WANG Lijing, HU Tongle, JI Lijing, CAO Keqiang

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

Abstract Replant diseases on strawberry caused by *Rhizoctonia solani, Fusarium oxysporum* and *Verticillium dahliae* are serious for the sustainable production of strawberry under continuous cropping. This research assayed the inhibition effect of calcium cyanamide against pathogenic fungi of replant disease of strawberry on Petri dishes and on sterilized soil. Results indicated that calcium cyanamide had obvious inhibitory effect on three pathogens on PDA plate. Among them, the inhibition effect on *Rhizoctonia solani* was the highest. As the concentrations of calcium cyanamide increased from 0.1 mg/mL to 10 mg/mL, the inhibition rate on mycelial growth reached from -1.43% to 100%. Inhibition effect on *Fusarium oxysporum* and *Verticillium dahliae* also existed on Petri dishes but with less 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* reached 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.* It indicated that the inhibitive effect of calcium cyanamide could be influenced greatly by 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 on strawberry caused by *Rhizoctonia solani, Fusarium oxysporum* and *Verticillium dahliae* solely or jointly has been a serious problem (Zhen et al., 2005). The pathogens can depress plant growth, decrease yield and cause economic loss to the strawberry industry, and this disease is also an epidemic in strawberry plantation worldwide.

At present, chemical control to replant disease is the main method to manage the problem. Soil fumigant such as methyl bromide is widely used in small fruit production in many countries (De Cal et al., 2004). However, because of the harmful impact on the ozone layer, methyl bromide will be totally banned in different countries not later than 2015 according to Montreal Protocol. These limitations laid much pressure for people searching for alternative products to substitute the use of 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 to control soil borne disease, has an efficiency on 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 virus in soil and reduce the extent of soil diseases. Although many studies on the effect of calcium cyanamide on

Wang Lijing, Hu Tongle, Ji Lijing, Cao Keqiang (author for correspondence)
Bio-control Center of Plant Disease and Pests of Hebei Province,
College of Plant Protection, Agricultural University of Hebei,
Baoding 071001, China
E-mail: ckq@hebau.edu.cn

soil sterilization have been reported, few were focused on related studies on variation of soil pathogens. Therefore, it is important to study the mechanisms of calcium cyanamide for disease control especially the impact on pathogens population in soil. In this paper, the inhibition effects of calcium cyanamide against pathogenic fungi on PDA plate as well as in sterilized soil under different soil water contents 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 *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℃ on potato dextrose agar (PDA). The fresh cultures of 7 to 10 days were used for all the testing.

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

Soil, collected from the strawberry plantation of Hebei Agricultural University, was dried by electric thermostatic drying oven and put into conical flask (250 mL). Each flask contained 100 g soil. Then the conical flasks were autoclaved three times at 121℃ for one hour on successive three days.

2.2 Methods

2.2.1 Inhibition effect of calcium cyanamide against the growth of pathogenic fungi on PDA plate

The *in vitro* test of calcium cyanamide on the growth of three pathogenic fungi included five different concentrations, i.e. 10.0, 5.0, 1.0, 0.5, and 0.1 mg•mL^{-1}. After the PDA media were melted and cooled to 45℃, 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 totally dissolved in the media, then the mixtures were plated in four sterilized Petri dishes (9.0 cm in diameter) immediately. The medium without calcium cyanamide served as control.

A 0.5 cm diameter agar disk, taken from one-week-old fungal culture, was placed with the fungal side upward in the center of each plate which was incubated in the dark at 25℃. Radial growth was determined by measuring the colony size along two diameters at right angles after 3 days for *Rhizoctonia solani*, 7 days for *Fusarium oxysporum* and 10 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×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 incubated at 25℃ for 10 days. Suspensions were filtered through four layers of cheesecloth to remove fungal mycelium. The population density of spores was adjusted to 10^6/mL measured by Hemacytometer. Each flask containing 100 g sterilized soil was inoculated with 6 mL spore suspension and the soil moisture content was regulated to 15% by adding 9 mL sterile distilled water. After maintained at 25℃ for three days, 1000 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. The flask without calcium cyanamide served as control. Four replicates for each treatment were prepared and all steps were operated in 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 rpm. 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 3 days and then the populations of soil fungi were measured by counting fungal colonies directly. The inhibition effect was determined relatively 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 millilitre of spore suspension was inoculated into 100 g sterilized dry soil and then stirred evenly by a rod. The pathogens were cultured for 3 days at 25℃ and then 100 mg of calcium cyanamide was added to each flask. The soil moisture content was regulated to 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 method above.

2.2.4 Data analysis

Data from the experiments were subjected to an analysis of 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 high inhibiton effect on mycelial growth of *Rhizoctonia solani*,*Fusarium oxysporum* and *Verticillium dahliae*. In general, the inhibition rates increased with the increment of 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 inhibiting rate reached 54.42%. According to statistic analysis the diameter of mycelium growth at this dosage was significantly less than that of control. Treatment with calcium cyanamide at 10 mg/mL had the highest inhibition rate (99.78%). Calcium

cyanamide also had stronger inhibiting 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 inhibiting rates reached 88.04% and 46.87%, respectively. The colony diameter of the two dosages were significantly less than that of control, but the colony diameter of the dosage at 0.1 mg/mL to 1.0mg/mL was at the same level with

Dosages of calcium cyanamide （mg/mL）	*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	

control. The growth of *Verticillium dahliae* was also suppressed but with less extent compared to the other two pathogens. The maximum inhibiting rate was 54.51% at the dosage of 10 mg/mL.

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

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

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

In this test, the inhibitive effects of calcium cyanamide on population of *Fusarium oxysporum* and *Verticillium dahliae* in the soil were simulated in flasks. To prevent the disturbance of other microorganisms, the soil was sterilized in advance. After treated for seven days by calcium cyanamide, the soil samples were taken, diluted and cultured on PDA Petri dishes. The numbers of colonies were recorded. Table 2 showed the inhibitive rates compared to check.

Table 2　The inhibitive effect of different dosage 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)	Inhibitive rate （%）	Number of colony (CFU/g)	Inhibitive rate （%）
1000	0 a	100.00	15 a	99.98
500	125 a	99.85	37 a	99.94
100	25625 b	68.46	13000 b	54.46
50	67500 c	16.92	17575 c	38.44
CK	81250 c	—	28550 d	—

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

From Table 2 both *Fusarium oxysporum* and *Verticillium dahliae* were significantly suppressed by different dosages of calcium cyanamide. With the dosage of 500 mg/100 g, the inhibitive rates reached as high as 99.9%. At the dosage of 1000 mg/100 g, the growth of both pathogens was absolutely suppressed. As the dosages getting less, the inhibitive effects were reduced quickly. When the dosage was at 50 mg/100 g, the inhibitive effects were rather low and could not play a role in killing the pathogen spores.

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

Fig. 1 showed the influence of soil moisture content on the inhibition effect of calcium cyanamide to *Fusarium oxysporum* and *Verticillium dahliae*. The results indicated that the inhibition effect of calcium cyanamide to both pathogens increased with the increasing of soil moisture content. No significant difference was found in inhibition rates between the moisture contents of 10% and 20% for *Fusarium oxysporum*, but the inhibitive rate at 40% was significantly higher than that of 20%. Once the moisture content reached 60%, the inhibitive effect of calcium cyanamide to *F. oxysporum* reached 76.4% which had no significant difference with the inhibition rate at 80%. *Verticillium dahliae* seemed to be more sensitive to calcium cyanamide as the moisture content increased. The inhibition rate soon reached to 95.2% as the moisture content in soil was 40%. The inhibition rates did not increase significantly further more as the moisture contents increased, while dropped greatly at the moisture contents at 20% and 10%.

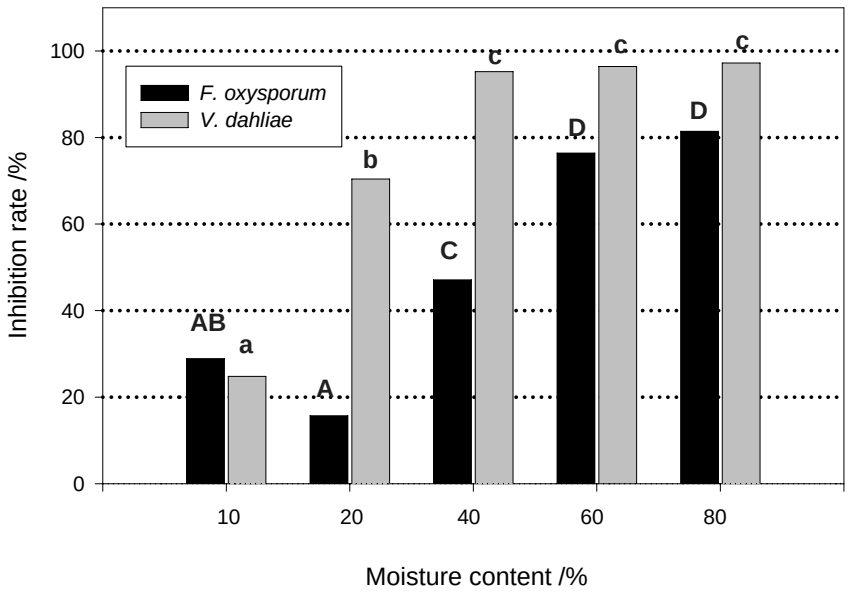

Fig. 1 Influence of soil moisture content on inhibition effects of calcium cyanamide to *Fusarium oxysporum* and *Verticillium dahliae*. (Note: The letters in the figure showed the statistical analysis by Duncans' test at P=0.05 level. The capital letters were for *F. oxysporum* and small letters were for *V. dahliae.*）

Results above showed that the inhibitive effect of calcium cyanamide was related with the moisture content of soil. Maybe the water of soil is helpful for producing the dicyandiamide -- a

poisonous gas, which can kill the pathogens in the soil. 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 concerned. Calcium cyanamide used in the experiment had significant effect to suppress mycelial growth on Petri dishes and fungal population in soil.

Based on the plate experiment, the inhibiting effect of calcium cyanamide was stronger on *Rhizoctonia solan*, than on *Fusarium oxysporum* and *Verticillium dahliae*. For 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 when calcium cyanamide reached certain dosages. Moreover, regulating moisture content of soil can promote the effect of calcium cyanamide. In fact soil is a complex environment, soil temperatures, humidity, acidity and microbes can influence the effect of calcium cyanamide directly or indirectly. This experiment is a kind of simulation test to prove the inhibiting effect of calcium cyanamide under rather stable temperature and without exist of other microorganisms. Further tests need to be done when natural soil was used and under different temperature and acidity. The inoculant of *Rhizoctonia solani* is 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 don't know yet whether *Rhizoctonia solani* survived after being inoculated into soil or they were totally killed by calcium cyanamide because they were the most sensitive fungus based on the experiment on Petri dishes. These difficulties prevented us from getting valuable results of this fungus in soil.

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

The basic dosages used in this experiment were decided based on the usage in practice 1125 kg/ha (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 soil also has to be kept in 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 by plastic is necessary when use it in field. This research also showed that calcium cyanamide had better inhibitive effect when the moisture content in soil is high, so a thorough irrigation under plastic is needed soon after the calcium cyanamide was used in soil.

References

Bletsos F A (2005). Use of grafting and calcium cyanamide as alternatives to methyl bromide soil fumigation and their effects on growth, yield, quality and fusarium wilt control in melon. Journal of Phytopathology, 153(3): 155—161

De Cal A, Martinez-Treceño A, Lopez-Aranda J M, and Melgarejo (2004). Chemical alternatives to methyl bromide in Spanish strawberry nurseries. Plant Disease, 88: 210—214

Gu Z R, Wei C M, Ma C Z (2005). Factors affecting inhibition of *Bacillus subtilis* G3 against sclerotial formation of *Rhizoctonia solani* in soil. Chinese Journal of Biological Control, 21(1): 33—36 (in Chinese)

Kurze S, Bahl H, Dahl R, Berg G (2001). Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Disease, 85: 529—534

Li L, Qi J S, Li C S, Xu Z T (2004). Studies on soil treated by CaCN2 and solarization to control meloidogyne spp. infecting vegetable crops. Journal of Laiyang Agricultural College, 21(2): 122—124 (in Chinese)

Pinkerton J N, Ivors K L, Reeser P W, Bristow P R, Windom G E (2002). The use of soil solarization for the management of soilborne plant pathogens in strawberry and red raspberry production. Plant Disease,. 86: 645—651

Rieder G (1981). Calcium cyanamide-fertilizer or pesticide? World crops, January : 17—21

Zhen W C, Cao K Q, Dai L, Hu T L (2005). Management of strawberry (*Fragria ananassa* Duch) replanting problem by soil amendments of medicinal herbs. Scientia Agricultura Sinica, 38(4): 730—735 (in Chinese)

Zhou E X, Yang M, Chen Y L (2002). The effects of soil environmental factors on the saprophytic colonization of *Rhizoctonia solani*. Acta Phytopathologica Sinica, 32(3): 214—218 (in Chinese)

Zhu B L, Ma J W, Ye X Z, Xia Z M (2001). Effects of lime-nitrogen on soil ameliorate and vegetables production. Journal of Zhejiang University (Agric. & Life Sci.), 27(3): 339—342 (in Chinese)