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DEVELOPMENT AND APPLICATION METHODS FOR POPLAR CANKER BIOCONTROL AGENTS

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Abstract

The joint application of *Trichoderma aureoviride* strain YGF9 and *Fusarium equiseti* strain LX6F2, isolated from poplar tissue and soil, respectively effectively controled the occurrence of poplar canker. The original biocontrol agent powders were prepared with maize flour and wheat bran as the raw materials by solid fermentation. The optimal formula of the YGF9 was 38% original powder, 38% kaolin, 13% sodium lignin sulfonate, 8.5% SDS and 2.5% humic acid. The optimal formula of LX6F2 was 42% original powder, 36% kaolin, 12% Twain-20, 8.0% nekal BX and 2.0% ascorbic acid. This will help to provide a new choice of the biological control of poplar canker.

Introduction

Poplar canker, caused by *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., is one of the leading diseases of the poplar, which could lead to seedling growth failure, afforestation failure as well as economic and ecological losses (Gong *et al*. 2009). Polar blister canker is common in the northeast and northwest of China (Wang and Wu 2008), and the incidence could be as high as 96.1% -100% in the worst areas and disease index could be 6.7 - 95.8% (Huang and Su 2003).

To control poplar blister canker, chemical fungicides were used for a long time. However, the application of fungicides may result in plant pathogenic fungi developing resistance (Agrios 2005, Benítez *et al*. 2004, Kim and Hwang 2004). Many of these chemicals are also too expensive for the resource-poor farmers (Raju *et al*. 2003). Many researches in the past two decades make biological control became an increasingly realistic option on disease management. Furthermore, biological pesticides become more popular than chemical pesticides in the prevention and control of plant diseases (Gu and Jiang 2000) since biological pesticides have fewer side-effects, like harmless to people and environmental friendly, etc.

Because of the wider application of biocontrol pesticides, it is very important to find suitable production methods of resistant biocontrol agent strains as antimicrobials being raw materials for making biocontrol bacterial agents, are an important part of biological pesticides. A key problem is the lack of knowledge about the production and long-term storage of biocontrol agents (Xue *et al*. 2014). In previous studies, we isolated and screened *Trichoderma viride* strain YGF9 (Yang *et al*. 2014) and *Fusarium equiseti* strain LX6F2 (Yang *et al*. 2015a) with *Botryosphaeria dothidea* as the target strain from poplar organization and poplar forests soil, respectively. They showed good biocontrol effects on poplar canker (Yang *et al*. 2015b). In this paper, these two biocontrol strains are taken as the original agents. Suitable solid fermentation substrates and conditions for the original powder were found using the single factor and orthogonal design patterns. Then, best

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carrier, wetting agents, dispersing agents and UV protecting agent type and ratio were screened and developed into poplar canker biocontrol agents which demonstrate good performance and researched techniques for their application for the forest. This study aims to lay the foundations for the further development and utilization of these two excellent biocontrol strains and provide a new biological control for poplar canker.

Materials and Methods

Botryosphaeria dothidea (Moug. *ex* Fr.) Ces. & De Not. (Fam.: Botryosphaeriaceae) collected from the forest protection key laboratory of China state forestry administration, number 72 (2) B. The YGF9 and LX6F2 strains were obtained through separation filter. Solid fermentation was done to prepare the original powder because solid-state fermentation have been considered as the best solid nutrient medium.

The appropriate microbial inoculum dosage forms were determined according to the biological properties of the biocontrol strains. Depending on these dosage forms, biocontrol agents were configured by adding optimal fillers, additives and protective agents.

The test agents were made by mixing the original powder and the selected carrier, dispersing agent, wetting agent and protective agent in a ratio of 40: 40: 10: 5: 5. The original powder without auxiliaries was used as the control, and the best auxiliaries were determined by measuring the antagonist activity and the number of viable cells after normal preparation at 30°C for 7 days.

The test carriers were kaolin, calcium carbonate and diatomaceous earth. The wetting agents used were SDS, Gleditsia powder and Nekal BX. The dispersants used were sodium lignosulfonate, CMC and Tween 20. The UV protecting agents used were humic acid, fluorescein sodium and ascorbic acid. The design of the auxiliaries selection experiment is shown in Table 1.

Table 1. The orthogonal experiment of auxiliaries selection.

Factors	Carriers	Wetting agents	Dispersants	UV protecting agents
Test 1	Kaolin	SDS	sodium lignosulfonate	Humic acid
Test 2	Kaolin	Gleditsia powder	CMC	Fluorescein sodium
Test 3	Kaolin	Nekal BX	Tween 20	Ascorbic acid
Test 4	Calcium carbonate	SDS	CMC	Ascorbic acid
Test 5	Calcium carbonate	Gleditsia powder	Tween 20	Humic acid
Test 6	Calcium carbonate	Nekal BX	sodium lignosulfonate	Fluorescein sodium
Test 7	Diatomaceous earth	SDS	Tween 20	Fluorescein sodium
Test 8	Diatomaceous earth	Gleditsia powder	Sodium lignosulfonate	Ascorbic acid
Test 9	Diatomaceous earth	Nekal BX	CMC	Humic acid

The performance of each antifungal agent group was comprehensively assessed with dispersion, wetting, suspension, viable cell count and inhibition activity.

After these three types of measurement were completed, the inhibition rate and live spore rate of the soluble powders were compared with different additives against CK (without additives).

The optimization of auxiliary proportions was conducted according to the following table:

Table 2. The optimization of auxiliary proportion.

Group	Original powder (%)	Carriers (%)	Wetting agents (%)	Dispersing agents (%)	Protecting agents (%)
1	50	32	10	7.0	1.0
2	46	34	11	7.5	1.5
3	42	36	12	8.0	2.0
4	38	38	13	8.5	2.5
5	34	40	14	9.0	3.0

Each component sample was configured as above. They were mixed evenly and then 1g of the agent was dissolved in 100 ml and thoroughly shaken. The number of viable cells and antagonist activity was then detected. Each procedure repeated for three times.

The configured agents were diluted 500-fold with water, mixed evenly and then applied on the poplar cutting seedlings. There were a set of 12 different application methods: A: Only sprayed with agent YGF9 (YP): a small sprayer was used to spray the agent diluted uniformly on the surface of cutting seedlings, and the surface was wet, but there were no significant drips. B: Only sprayed with agent LX6F2 (LP). C: Sprayed with agentsYGF9 and LX6F2 simultaneously (YP+LP). D: Roots were irrigated with agent YGF9 (YG) only: root irrigating device was used in order to irrigate the cutting seedlings with the diluted agent, 50-100 ml/strain, and the rhizosphere of soil appropriate. E: Roots were irrigated with agent LX6F2 (LG) only. F: Roots were irrigated with agents YGF9 and LX6F2 simultaneously (YG+ LG). G: Cutting seedlings were sprayed with agent YGF9 and roots were irrigated with agent LX6F2 (YP+ LG). H: Cutting seedlings were sprayed with agent LX6F2 and roots were irrigated with agent YGF9 (YG + LP). I: Cutting seedlings were sprayed with agent YGF9 simultaneously and roots were irrigated with agent YGF9 (YG+ YP). J: Cutting seedlings were sprayed with agent LX6F2 simultaneously and roots were irrigated with agent LX6F2 (LG+ LP). K: Roots were irrigated and cutting seedlings were sprayed with water simultaneously as a negative control. L: Roots were irrigated and cutting seedlings were sprayed with carbendazim simultaneously, diluted 500-fold, as a positive control.

B. dothidea was inoculated the on the healthy poplar seedling limb. The incidence and disease indices of the seedling canker were observed after 20 days. The scabs were divided into five classes, according to the expansion in the diameter of the scab, as shown in Table 3.

The application concentration was setting as follows: Experimental treatments A (the blank control): water spray and irrigation of roots. Experimental treatments B: biocontrol bacterial liquid which was diluted 100-fold with water spray and irrigation of roots. Experimental treatments C: biocontrol bacterial liquid which was diluted 500-fold with water spray and irrigation of roots. Experimental treatments D: biocontrol bacterial liquid which was diluted 1000-fold with water spray and irrigation of roots. Experimental treatments E (determination of chemical pesticide control efficacy): 75% carbendazim, diluted 500-fold with water spray and irrigation of roots.

Each experimental treatment used a selection of nine potted cutting seedlings which had the same growing trend. The agents were applied once every 15 days, three times in total. A survey was conducted at 10 days after the last treatment. The disease index was documented and the final control efficacy was calculated. There was a simultaneous determination at the impact of the biocontrol agents on plant growth of the survey, based mainly on the measurement of the height and stem diameter of each seedling.

Table 3. Disease grading standards of the seedling morbidity experiment.

Scab diameter (mm)	Grading	Central value
0	0	0
1 - 4.99	I	1
5 - 9.99	II	2
10 - 14.99	III	3
15 - 19.99	IV	4
≥20	V	5

The disease index is given by Σ (Disease stage number × Representative values)/Total plants × incidence of the most important level representative value×100. The control efficiency = (disease index of control - disease index of test group)/Disease index of the control × 100.

Results and Discussion

When the solid culture substrate screening results and fermentation conditions are integrated, the most suitable method of preparation of the original powder of the YGF9 strains was: 30 ml of water in a mixed substrate of 35 g corn flour and 35 g wheat bran with 20 ml YFG9 strain seed fermentation liquid, cultured for 7 days at 30°C dried naturally. The most suitable method of preparation for the original powder of the LX6F2 strains was: 50 ml of water in a mixed substrate of 25 g corn flour and 25 g wheat bran and 10 ml LX6F2 strain seed fermentation liquid, cultured for 7 days at 30°C, dried naturally.

Table 4. Results of auxiliary agent selection.

Group	YGF9		LX6F2	
	Number of spores (cfu/g)	Inhibitory rate (%)	Number of spores (cfu/g)	Inhibitory rate (%)
1	5.66×10^{10}	87.39	1.50×10^{10}	86.53
2	1.58×10^{10}	86.21	6.72×10^{9}	79.84
3	1.18×10^{10}	85.97	1.95×10^{10}	87.21
4	0.81×10^{10}	83.09	7.52×10^{9}	80.87
5	0.92×10^{10}	84.12	8.16×10^{9}	82.32
6	6.08×10^{9}	77.47	3.44×10^{9}	71.09
7	0.86×10^{10}	83.91	3.12×10^{9}	69.86
8	5.44×10^{9}	74.38	3.68×10^{9}	76.32
9	3.12×10^{9}	71.83	4.32×10^{9}	77.35

The results of the orthogonal experiment on the biocontrol agent auxiliaries showed that the most effective of the YGF9 auxiliary agents in the first measured groups was used kaolin as the carrier, SDS as the wetting agent, sodium lignosulfonate as the dispersing agent and humic acid as the UV protective agent. In this group, the number of viable spores was 5.66×10^{10} cfu/g and the

inhibitory rate was 87.39%. The most effective of the LX6F2 auxiliary agents in the third measured groups was used kaolin as the carrier, Nekal BX as the wetting agent, Tween 20 as the dispersing agent and ascorbic acid as the UV protective agent. In this group, the number of viable spores was 1.95×10^{10} cfu/g and the inhibitory rate was 87.21% (Table 4).

The dispersity, wettability and suspension properties of these nine groups were determined, and the results are shown in Table 5. A comprehensive assessment showed that the results of the first group were much better than the other groups. After comprehensive consideration of the number of viable cells, inhibitory effects, costs of materials and other factors that result from the addition of different auxiliaries biocontrol agents, it was ultimately determined that the two biocontrol agents should both use the first group of components as auxiliaries, i.e. kaolin as the carrier, SDS as the wetting agent, sodium lignosulfonate as the dispersing agent and humic acid as the UV protective agent.

Table 5. Capabilities of biocontrol agents.

Group	Dispersity	Wettability	Suspension properties	Comprehensive assessment
1	++	++	++	6+
2	++	+	-	4+
3	+	++	++	5+
4	+	+	+	3+
5	+	+	+	3+
6	+	-	-	1+
7	+	-	+	2+
8	-	-	-	-
9	+	-	-	1+

+ and ++ denotes good and excellent, - indicates poor performance.

The formulation optimization results showed that the best formulation of the YGF9 agents was the fourth group, which had 38% original powder, 38% kaolin, 13% sodium lignosulfonate, 8.5% SDS and 2.5% humic acid. In this case, the number of viable spores was 6.25×10^{10} cfu/g and the inhibitory rate was 89.12%. The best formulation for the LX6F2 agents was the third group, which had 42% original powder, 36% kaolin, 12% Tween 20, 8.0% Nekal BX and 2% ascorbic acid. In this case, the number of viable spores was 2.26×10^{10} cfu/g and the inhibitory rate was 87.21% (Table 6).

As can be seen from the results, not only did the increment of stem height and stem diameter of the poplars significantly improve, the disease index was also significantly reduced after application of the agents which were diluted 100 and 500 times. For agents diluted 1000 times, the increment of stem height and stem diameter of poplar was increased slightly compared to the water control, and the reduction level of the disease grade and index compared fairly to 500-fold carbendazim. Furthermore, the prevention effect of the 1000-fold diluent reached 70.00%, slightly higher than the carbendazim control (Table 7). The results showed that the joint administration of both biocontrol agents could not only effectively control the occurrence of disease, but also play a facilitating role in promoting the growth of poplar.

Table 6. Results of optimization of the auxiliary proportions.

Group	YGF9		LX6F2	
	Number of spores (cfu/g)	Inhibitory rate (%)	Number of spores (cfu/g)	Inhibitory rate (%)
1	5.96×10^{10}	85.34	1.73×10^{10}	86.53
2	5.58×10^{10}	83.29	1.24×10^{9}	79.84
3	5.03×10^{10}	85.88	2.26×10^{10}	87.21
4	6.25×10^{10}	89.12	9.52×10^{9}	80.87
5	4.69×10^{10}	85.37	8.16×10^{9}	82.32

Table 7. Effects of application concentration on plant growth and control efficiency.

Process modes	Stem height (cm)	Stem diameter (mm)	Scab diameter (mm)	Grade	Disease index	Control effect (%)
CK1(water)	8.48	0.62	18.68	IV	88.90	
100-folds diluent	14.67	1.13	4.29	I	20.00	77.50
500-folds diluent	12.88	0.93	4.87	I	24.44	72.50
1000-folds diluent	9.79	0.81	5.13	II	26.67	70.00
CK2(500-folds carbendazim)	6.47	0.58	7.28	II	28.89	67.50

The synthesized results of these three tests indicate that the most suitable woodland application technique is: dilute the agents 500 times and combine sprayed YGF9 agent with a poured root LX6F2 agent in March and November each year. Doing this twice in each month at an interval of 15 days, gives a total of four applications per year.

The choice of microbial agent dosage forms is decided by the physiological characteristics and demands of the practical application of biocontrol strains. Currently, the four main kinds of microorganism' dosage are solid agents, liquid agents, freeze-dried agents and microencapsulated agents. At present, the liquid dosage is rarely used due to the fast decline in the number of viable cells, short storage period, inconvenient to transport, susceptibility to contamination and other shortcomings (Wu 2003). Freeze-dried agents are not suitable for this type of microbial pesticide agents because of the complex production process and higher costs. Microencapsulated agents are also excluded because the production technology is still at an experimental stage. The production process is complex, and they require more production equipment, which mainly used for the preservation of the intestinal bacteria at present (Escande and Echandi 1991, Li 2013). Currently, only solid agents are widely used microbial pesticides due to their high number of viable cells, simple production process, low cost, and ease of storage, transport and field application, etc.

Applying a solid fermentation approach to propagate the strains has more potential than any other technique for the production of agents for many filamentous fungi (Escande and Echandi 1991, Lewis and Papavizas 1993), for example, Susana *et al.* (1999) used solid fermentation to produce black epicoccum biocontrol agents. Jin's (2010) research indicates that the use of solid-state fermentation to manufacture biocontrol agents which have a one-year high viable content and physiological activity at room temperature. In this paper, the manufacture of the original agents'

powder uses optimized liquid fermentation of the two biocontrol strains followed by solid-state fermentation, which gives a better environment for the growth and storage of viable bacteria in the agents' original powder. In addition, the cost of production of the agents reduced due to the low cost of solid fermentation substrates.

Previous studies have shown that *Trichoderma aureoviride* YGF9 and *Fusarium equiseti* LX6F2 are two strains which have a good inhibitory potential as biocontrols of poplar canker. In this paper, jointly applied viable preparations of *Trichoderma aureoviride* and *Fusarium equiseti* obtained by artificial culture are developed for use on poplar under natural conditions. Thus, the viable bacteria of the agents can colonize the poplar successful and can continue to multiply in the soil of poplar plantations. This forms a virtuous cycle of beneficial microorganisms in the host–pathogen–soil, which is conducive to maintain the microecology balance of the host plants and rhizosphere soil and so achieving sustainable control of the disease. In addition, the study found that the administration of biocontrol agents can control the occurrence of poplar canker effectively and also has a role in promoting the growth fluid of poplar, which could be connected with the effects of the biocontrol agents in improving the microenvironment and enhancing the resistance and vitality of the host. Galletti found (Stefania *et al*. 2008) that biocontrol strain of *Trichoderma* can control the incidence of beet brown spot, and can improve the sugar yield of beet, as well as difficult to produce drug resistance. Therefore biocontrol strain of *Trichoderma* can be popularized and applied in vegetable crop production, and the prospects are bright (Gamal *et al*. 2007).

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