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RESEARCH ARTICLE

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Inhibitory efficacy of calcium cyanamide on the pathogens of replant diseases in strawberry

Abstract Replant diseases in strawberry caused by *Rhizoctonia solani, Fusarium oxysporum* and *Verticillium dahliae* are serious problems for its sustainable production under continuous cropping. This research studied the inhibitory effect of calcium cyanamide on pathogenic fungi in Petri dishes and on sterilized soil. Results indicated that calcium cyanamide had an obvious inhibitory effect on three pathogens on potato dextrose agar (PDA) plates. Among them, the inhibitory effect on *Rhizoctonia solani* was the highest. As the concentrations of calcium cyanamide was increased from 0.1 to 10 mg/mL, the inhibition rate on mycelial growth increased from –1.43% to 100%. Inhibitory effects on *Fusarium oxysporum* and *Verticillium dahliae* also existed on Petri dishes but to a lesser extent. Similar results were also observed in sterilized soil. When the concentration of calcium cyanamide in sterilized soil was 0.1%, the inhibitory effect on *Fusarium oxysporum* and *Verticillium dahliae* was 68.46% and 54.46%, respectively. The inhibitive effect of calcium cyanamide on *Fusarium oxysporum* and *Verticillium dahliae* increased quickly as the soil moisture changed from 10% to 40% for *Verticillium dahliae* and from 10% to 60% for *Fusarium oxysporum*. This indicated that the inhibitive effect of calcium cyanamide could be influenced greatly by the moisture content in the soil.

Keywords calcium cyanamide, replant disease of strawberry, inhibitory efficacy

1 Introduction

With the rapid development of strawberry production, replant disease in strawberry caused by *Rhizoctonia solani*, *Fusarium oxysporum* and *Verticillium dahliae* individually or jointly has been a serious problem (Zhen et al., 2005). The pathogens can slow down plant growth and yield and cause economic losses to the strawberry industry. This disease is also an epidemic in strawberry plantations worldwide.

At present, chemical control of replant diseases is the main method to manage the problem. Soil fumigants such as methyl bromide are widely used in small fruit production in many countries (De Cal et al., 2004). However, because of its harmful impact on the ozone layer, methyl bromide will be totally banned in different countries by 2015 according to the Montreal Protocol. These limitations have put much pressure on people searching for alternative products to substitute for methyl bromide. Calcium cyanamide has a long history in agriculture as a fertilizer. It can meliorate the soil, prevent soil acidification, raise yield and improve quality. In addition, calcium cyanamide as a soil fumigant for controlling soil-borne diseases is effective in suppressing clubroot disease in cabbage, fusarium wilt in cucumber and melon, etc. It has been reported that soil sterilization with calcium cyanamide has positive effects on growth, production and fusarium wilt control in melon compared with methyl bromide soil fumigation (Bletsos, 2005). Zhu et al. (2001) showed that calcium cyanamide could effectively control spinach rhizoctonia rot and strawberry fusarium wilt, kill some pest and viruses in the soil and reduce the extent of soil diseases. Although many studies on the effect of calcium cyanamide on soil sterilization have been reported, few were focused on related studies on different soil pathogens. Therefore, it is important to study the mechanisms of calcium cyanamide for disease control, especially its impact on pathogen population in the soil. In this paper, the inhibitory effects of calcium cyanamide against pathogenic fungi on potato dextrose agar (PDA) plates as well as in sterilized soil under different soil water content were assessed.

2 Materials and methods

2.1 Materials

Three pathogenic fungi, *Rhizoctonia solani* Kühn, *Fusarium oxysporum* Schl. f. sp. *fragariae* Winks et Williams and

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Verticillium dahliae Kelb were provided by the lab of Plant Disease Epidemiology and Integrated Control, Agricultural University of Hebei. The three fungi were incubated at 25°C on PDA. The fresh cultures of seven to ten days were used for all the testing.

Calcium cyanamide, a black powder with 20% nitrogen content, was provided by Ningxia Darong Group.

Soil collected from the strawberry plantation of Hebei Agricultural University was dried in an electric thermostatic drying oven and put into conical flasks (250 mL). Each flask contained 100 g of soil. The conical flasks were then autoclaved three times at 121°C for one hour for three consecutive days.

2.2 Methods

2.2.1 Inhibitory effect of calcium cyanamide against the growth of pathogenic fungi on PDA plates

The invitro test of calcium cyanamide on the growth of three pathogenic fungi included five different concentrations: 10.0, 5.0, 1.0, 0.5, and 0.1 mg/mL. After the PDA media were melted and cooled to 45°C, 1 000 mg, 500 mg, 100 mg, 50 mg and 10 mg of calcium cyanamide, sterilized under ultraviolet radiation for half an hour, were added into each flask containing 100 mL PDA. The mixtures were shaken until the calcium cyanamide had totally dissolved in the media, then the mixtures were plated on four sterilized Petri dishes (9.0 cm in diameter) immediately. A medium without calcium cyanamide served as the control.

A 0.5 cm diameter agar disk, taken from one-week-old fungal cultures, was placed with the fungal side up in the center of each plate then incubated in the dark at 25°C. Radial growth was determined by measuring the colony size along two diameters at right angles after three days for *Rhizoctonia solani*, seven days for *Fusarium oxysporum* and ten days for *Verticillium dahliae*. Fungitoxicity was expressed in terms of percentage of mycelial growth inhibition and calculated according to the formula of Pandey et al. (1982)

$$(dc - dt)/dc \times 100,$$

where dc = average diameter of fungal colony with control and dt = average diameter of fungal colony with treatment.

2.2.2 Effect of calcium cyanamide on pathogenic fungi in sterilized soil

Spore suspensions of *Fusarium oxysporum* and *Verticillium dahliae* were prepared by adding sterile distilled water directly into the Petri dishes which were then incubated at 25°C for ten days. Suspensions were filtered through four layers of cheesecloth to remove fungal mycelium. The population density of spores was adjusted to 10^6/mL as measured by a hemacytometer. Each flask containing 100 g sterilized soil was inoculated with 6 mL spore suspension and the soil moisture content was kept at 15% by adding 9 mL sterile distilled water. After storing at 25°C for three days, 1 000 mg, 500 mg, 100 mg, 50 mg, and 10 mg of calcium cyanamide was added to each flask. For better mixing, each flask was shaken for half a minute. A flask without calcium cyanamide served as the control. Four replicates for each treatment were prepared and all steps were operated in a laminar flow cabinet.

After seven days, 10 g of the soil mixture was taken from each flask and suspended in 90 mL of sterilized water in 250 mL flasks and shaken for 30 min at 150 r/min. Then 10 to 100 fold dilutions were made, and 20 μL aliquots from undiluted and diluted suspensions were spread onto Petri dishes containing PDA amended with streptomycin at 0.5 g/L. Three replicates were used for each dilution. Petri dishes were kept at 25°C for three days and then the populations of soil fungi were measured by counting fungal colonies directly. The inhibitory effect was determined relative to the control and calculated by the following equation

Inhibition rate (%) = (Colony number of control − colony number of treatment)/(colony number of control) × 100.

2.2.3 Influence of different soil moisture on the function of calcium cyanamide

Ten millilitres of spore suspension was inoculated into 100 g sterilized dry soil and then stirred evenly by a rod. The pathogens were cultured for three days at 25°C and then 100 mg of calcium cyanamide was added to each flask. The soil moisture content was kept at 10%, 20%, 40%, 60% and 80% by adding 0 mL, 10 mL, 30 mL, 50 mL and 70 mL sterilized water, respectively. After seven days, populations of soil fungi and the inhibition rate of each treatment were estimated and calculated by the same equation in 2.2.2.

2.2.4 Data analysis

Data from the experiments were analysed using DPS software (Zhejiang University, version 3.01, Tang, NC). Duncan's method was used to determine significant differences at $P = 0.05$ level.

3 Results and analysis

3.1 Influence of calcium cyanamide on mycelial growth of pathogens

The results presented in Table 1 indicated that calcium cyanamide had a high inhibitory effect on the mycelial growth of *Rhizoctonia solani*, *Fusarium oxysporum* and *Verticillium dahliae*. In general, the inhibition rates increased with an increase in calcium cyanamide concentration.

Among the three pathogens, *Rhizoctonia solani* seemed to be the most sensitive one. When the dosage of calcium cyanamide was 0.5 mg/mL, the inhibition rate reached

Table 1 Inhibitory effect of different dosages of calcium cyanamide on three pathogens of strawberry

Dosages of calcium cyanamide/mg · mL^{-1}	*Rhizoctonia solani*		*Fusarium oxysporum*		*Verticillium dahliae*	
	Colony diameter/cm	Inhibitive rate/%	Colony diameter/cm	Inhibitive rate/%	Colony diameter/cm	Inhibitive rate/%
10.0	0.52 a	99.78	1.27 a	88.04	2.95 a	54.51
5.0	1.27 b	89.90	3.93 b	46.87	4.20 b	31.31
1.0	2.45 c	74.37	5.55 c	21.86	4.99 c	16.71
0.5	3.97 d	54.42	6.11 c	13.14	5.06 c	15.36
0.1	8.22 e	–1.43	6.92 c	0.62	5.36 c	9.70
CK	8.11 e		6.96 c		5.89 d	

Note: The same letter in each column means there is no significant difference according to Duncan's test at $P = 0.05$.

54.42%. According to the statistical analysis, the diameter of mycelium growth at this dosage was significantly less than that of the control. Treatment with calcium cyanamide at 10 mg/mL had the highest inhibition rate (99.78%). Calcium cyanamide also had a stronger inhibitory effect against mycelial growth of *Fusarium oxysporum* on PDA Petri dishes. At the dosages of 10.0 mg/mL and 5.0 mg/mL, the inhibition rates reached 88.04% and 46.87%, respectively. The colony diameter of the two dosages was significantly less than that of the control, but at the dosages of 0.1 to 1.0 mg/mL, it was the same size as the control. The growth of *Verticillium dahliae* was also suppressed, but to a lesser extent compared with the other two pathogens. The highest inhibition rate was 54.51% at the dosage of 10 mg/mL.

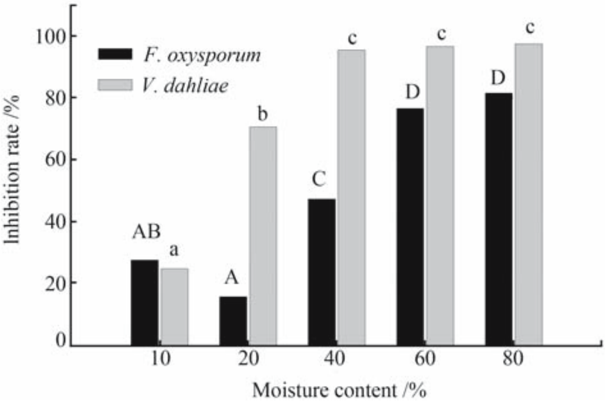
3.2 The inhibitory effect of calcium cyanamide on the pathogens in sterilized soil

In this test, the inhibitory effects of calcium cyanamide on populations of *Fusarium oxysporum* and *Verticillium dahliae* in the soil were simulated in flasks. To prevent disturbance from other microorganisms, the soil was sterilized in advance. After treatment with calcium cyanamide for seven days, the soil samples were taken, diluted and cultured on PDA Petri dishes. The numbers of colonies were recorded. Table 2 shows the inhibition rates compared with the control.

In Table 2, both *Fusarium oxysporum* and *Verticillium dahliae* were significantly suppressed by different dosages of calcium cyanamide. At the dosage of 500 mg/100 g, the inhibition rates reached as high as 99.9%. At the dosage of 1 000 mg/100 g, the growth of both pathogens was completely suppressed. As the dosages were decreased, the inhibitory effects also reduced quickly. When the dosage was at 50 mg/100 g, the inhibitory effects were rather low and could not play a role in killing the pathogen spores.

3.3 Influence of soil moisture content on inhibitory effects of calcium cyanamide to the pathogens

Fig. 1 shows the influence of soil moisture content on the inhibitory effect of calcium cyanamide to *Fusarium oxysporum* and *Verticillium dahliae*. The results indicated that the inhibitory effect of calcium cyanamide to both pathogens increased with the increase in soil moisture content. No significant difference was found in inhibition rates between the moisture contents of 10% and 20% for *Fusarium*



Note: The letters in the figure show the statistical analysis by Duncans' test at $P = 0.05$ level. The capital letters are for *F. oxysporum* and small letters are for *V. dahliae*.

Fig. 1 Influence of soil moisture content on inhibitory effects of calcium cyanamide on *Fusarium oxysporum* and *Verticillium dahliae*

Table 2 The inhibitory effect of different dosages of calcium cyanamide on *Fusarium oxysporum* and *Verticillium dahliae*

Dosages of calcium cyanamide (mg/100 g soil)	*Fusarium oxysporum*		*Verticillium dahliae*	
	Number of colony/CFU · g^{-1}	Inhibitive rate/%	Number of colony/CFU · g^{-1}	Inhibitive rate/%
1 000	0 a	100.00	15 a	99.98
500	125 a	99.85	37 a	99.94
100	25 625 b	68.46	13 000 b	54.46
50	67 500 c	16.92	17 575 c	38.44
CK	81 250 c	—	28 550 d	—

Note: The same letter in each column means there is no significant difference according to Duncan's test at $P = 0.05$.

oxysporum, but the inhibition rate at 40% was significantly higher than that at 20%. When the moisture content was 60%, the inhibitory effect of calcium cyanamide on *F. oxysporum* reached 76.4%, but this had no significant difference with the inhibition rate at 80%. *Verticillium dahliae* seemed to be more sensitive to calcium cyanamide as the moisture content was increased. The inhibition rate reached 95.2% even when the moisture content in soil was only 40%. The inhibition rates dropped greatly at the moisture contents of 20% and 10%, but it did not increase significantly any further when the moisture content was increased.

The results above show that the inhibitory effect of calcium cyanamide was related to the moisture content of soil. Perhaps the water in the soil is helpful for producing dicyandiamide—a poisonous gas, which can kill pathogens. Moreover, high moisture content could also suppress the growth of the fungi.

4 Discussion

In order to control replanting diseases in a more sustainable and environment friendly way, seeking for alternative materials instead of commonly used soil fumigants is getting much attention. Calcium cyanamide, used in this study, had a significant effect in suppressing mycelial growth on Petri dishes and fungal population in soil.

Based on the plate experiment, the inhibitory effect of calcium cyanamide was stronger on *Rhizoctonia solani* than on *Fusarium oxysporum* and *Verticillium dahliae*. At the same dosage of calcium cyanamide, the mycelium growth of *Verticillium dahliae* was the least sensitive one.

Experiment results showed that calcium cyanamide can significantly reduce the population of pathogenic fungi in the soil at certain dosages. Moreover, regulating the moisture content in the soil can promote the effect of calcium cyanamide. In fact, soil is a complex environment and soil temperature, humidity, acidity and microbes can influence the effect of calcium cyanamide directly or indirectly. This experiment is a kind of a simulation test to prove the inhibitory effect of calcium cyanamide under rather stable temperatures and without the presence of other microorganisms. Further tests need to be done on natural soil under different temperature and acidity.

The inoculant of *Rhizoctonia solani* is a hyphae, which is difficult to distribute uniformly in soil. Another problem in the experiment was to re-isolate *Rhizoctonia solani* from the treated soil. We do not know yet whether *Rhizoctonia solani* survived after being inoculated into the soil or they were totally killed by calcium cyanamide because they were the most sensitive fungus on the basis of the experiment on Petri dishes. These difficulties prevented us from getting valuable results of this fungus in the soil.

Calcium cyanamide originally was used as a fertilizer to supply nitrogen to soil. Later, people found that it could control soil-borne diseases, because during the decomposing process, calcium cyanamide produced dicyandiamide in water (Rieder, 1981). Since then it has been used quite often on strawberry in Spain, on vegetables in Japan and in all EU countries to overcome the problems of insufficient crop rotation (De Cal, 2004). Currently in China, people have started to use it to control soil-borne disease on vegetables, such as on celery root knot nematode in Shandong, and on spinach and soybean in Zhejiang, etc. (Li et al., 2004; Zhu et al., 2001). However, there are few reports of its use on strawberry. Strawberry is an important fruit in early spring in the northern part of China. Replant disease in strawberry is one of the most severe problems compared with other crops. For this reason, it has caused almost a quarter of the methyl bromide use in agriculture in China. Finding a successful alternative to methyl bromide on replant disease control on strawberry could bring much benefit not only to the economy but also to the environment. This research proved that calcium cyanamide could inhibit the growth of the three major pathogens of strawberry on Petri dishes and the populations in soil, which formed the theoretical bases for using it in replant disease control on strawberry.

The basic dosages used in this experiment were decided on the basis of the usage in practice (1 125 kg/hm^2, equal to 0.05% of weight of cultivated surface soil). Results in this research showed that in order to get a high control effect, the dosage must be enough and the soil also has to be kept on a better moisture condition. Although the price at a dosage of 0.5% (500 mg/100 g soil) was almost the same as that for methyl bromide, considering it could reduce the use of fertilizer and be environment friendly, it has much potential to be used in strawberry production in the near future. Because dicyandiamide is an evaporable gas, a better covering with plastic film is necessary when using it in the field. This research also showed that calcium cyanamide had a better inhibitory effect when the moisture content in soil was high. A thorough irrigation under plastic film is therefore needed as soon as the calcium cyanamide is used in soil.

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