brassica* crops and overcoming of resistance in some hosts, highlights the importance of comparative analyses. Using diverse hosts to accurately identify the *P. brassicae* pathotypes can assist farmers to select crop varieties that are less susceptible to the dominant pathotypes in their regions, thereby reducing anticipated crop losses. Furthermore, the utilization of differentiated hosts provides valuable resources for clubroot resistance breeding programs, aiming to enhance the resistance of crops to clubroot disease [13,14].

Clubroot management involves physical, biological, agricultural, and chemical methods. Physical methods have achieved some success. Research indicates that covering the soil with totally impermeable films for two weeks can reduce clubroot severity from 82% in the untreated check to 35% [15]. Agricultural methods comprise soil alkalinity improvement, crop rotation, and the development of resistant varieties. Host resistance is an effective means for clubroot control. 'Mendel' is the first CR *B. napus* canola cultivar, released in 2001, demonstrating resistance to multiple pathotypes of *P. brassicae*. Its resistance is derived from a resynthesized *B. napus* line, which was the hybrid offspring of the *B. oleracea* line 'ECD-15' and the *B. rapa* line 'ECD-04' [16,17]. However, researchers have indicated that the CR of 'Mendel' is being eroded due to the high pathogenic variability of *P. brassicae* populations. Fredua-Agyeman et al. [18] assessed *P. brassicae* virulence in Alberta, Canada, revealing that while 'Mendel' was resistant to all old pathotypes, it resisted only around 50% of new strains. Chemical control remains a crucial and cost-effective method for clubroot management in the field. Chemical agents for clubroot primarily consist of fungicides that act by inhibiting lipid and membrane formation, respiratory processes, mitosis, and cell division in the pathogenic fungus. These agents can also in-

duce resistance in host plants and exhibit multi-site joint activity. Studies have shown that fluazinam (chemical formula: $C_{13}H_4C_{l2}F_6N_4O_4$) and cyazofamid (chemical formula: $C_{13}H_{13}ClN_4O_2S$) can significantly reduce clubroot severity and the disease index in host plants [19,20]. However, due to health and environmental concerns, many countries have restricted or prohibited the use of previously employed chemicals for clubroot control, such as methyl bromide and mercurous chloride. Consequently, finding environmentally friendly, cost-effective alternatives against various *P. brassicae* pathotypes is essential for ensuring sustainable and green agricultural practices.

Calcium cyanamide (chemical formula: $CaCN_2$) is widely used for soil pH regulation, which inhibits the germination of the resting spores of *P. brassicae* [21]. Dazomet (chemical formula: $C_5H_{10}N_2S_2$), a broad-spectrum fumigant, primarily degrades into methyl isothiocyanate with a chemosterilizing effect in moist soil [22]. Hwang et al. [23] reported that pre-treatment with dazomet (0.15 and 0.20 g a.i. L^{-1} soil) increased seedling emergence and plant height in greenhouses, while reducing the severity of clubroot infection. However, despite being considered environmentally friendly chemicals, ammonium bicarbonate (chemical formula: NH_4HCO_3) and chloroisobromine cyanuric acid (chemical formula: $C_3HO_3N_3ClBr$) have not been reported for clubroot control. Ammonium bicarbonate, a common agricultural fertilizer, has shown inhibitory effects on pathogens. Arslan et al. [24] found that 0.5% and 1% ammonium bicarbonate effectively suppressed the incidence and severity of apple scab. Chloroisobromine cyanuric acid is a systemic fungicide, playing a chemosterilizing role through the release of hypobromous acid and hypochlorous acid. Studies indicate that 50% chloroisobromine cyanuric acid exhibits high efficacy against various soil-borne diseases in multiple crops [25,26]. These research findings suggest potential applications for these two agents in clubroot control. 1-napthaleneacetic acid (NAA) is a lipophilic and membrane-permeable form of auxin, capable of penetrating cellular membranes independent of pH changes [27]. Research has indicated that NAA application can inhibit primary root elongation in *Arabidopsis thaliana* and increase the lateral root primordia density and lateral root number [28]. Therefore, NAA was selected to investigate whether the increase in lateral root numbers in plants could work against *P. brassicae* infection.

Therefore, the objectives of this study were as follows: (i) employ the Williams and ECD systems for the pathotype identification of *P. brassicae* isolates from Hangzhou, Yuhang and Quzhou, Kaihua in Zhejiang Province; (ii) investigate the efficacy and potential mechanisms of plastic film covering and chemical agents including calcium cyanamide, chloroisobromine cyanuric acid, dazomet, and ammonium bicarbonate in clubroot management; and (iii) assess the feasibility of field applications involving plastic film covering and these chemical agents, and to identify the most suitable method for clubroot management in Zhejiang Province.

2. Materials and Methods

2.1. Plant Materials

The 15 ECD hosts, comprising 5 accessions of *B. campestris*, 5 accessions of *B. napus*, and 5 accessions of *B. oleracea*, are documented in the research of Buczacki et al. [12]. The ECD hosts were provided by Professor Dixon G. R. at the University of Reading, UK.

To test the effectiveness of various chemical agents against clubroot, we used the Chinese cabbage cultivar 'Granaat' as the test plant material, which is universally susceptible to all *P. brassicae* strains included in the ECD set.

2.2. Identification of the Pathotype of P. brassicae Using the ECD and Williams Systems

According to the method of Liu et al. [29], a suspension of resting spores of *P. brassicae* was prepared. Twenty grams of galled root tissue was homogenized with 200 mL of distilled water in a blender. The homogenate was filtered through eight layers of gauze, and the concentration of resting spores in the filtrate was estimated using a hemocytometer and adjusted to 10^7 spores/mL with distilled water. Seedlings were inoculated using the

injection method of Pang et al. [13]. Briefly, seeds were soaked in a water bath at 55–60 °C for 8 min, placed on a single layer of moistened filter paper in a petri dish and germinated at 28 °C for 2–3 days. The seedlings were then planted into trays (length × width × height was 55 × 28 × 6 cm) filled with a sterilized potting mix (6 parts peat soil: 3 parts vermiculite: 1 part perlite). Five days after seeding, the resting spore suspension of 10^7 spores/mL was injected into the roots of the plant using 1 mL syringes without needles to ensure that the resting spore suspension was in full contact with the roots. Each seedling was injected with 1 mL. All plants grew under controlled conditions (22 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}) in a greenhouse, with watering and fertilization as needed. Each biological replicate comprised 12 plants of each host crop, and the experiment was replicated three times.

After 6 weeks, the severity of clubroot disease was determined based on disease grading standards. Clubroot severity was assessed on a 0–3 scale, where 0 = no clubs, 1 = a few small clubs, 2 = moderate clubbing, and 3 = severe clubbing [30].

An analysis of the disease condition and the calculation method are as follows [31]:

$$\text{Disease index(DSI)} = \frac{\sum(n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3)}{N \times 3} \times 100$$

where n_0, n_1, n_2, and n_3 are the numbers of plants in each class; 0, 1, 2, and 3 are the symptom severity classes; and N is the total number of plants.

2.3. Chemical Treatment in Greenhouse Experiment

In the greenhouse trials, the potting mix was artificially infested with *P. brassicae* obtained from Quzhou city, Kaihua county (1×10^7 resting spores per g of potting mix). Each plastic pot (length × width × height was 5.5 × 5.5 × 6 cm) was filled with 40 g of infested potting mix, and each biological replicate for each treatment comprised 32 pots. The experiment was performed with 3 biological replications. The greenhouse experiment included the following treatments: a control group without any treatment (CK); a group with black plastic film covering (T1); and chemical treatments with calcium cyanamide (90 g/m^2, T4), chloroisobromine cyanuric acid (4.5 g/m^2, T9), dazomet (37.5 g/m^2, T14), and ammonium bicarbonate (150 g/m^2, T19) (Table 1). Black plastic film was applied to all pots after the chemical treatments. All treatments were placed in a greenhouse, and the environmental conditions were the same as described in Section 2.2. An additional ammonium bicarbonate treatment, marked as T22, was implemented at a higher temperature (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}) due to its thermolability ($NH_4HCO_3 \xrightarrow{\Delta} NH_3 \uparrow + H_2O + CO_2 \uparrow$) and the hot weather conditions prevailing in Zhejiang Province. After 14 days of treatment, the plastic film was removed and ventilated for 7 days. An appropriate amount of infested potting mix was taken from each treatment for the qPCR experiment, and then, the seedlings of the clubroot-susceptible Chinese cabbage cultivar 'Granaat' were planted. Plant height and clubroot severity were recorded 6 weeks after seeding.

Table 1. Chemical agents selected for the assessment of the clubroot control, *Plasmodiophora brassicae*, under greenhouse and field conditions.

Treatment [1]	Common Name	Formulation [2]	Rate (a.i. m^{-2}) [3]	Registrant Information
CK	Without treatment	-	-	-
T1	Black plastic film	-	-	Jiangsu Taika Greenhouse Co., Ltd. (Suqian City, Jiangsu Province, China)
T2	Calcium cyanamide (chemical formula: $CaCN_2$)	GR $N \geq 21\%$ $CaCN_2 \geq 55\%$	60 g	Ningxia Jiafeng Chemicals Co., Ltd. (Shizuishan City, Ningxia Hui Autonomous Region, China)
T3			75 g	
T4			90 g	
T5			105 g	
T6			120 g	
T7	Chloroisobromine cyanuric acid (chemical formula: $C_3HO_3N_3ClBr$)	SP $C_3HO_3N_3ClBr = 50\%$	1.5 g	Hebei Shangrui Chemicals Co., Ltd. (Shijiazhuang City, Hebei Province, China)
T8			3.0 g	
T9			4.5 g	
T10			6.0 g	
T11			7.5 g	
T12	Dazomet (chemical formula: $C_5H_{10}N_2S_2$)	MG $C_5H_{10}N_2S_2 = 98\%$	27.5 g	Zhejiang Haizheng Chemicals Co., Ltd. (Taizhou City, Zhejiang Province, China)
T13			32.5 g	
T14			37.5 g	
T15			42.5 g	
T16			47.5 g	
T17	Ammonium bicarbonate (chemical formula: NH_4HCO_3)	- $N \geq 17.1\%$ $H_2O \leq 3.5\%$	120 g	Weihui Yubei Chemicals Co., Ltd. (Xinxiang City, Henan Province, China)
T18			135 g	
T19			150 g	
T20			165 g	
T21			180 g	
T22			150 g [4]	

[1] CK represents the group without treatment; T1 was the plastic film covering treatment; T2–T6 were calcium cyanamide with 60, 75, 90, 105, and 120 g/m^2; T7–T11 were chloroisobromine cyanuric acid treatment with 1.5, 3.0, 4.5, 6.0, and 7.5 g/m^2; T12–T16 were dazomet treatment with 27.5, 32.5, 37.5, 42.5, and 47.5 g/m^2; T17–T22 were ammonium bicarbonate treatment with 120, 135, 150, 165, and 180 g/m^2. [2] Product formulation: GR—Granules; SP—Soluble power; MG—Micro-granules. [3] Rates were based on the active ingredient (a.i.) recommended by manufacturers. [4] T22 was an ammonium bicarbonate treatment conducted at higher temperatures (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}) under greenhouse conditions. No data available.

2.4. Quantitative Polymerase Chain Reaction (qPCR) Analysis of Infected Soil

For a qPCR analysis, plant materials were removed, and the infested potting mix of each treatment was mixed and air-dried at room temperature (26 °C) before sieving through a 1 mm mesh. A DNA extraction from the soil was performed following the instruction manual of the SPINeasy DNA Kit for Soil (MP Biomedicals, Irvine, CA, USA). The sequences of primers and probes for the qPCR, as well as qPCR conditions, were modified based on the method by Czubatka-Bienkowska et al. [32]. The primers Pb-in3-F 5′-TACAGGAGCTGGTCCTTCCA-3′ and Pb-in3-R 5′-CGCCACACTAGCATTCAAGC-3′ were used to amplify a 114 bp fragment of the 18S ribosomal DNA sequence (GenBank accession number MN860572.1). A Pb-in3-Probe 5′-TCATCGCCCGGGAAGCCTTAGCAGCC-3′ probe with FAM as the 5′ terminal reporter dye and BHQ1 as the 3′ quencher was used to improve the qPCR specificity. Real-time quantitative PCR was performed using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hong Kong, China). The qPCR system and procedure were followed according to the instruction manual of Probe qPCR Mix, with UNG (Takara Biomedical Technology (Beijing) Co., Ltd. Beijing, China). For each sample, 20 μL of reaction mix containing 10 μL of probe qPCR mix, 0.4 μL each of forward and reverse primers (10 μM), 0.8 μL of probe (10 μM), 2 μL of sample complementary DNA, and 6.4 μL sterile distilled water. The qPCR thermocycling conditions for all reactions were 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Three technical replicates were performed for each sample. Pathogen copy numbers were

quantified using a standard curve generated from a 10-fold dilution series of the plasmids containing *P. brassicae* target sequences.

2.5. Soil pH Determination

The determination of the soil pH was conducted according to the standards set by the Ministry of Ecology and Environment of the People's Republic of China. Briefly, the soil pH was measured using the potentiometric method, and the pHS-3C acidometer was calibrated with 4.00, 6.86, and 9.18 standard buffer solutions before pH determination. Five grams of air-dried soil was sieved through a 1 mm mesh and mixed with 50 mL of deionized water. The mixture was stirred with a glass rod for 1 min and then allowed to stand at room temperature for 30 min, followed by the pH determination. The experiment was replicated three times.

2.6. NAA Treatment

The plant material used in the experiment was the Chinese cabbage cultivar 'Granaat', susceptible to *P. brassicae*. The growth medium consisted of the basic MS medium supplemented with 0, 0.04, 0.08, 0.12, 0.16, and 0.20 mg/L of NAA. Seeds of 'Granaat' were treated with 70% ethanol for 1 min, surface-disinfected with 0.1% $HgCl_2$ for 10 min, rinsed 3–4 times with sterile water, and then germinated on NAA-MS medium, following the seeding procedure proposed by Yu et al. [33]. The cultivation conditions were as follows: a light intensity of 2250 lx in a growth chamber, a photoperiod of 16 h, and a temperature of 25 ± 1 °C. Each biological replicate included 8 plant materials for each NAA treatment concentration, and the experiment was carried out in three biological replicates. The root phenotypes of the 'Granaat' were observed two weeks after seeding.

The measurement of the root length of 'Granaat' was conducted using ImageJ 1.53k (National Institutes of Health, Bethesda, MD, USA), and a visualization was performed using GraphPad Prism version 8.0.2 (GraphPad Software, Inc., Boston, MA, USA).

To investigate the impact of NAA treatment on the infection of 'Granaat' by *P. brassicae*, a greenhouse experiment was conducted. The preparation of the infested soil, specifications of plastic pots, seed germination method, and plant cultivation conditions were consistent with those described in Section 2.3. The treatments were as follows: seedlings were planted in plastic pots containing infested soil, and each seedling in the experimental group was inoculated with 1 mL of NAA at a concentration of 0.20 mg/L; the CK remained untreated with NAA. After 6 weeks, the root phenotypes of 'Granaat' were observed.

2.7. Field Trials

The effect of chemical agents on plant growth parameters and clubroot severity on the clubroot-susceptible Chinese cabbage cultivar 'Granaat' was investigated in a field naturally infested with *P. brassicae* at Hangzhou, Yuhang (30°42′ N, 120°30′ E), in mid-September 2020 and 2021. Meteorological data recorded a lower air temperature (<30 °C) during the treatment period in 2020 (which had 17 days with an average air temperature above 30 °C during the treatment period) compared to 2021 (26 days). Before treatment, the field was ploughed, and residual crops and weeds were removed. The treatments in field trials were as follows: a control group without any treatment (CK); a group with black plastic film covering (T1); and 5 chemical treatments each with calcium cyanamide (60–120 g/m^2, T2–T6), chloroisobromine cyanuric acid (1.5–7.5 g/m^2, T7–T11), dazomet (27.5–47.5 g/m^2, T12–T16), and ammonium bicarbonate (120–180 g/m^2, T17–T21) (Table 1). Black plastic film was applied to all blocks after the chemical treatments. The humidity of the field was kept at 40–70%. The plastic film was removed after 14 days and ventilated for 7 days. In total, 40 to 50 seeds of 'Granaat' were planted directly in each block. The experiment was arranged in a randomized complete block design with 3 biological replications, with each block measuring 2.5 m^2. The planting density was 16–20 plants/m^2. Six weeks after seeding, all the plants were uprooted. The roots were washed under tap water, and clubroot severity was recorded for each plant. A DSI analysis was performed as described above.

2.8. Statistical Analysis

The experimental data obtained above were first processed in MS-Excel™. An independent sample *t*-test in IBM SPSS Statistics v23 (IBM Inc., Armonk, NY, USA) was performed to compare CK and T1 in both greenhouse and field trials, as well as T19 and T22 in the greenhouse trials. An analysis of variance was performed on T1–T19 in the greenhouse trials and T1–T21 in the field trials using One-Way ANOVA in IBM SPSS Statistics v23 (IBM Inc.). Significant differences among mean values were determined using Duncan's Post Hoc Multiple Comparison at $\alpha = 0.05$. The correlation between *P. brassicae* resting spore density in soil and the DSI was evaluated by performing simple linear regression analysis in Prism (GraphPad Software Inc., Boston, MA, USA).

3. Results

3.1. Identification of the Pathotypes of P. brassicae with the Williams and ECD Systems

The pathotypes of *P. brassicae*, collected from Hangzhou, Yuhang and Quzhou, Kaihua in Zhejiang Province, were identified using the Williams system and the ECD system. The results indicated that the pathotype of *P. brassicae* infecting purple cai-tai in Hangzhou city, Yuhang district was identified as pathotype 1 (P1), ECD20/31/12. Similarly, the pathotype infecting canola in Quzhou city, Kaihua county was identified as P3, ECD20/15/4 (Table 2).

Table 2. Pathotypes of *P. brassicae* identified using the Williams and ECD systems.

Sampling Location	Host Origin	Pathotype	
		Williams System	ECD System
Hangzhou, Yuhang	Purple cai-tai	P1	ECD20/31/12
Quzhou, Kaihua	Canola	P3	ECD20/15/4

3.2. Effects of Different Chemical Treatments on Clubroot Control in the Greenhouse Experiments

The pathogen isolated from Quzhou, Kaihua, canola as the host were used in the greenhouse experiment. Plant height and DSI were indicators used to evaluate the control effect of treatments.

The results indicated that 'Granaat' treated with ammonium bicarbonate under both normal and high-temperature conditions exhibited lush and dark green leaves, surpassing the effects of calcium cyanamid and dazomet treatments. In contrast, 'Granaat' in the CK, black plastic film covering, and chloroisobromine cyanuric acid treatment trials showed stunted growth and sparse and yellow leaves (Figure S1).

Measuring the plant height revealed that 'Granaat' in the CK and black plastic film covering groups were stunted, with heights of 14.63 cm and 14.30 cm, respectively, showing no significant difference between them (Figure 1a,b,h and Table S1). Among the four chemical treatments (calcium cyanamid, chloroisobromine cyanuric acid, dazomet, and ammonium bicarbonate), the heights of 'Granaat' were 18.33 cm, 13.63 cm, 18.74 cm, and 21.10 cm, respectively (Figure 1h and Table S1). A significant difference in plant height was observed between the ammonium bicarbonate and plastic film covering treatment, indicated a positive effect of ammonium bicarbonate (Figure 1f,h and Table S1). The plant height of 'Granaat' after ammonium bicarbonate treatment under high-temperature conditions was 20.02 cm, with no significant difference between the two ammonium bicarbonate treatments at different temperatures (Figure 1g,h and Table S1).

Figure 1. Phenotypic observations (**a**–**g**) and plant height measurements (**h**) of the Chinese cabbage cultivar 'Granaat' under greenhouse conditions. The measurement of plant height was performed 42 days after the 'Granaat' planting. CK represented the group without treatment (**a**); T1, T4, T9, T14, and T19 were treatments of plastic film covering (**b**); calcium cyanamide at 90 g/m^2 (**c**); chloroisobromine cyanuric acid at 4.5 g/m^2 (**d**); dazomet at 37.5 g/m^2 (**e**); and ammonium bicarbonate at 150 g/m^2 (**f**) (22 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}). T22 was a 150 g/m^2 ammonium bicarbonate treatment (**g**) conducted at higher temperatures (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}). Scale bars, 4 cm. Bars represent the means ± SEM (n = 3). The ns represents no significance (Student's t-test). Different letters indicate samples with statistical differences ($p < 0.05$, Duncan's test).

Both the CK and plastic film mulching treatments suffered from severe disease symptoms with a high DSI (67.08 and 69.08, respectively), showing no significant difference between them. Therefore, the individual plastic film cover treatment showed no significant effect on clubroot. Three chemical treatments, calcium cyanamide, dazomet, and ammonium bicarbonate, significantly reduced disease symptoms and the DSI. Their average DSI values were 24.24, 1.52, and 40.40, respectively. Dazomet exhibited the best control effect on clubroot severity. However, 'Granaat' plants treated with chloroisobromine cyanuric acid displayed the highest DSI (75.05), indicating a significantly susceptible phenotype. The ammonium bicarbonate treatment under high-temperature conditions showed a significant positive effect on clubroot management, with reduced disease symptoms and displayed a DSI of 1.29, significantly lower than that of conventional ammonium bicarbonate treatment (Figure 2a and Table S1). It is noteworthy that, with both temperature variants of ammonium bicarbonate, both infested and healthy plants produced more lateral roots compared to plastic film cover treatments (Figure 2b).

3.3. The Resting Spore Density in the Soil and the Soil pH in Greenhouse Experiments

To explore the possible mechanisms of the chemical treatments on clubroot, two experiments were carried out: a qPCR analysis of the resting spore density in the soil and a pH measurement of soil samples. The plasmid concentration of *P. brassicae* used in this experiment was 4.35 ng/μL, corresponding to a resting spore copy of 1.98 × 10^9/μL. It was serially diluted in a 10-fold gradient to create a range of concentrations as DNA templates. The Ct values obtained through qPCR showed a strong linear correlation with the logarithm of plasmid DNA copy numbers, represented by the standard curve equation: $y = -3.172\,x + 42.462$, $R^2 = 0.998$ (Figure 3a).

The qPCR results revealed that the copies of resting spores in the soil subjected to plastic film covering were significantly higher than those in the CK, with values of 2.92 × 10^7 and 2.13 × 10^7 g per soil, respectively. The copies of resting spores in the four chemical treatments, calcium cyanamide, chloroisobromine cyanuric acid, dazomet, and ammonium bicarbonate, were significantly lower than those in the plastic film covering

treatment, with values of 1.09×10^7, 2.39×10^7, 2.67×10^5, and 1.59×10^7 g per soil, respectively. The resting spore copies in the soil treated with ammonium bicarbonate under high-temperature conditions were 1.26×10^7 copies per gram of soil, significantly lower than those in the conventional ammonium bicarbonate treatment (Figure 3b and Table S1). Additionally, an analysis of the correlation between the copies of resting spores in the soil under plastic film covering, ammonium bicarbonate, calcium cyanamide, and dazomet treatments and the DSI for 'Granaat' was conducted. The results demonstrate a linear correlation between the soil resting spore density of these four treatments and DSI, with a correlation coefficient (R^2) of 0.925 (Figure 3c).

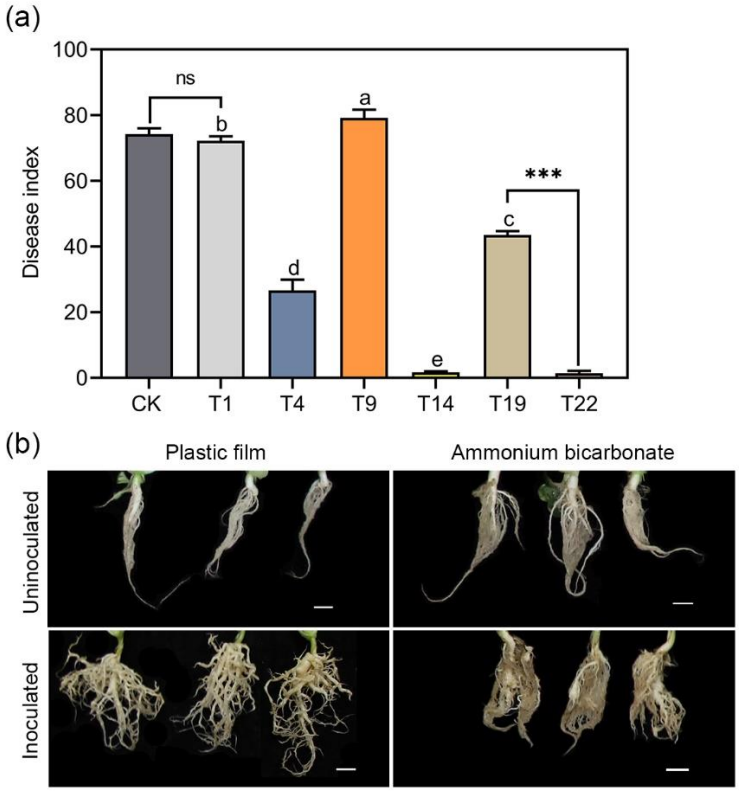

Figure 2. Evaluation of disease index (DSI) in Chinese cabbage cultivar 'Granaat' under different treatments (**a**) and observation of root phenotypes in both uninoculated and *Plasmodiophora brassicae*-inoculated plants exposed to plastic film covering and ammonium bicarbonate treatments (**b**) in greenhouse conditions. The experiments were performed 42 days after 'Granaat' planting. CK represents the group without treatment; T1, T4, T9, T14, and T19 were the treatments of plastic film covering, calcium cyanamide at 90 g/m^2, chloroisobromine cyanuric acid at 4.5 g/m^2, dazomet at 37.5 g/m^2, and ammonium bicarbonate at 150 g/m^2, respectively (22 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}). T22 was a 150 g/m^2 ammonium bicarbonate treatment conducted at higher temperatures (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}). Bars represent the means ± SEM (n = 3). The ns and asterisks represent no significance and significant differences (*** $p < 0.001$, Student's t-test), respectively. Different letters indicate samples with statistical differences ($p < 0.05$, Duncan's test). Scale bars, 1 cm.

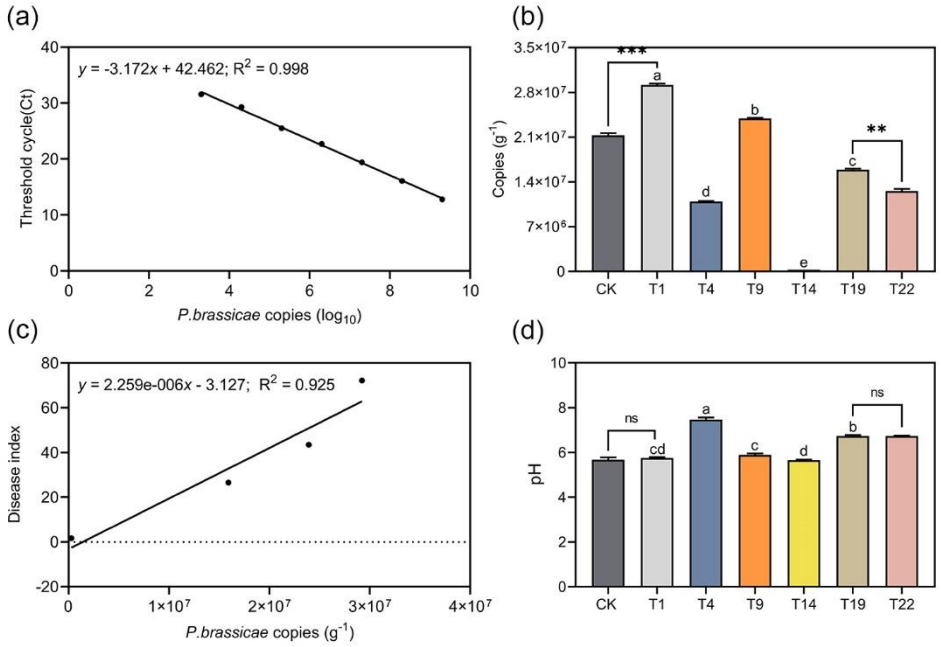

Figure 3. Analysis of the standard curve (**a**), copies of *P. brassicae* resting spores in soil (**b**), correlation between the *P. brassicae* resting spore density in soil and the DSI (**c**), and soil pH (**d**) under greenhouse conditions. The quantification of *P. brassicae* resting spores in soil and measurement of soil pH were performed after 14 days of treatment and ventilated for 7 days. A correlation analysis was performed with the treatments of dazomet, calcium cyanamide, ammonium bicarbonate, and plastic film covering (from left to right). CK represents the group without treatment; T1, T4, T9, T14, and T19 were treatments of plastic film covering, calcium cyanamide at 90 g/m^2, chloroisobromine cyanuric acid at 4.5 g/m^2, dazomet at 37.5 g/m^2, and ammonium bicarbonate at 150 g/m^2, respectively (22 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 µmol·m^{-2}·s^{-1}). T22 was a 150 g/m^2 ammonium bicarbonate treatment conducted at higher temperatures (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 µmol·m^{-2}·s^{-1}). Bars represent the means ± SEM (n = 3). The ns and asterisks represent no significance and significant differences (** $p < 0.01$, *** $p < 0.001$, Student's t-test), respectively. Different letters indicate samples with statistical differences ($p < 0.05$, Duncan's test).

The soil pH measurement results indicate that the pH value of CK was 5.67, with no significant difference compared to the plastic film covering, which had a pH value of 5.76. Following the chloroisobromine cyanuric acid and dazomet treatments, the soil pH values showed no significant difference compared to those of the plastic film covering, measuring 5.89 and 5.64, respectively. However, the soil pH values after calcium cyanamide and ammonium bicarbonate treatments were significantly higher than those under plastic film covering, measuring 7.46 and 6.73, respectively. Notably, the pH value of ammonium bicarbonate under high-temperature conditions was 6.73, showing no significant difference compared to the conventional ammonium bicarbonate treatment (Figure 3d and Table S1).

3.4. The Effect of NAA on 'Granaat' Root System and Clubroot Control

To investigate the response of 'Granaat' roots to NAA and its potential in controlling clubroot, seed germination experiments were conducted on MS culture media with varying NAA concentrations. Additionally, greenhouse experiments with NAA treatments were also performed.

The seed germination experiment results indicate that on a MS culture medium without NAA, 'Granaat' developed elongated primary roots with minimal lateral roots. As the NAA concentration increased, primary root growth in 'Granaat' was suppressed, leading to a gradual decrease in length. Conversely, lateral roots initiation was induced, resulting in an increasing number of lateral roots (Figure 4a and Table S2). An ANOVA revealed that after reaching an NAA concentration of 0.08 mg/L, the length of the primary root

in 'Granaat' ceases to significantly decrease with further increases in NAA concentration (Figure 4b).

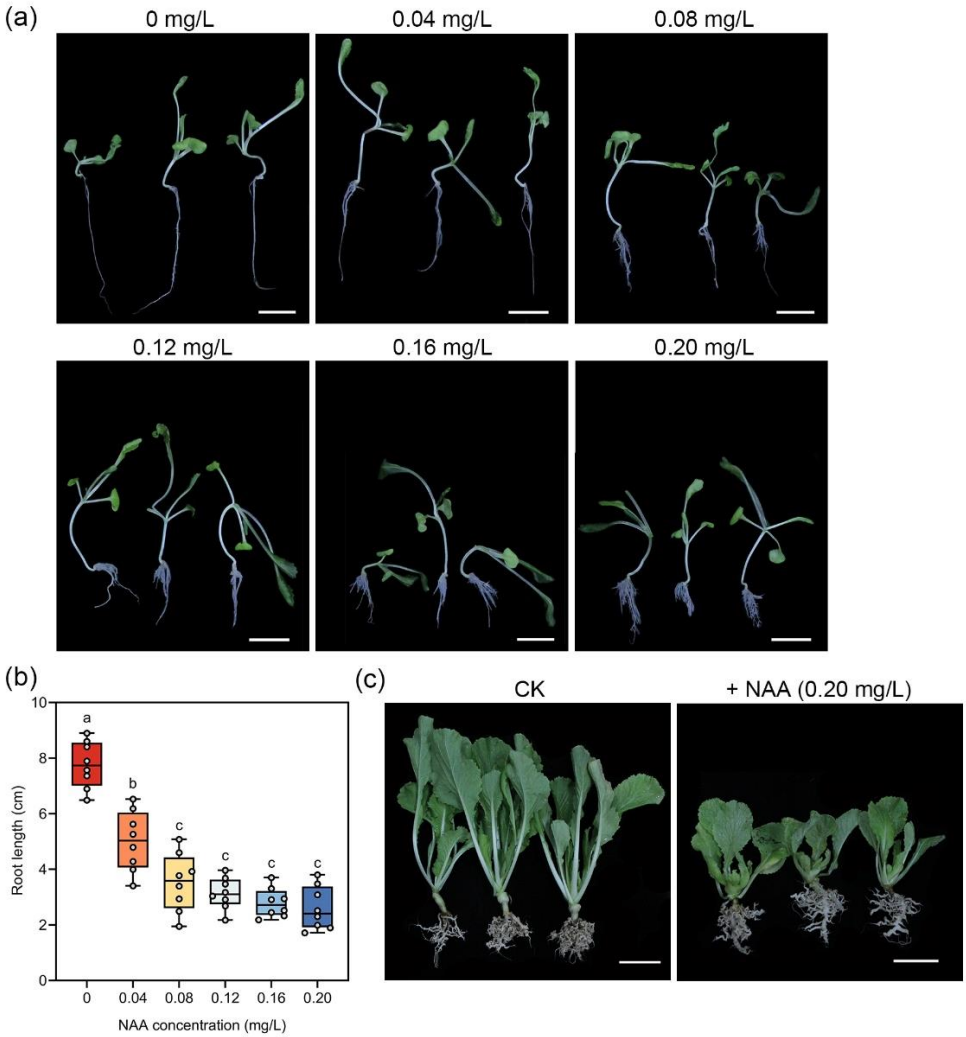

Figure 4. Observations of 'Granaat' phenotypes in MS media with different concentrations of NAA (**a**), measurement of root length (**b**), and an analysis of the root phenotypes of 'Granaat' infected by *P. brassicae* under greenhouse conditions (**c**). The cultivation conditions for 'Granaat' in MS media included a light intensity of 2250 lx in a growth chamber, a photoperiod of 16 h, and a temperature of 25 ± 1 °C, and phenotypes were observed two weeks after seeding. A root phenotype analysis was performed 42 days after 'Granaat' planting. Different letters indicate samples with statistical differences ($p < 0.05$, Duncan's test). Scale bars in (**a**) and (**c**), 2 cm and 4 cm, respectively.

An NAA concentration of 0.20 mg/L was selected for greenhouse experiments. However, the results reveal that, during the early stages of growth, the induction of lateral root initiation and an increase in lateral root number alone did not mitigate the impact of *P. brassicae* on 'Granaat'. In comparison to the CK group, NAA-treated plants, despite exhibiting distinct primary and lateral root structures, manifest more severe symptoms, including stunted growth and extensive swelling of the entire root system (Figure 4c).

3.5. Effects of Different Chemical Treatments on Clubroot Control in the Field Trials

Field trials were conducted in Hangzhou, Yuhang, in 2020 and 2021. In the 2020 field trial, the chemical treatments were applied on September 11th, followed by plastic film covering. The film was removed for ventilation on September 27th, and seeding took place on October 6th. In the 2021 field trials, these three time points were September

9th, September 25th, and October 4th, respectively. The weather conditions in Hangzhou, Yuhang District, during the two-year experimental period revealed that no days exceeded 30 °C throughout the entire experiment period in 2020. In contrast, 2021 experienced 10 days exceeding 30 °C (including 30 °C itself) during the 17 days from chemical treatment to film removal. Furthermore, only 1 day during the period from film removal to seeding had a temperature below 30 °C (https://data.cma.cn/, accessed on 15 February 2022) (Figure 5a).

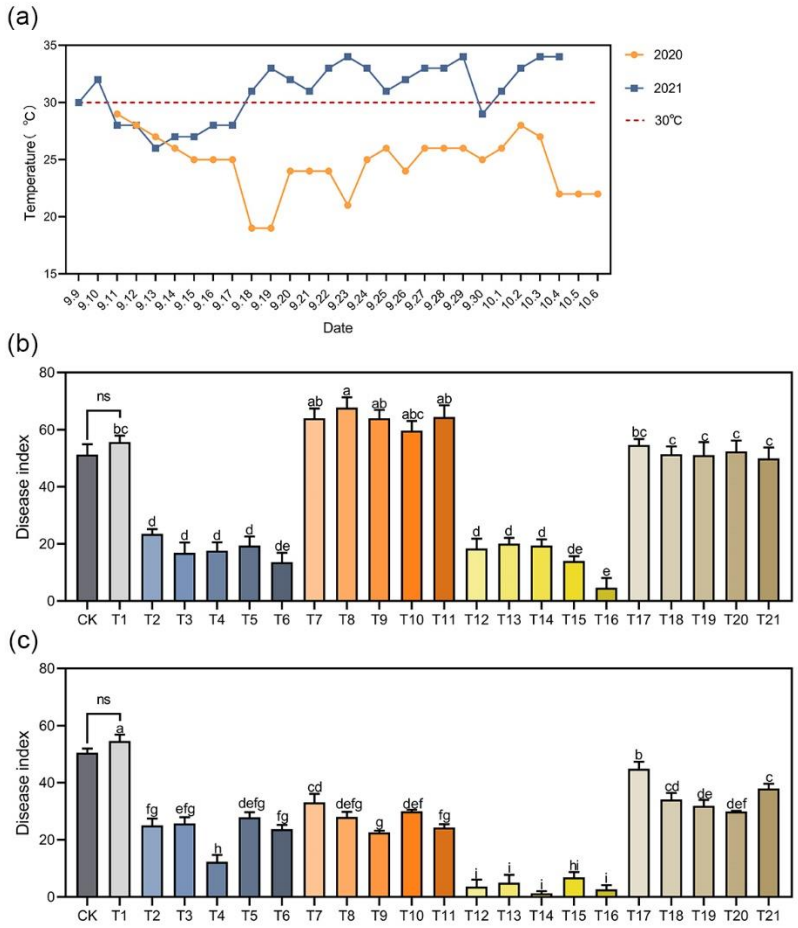

Figure 5. Meteorological data in the treatment period (**a**), and DSIs of the 'Granaat' subjected to different treatments for clubroot control in the yield trials in 2020 (**b**) and 2021 (**c**). CK represents the group without treatment; T1 was the plastic film covering treatment; T2 to T6 were treated with calcium cyanamide at rates of 60, 75, 90, 105, and 120 g/m^2, respectively; T7 to T11 were treated with chloroisobromine cyanuric acid at rates of 1.5, 3.0, 4.5, 6.0, and 7.5 g/m^2, respectively; T12 to T16 received dazomet at rates of 27.5, 32.5, 37.5, 42.5, and 47.5 g/m^2, respectively; and T17 to T21 were treated with ammonium bicarbonate at rates of 120, 135, 150, 165, and 180, respectively. Different letters indicate samples with statistical differences ($p < 0.05$, Duncan's test).

The results of the 2020 field trial reveal that the DSI of 'Granaat' under a plastic film cover showed no significant difference compared to that of the CK, with values of 51.27 and 55.64, respectively. Among the four chemical treatments, all application gradients of calcium cyanamide and dazomet significantly reduced the DSI compared to the plastic film cover. There were no significant differences in the DSI among different application rates of calcium cyanamide, ranging from 12.27 to 21.17. Dazomet at an application rate of 47.5 g/m^2 exhibited the most effective treatment against clubroot, resulting in a DSI of 4.21. The DSI of 'Granaat' treated with chloroisobromine cyanuric acid and ammonium

bicarbonate showed no significant difference compared to plastic film cover, ranging from 59.67 to 67.73 and 49.92 to 54.64, respectively (Figure 5b and Table S3).

In the 2021 field trial, the DSI of 'Granaat' under plastic film cover showed no significant difference compared to that of the CK, with values of 54.63 and 50.51, respectively. However, the DSIs for all four chemical treatments were significantly lower than that under plastic film cover. The DSI of 'Granaat' after calcium cyanamide treatment ranged from 12.35 to 27.91. For the chloroisobromine cyanuric acid treatment, the DSI ranged from 22.59 to 33.14, while the dazomet treatment showed the lowest DSI, ranging from 1.31 to 6.86. Ammonium bicarbonate treatment resulted in a DSI of 29.95 to 44.89 (Figure 5c and Table S3).

4. Discussion

Our laboratory previously conducted sampling investigations on the clubroot pathogen in various locations within Zhejiang Province. Using the Williams and ECD systems, P1 (ECD20/31/12, ECD24/16/30), P2 (ECD17/15/15), P3 (ECD20/15/4), and P8 (ECD16/0/0, ECD24/0/0) were identified within the province. For our materials, we selected the less virulent strain (P3, ECD20/15/4) and the most virulent strain (P1, ECD20/31/12) found in Zhejiang Province. This choice was made for two reasons. First, our sampling was conducted in commercial fields, where farmers, who suffered economic losses due to clubroot in cruciferous crops, usually shift to planting non-host crops in subsequent rotations. Finding field conditions with the same pathotype as the strain used in greenhouse experiments is therefore difficult. This resulted in differences in the *P. brassicae* pathotypes used in the greenhouse and field experiments in this study. Second, the ultimate aim of this research was to be applicable in field production, effectively aiding farmers in preventing and controlling clubroot disease and reducing associated economic losses. Our results indicate that three chemical agents that we used, calcium cyanamide, dazomet, and ammonium bicarbonate, exhibit excellent control efficacy against the less virulent pathotype, P3, in greenhouse experiments. Excitingly, these chemical agents also demonstrated effectiveness in the field trials against the most virulent pathotype, P1, identified within Zhejiang Province. This suggests that the screened chemical agents possess high practical value, with their effectiveness not limited to less virulent clubroot strains.

Previous studies by Dixon [34] indicated that the application of calcium cyanamide promoted diversity and richness within the soil bacterial community and suppresses soil-borne pathogens. Additionally, Ca^{2+} may be released from calcium cyanamide, subsequently elevating the pH of the soil. The impacts of Ca^{2+} and alkaline soil pH on clubroot have been reported by many researchers [35–37]. Dazomet is a highly effective soil fumigant for managing various soil-borne diseases, nematodes, insects, and weeds. Its main degradation product in moist soil is methyl isothiocyanate, a nonselective toxin [22]. In this study, we confirmed that calcium cyanamide and dazomet effectively controlled clubroot by increasing plant height and reducing symptoms, aligning with findings from previous studies [38–40]. Furthermore, the qPCR analysis showed that both agents significantly reduced the resting spore density, strongly correlating with the plant disease index. The pH measurements revealed that, compared to the plastic film mulching treatment, soil alkalinity significantly increased after the calcium cyanamide treatment, while the soil pH remained unchanged after the dazomet treatment. Therefore, we hypothesize that the mode of action of both chemical agents on clubroot involves killing resting spores, leading to their DNA degradation in the soil. This process reduces infection in plant roots, resulting in a significant reduction in the disease index of plants. The distinction in their actions lies in the fact that calcium cyanamide operates through chemical toxicity and pH elevation in alkaline soil, whereas dazomet, without requiring alkaline soil pH mediation, directly eradicates resting spores through the chemical toxicity of its degradation products. In addition, a linear correlation emerged between the DSI and the copies of resting spores in the soil for the treatments of plastic film covering, ammonium bicarbonate, calcium cyanamide, and dazomet. This correlation demonstrates a reduction in plant disease severity as the

density of resting spores in the soil declines. Therefore, based on the results of the dazomet treatment, it can be inferred that *Brassica* crops can evade clubroot colonization when the soil resting spore density drops below the level of 10^5 g per soil.

However, despite its effectiveness as a broad-spectrum agent for clubroot control, dazomet has certain impacts on non-target soil organisms. Studies have shown that soil fumigants can cause a decline in soil microbial activity, leading to reduced soil enzyme activity [41]. Additionally, dazomet disrupts the balance of soil biological communities, with varying sensitivities among soil organisms, ranging from high to low in the order of microarthropods, nematodes, fungi, and bacteria [42].

The two environmentally friendly agents selected in this study, ammonium bicarbonate and chloroisobromine cyanuric acid, which have not been reported for clubroot management, exhibited contrasting effects on *P. brassicae* control. Ammonium bicarbonate demonstrated promising efficacy, while chloroisobromine cyanuric acid proved unsuitable for clubroot management. The greenhouse experiments indicated that the disease index and resting spore copy number of 'Granaat' treated with ammonium bicarbonate at high temperatures (32 °C/20 °C) were significantly lower than those treated with ammonium bicarbonate at normal temperatures (23 °C/20 °C). However, it was worth noting that the resting spore copies, despite being significantly reduced, remained relatively high. The soil pH for both treatments remained at 6.73, showing no significant difference. Due to the thermal instability of ammonium bicarbonate (it undergoes significant decomposition at temperatures above 30 °C, releasing ammonia and carbon dioxide gases), we attribute the differential efficacy of ammonium bicarbonate treatments at different temperatures to the release of ammonia and carbon dioxide gases. The destructive impact of these gases on the structure of resting spores is limited, suggesting that they may not directly kill them and induce DNA degradation. Instead, they likely act by suppressing the vitality of *P. brassicae* resting spores, reducing their ability to infect host plants, thereby achieving clubroot disease control. These gases may also potentially influence the pathogen in the soil through interaction with soil pH values.

In the field trials conducted in 2020 and 2021, the impact of ammonium bicarbonate on clubroot management showed significant differences. These outcomes corresponded respectively with the results obtained from greenhouse experiments involving conventional and high-temperature conditions with ammonium bicarbonate treatments. Weather data revealed that during the field treatment period in 2020, there were no days that exceeded 30 °C. Conversely, during the treatment period in 2021, temperatures exceeding 30 °C occurred for approximately 70% of the time (including 30 °C). Therefore, we attribute the improved efficacy of ammonium bicarbonate against clubroot disease in 2021 to the elevated temperatures during the treatment period. Temperatures exceeding 30 °C facilitated the decomposition of ammonium bicarbonate, leading to the release of ammonia and carbon dioxide, which suppresses the activity of resting spores of *P. brassicae* in the soil and results in a more robust control effect. We recommend conducting field applications of ammonium bicarbonate during periods of elevated temperatures to optimize its effectiveness in clubroot disease control.

An intriguing observation emerged from our greenhouse experiment: regardless of whether ammonium bicarbonate treatment was conducted under high-temperature or standard conditions, both diseased and healthy plants exhibited a greater number of lateral roots compared to other treatments. Consequently, even plants severely affected with third-grade clubroot infection, due to their abundant lateral roots for water and mineral nutrients absorption from the soil, their above-ground growth conditions significantly surpassed those subjected to the control treatment (plastic film mulching). Meier et al. [27] provided a theoretical foundation for this observation by demonstrating the mechanism by which ammonium nitrogen influences plant root system development. Their research indicated that ammonium uptake mediated by ammonium transporters promotes the accumulation of auxin in the shoot to the root system This leads to root apoplast acidification, subsequently increasing the pH-dependent import of protonated auxin into the cortical and epidermal

cells overlaying lateral root primordia, thereby promoting lateral root initiation. Based on this, we proposed a novel approach for the host plant to counteract clubroot: induce the emergence of more lateral roots in infested host plants to maintain their growth in situations where the complete eradication of *P. brassicae* is challenging and where the resistance of CR varieties is difficult to sustain. By doing so, we can mitigate the impact of *P. brassicae* on Brassicaceae crops, ultimately achieving a balanced coexistence between the host plant and the pathogen.

Additionally, Meier et al. [27] found that the mutant *qko*, which loses over 90% of its ammonium absorption capacity, cannot induce lateral root formation with the ammonium supply, or stimulate lateral root development with the IAA supply. However, the application of NAA (a lipophilic and membrane-permeable form of auxin), independent of pH changes, can restore the lateral root phenotype in *qko*. To validate our proposed approach, we treated 'Granaat' with NAA through a series of concentration gradients. The results revealed that increasing the NAA concentration resulted in increased lateral root numbers and reduced root lengths in 'Granaat' grown on an NAA-supplemented MS medium. Unfortunately, subsequent experiments indicated that while NAA treatment increased the number of lateral roots in plants, both primary and lateral roots developed severe gall, inhibiting plant growth. Therefore, we posit that this represents a dynamic competitive relationship between lateral root formation and resting spore infection. If the rate of lateral root initiation is slower than the infection rate of the pathogen, plants are more severely affected. Conversely, if the lateral root initiation rate exceeds the pathogen's invasion rate, the plant can ensure sufficient healthy lateral roots to sustain water and nutrient uptake during the growth process.

5. Conclusions

Our research indicates two approaches for managing clubroot in field production: first, reduce the *P. brassicae* resting spore density in the soil to levels below 10^5 spore/g, as achieved through dazomet; second, induce host crops to develop more lateral roots during the growth period as a resistance strategy against *P. brassicae* infestation, exemplified by ammonium bicarbonate.

Currently, chemical control methods dominate the field production in combating clubroot. However, considering the potential ecological impact of chemical agents and their associated costs in field applications, we propose that ammonium bicarbonate's environmentally friendly and cost-effective characteristics make it more suitable for assisting farmers in mitigating the impact of clubroot on cruciferous vegetables in the field. This approach aims to maximize crop yield and minimize economic losses associated with *P. brassicae* infestation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy14020377/s1, Figure S1: Clubroot resistance assay on Chinese cabbage 'Granaat' under CK (a) and treatments of plastic film covering (b), calcium cyanamide at 90 g/m^2 (c), chloroisobromimne cyanuric acid at 4.5 g/m^2 (d), dazomet at 37.5 g/m^2 (e), and ammonium bicarbonate at 150 g/m^2 (f) in greenhouse conditions (22 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}). (g) represents a 150 g/m^2 ammonium bicarbonate treatment at a higher temperature (32 ± 2 °C day/18 ± 2 °C night, 16 h photoperiod with 300 μmol·m^{-2}·s^{-1}); Table S1: Evaluation of parameters in Chinese cabbage cultivar 'Granaat' under different treatments for clubroot control in greenhouse conditions; Table S2: Measurements of the root length of 'Granaat' in MS medium with different concentrations of NAA; Table S3: Disease indices of 'Granaat' under different treatments for clubroot control in field conditions.

Author Contributions: Conceptualization, Y.Z. and X.Y.; Methodology, Y.Z. and H.Y.; Software and Validation, T.Z., X.M. and Y.W.; Formal Analysis, Y.Z. and H.Y.; Investigation, Y.Z., H.Y., T.Z., X.M., Y.W., K.L., N.S. and X.Y.; Writing—Original Draft Preparation, Y.Z.; Writing—Review and Editing, I.H.; Visualization, Y.Z. and I.H.; Supervision, Project Administration, and Funding Acquisition, X.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available in the main text and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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