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Research Article

STUDY ON PREPARATION AND THE ACTION MECHANISM OF A NOVEL COTTON SEED COATING AGENT

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ABSTRACT
It is the O-carboxymethyl chitosan as the main raw material, compound with the constant element, nutrient elements, plant growth regulators and auxiliary components that prepares the novel environmental friendly cotton seed coating agent. A series of indicators of the novel environmental friendly cotton seed coating agent has been investigated by experiments. We have studied the promoting germination effect, disease and pest resistance and yield quality increasing effect through laboratory germination experiments, antibacterial experiments and field trials. The results show that compared with traditional pesticides seed coating agent, this novel cotton seed coating agent can control cotton pests effectively, improve cotton seedling emergence, and promote plant growth, increase cotton production, high efficiency, safety and environmental friendly.

Keywords: *Cotton Seed Coating Agent; O-carboxymethyl Chitosan; Antibacterial; Germination Rate; Yield*

INTRODUCTION
Cotton is an important economic crop in our country in a very extensive planting area. However, pests have serious impact on the yield and quality during the growth of cotton.

By using seed coating agent, we can promote seed germination and emergence, improve cotton quality and increase cotton production. However, the seed coating agent currently used mostly contains of Duo Kefu, carbendazim, carbofuran and other toxic ingredients, which will leave persistent contamination in the soil and endanger the safety of humans and animals, causing serious environmental issues that hinder the development of eco-agriculture (Zhaofang, 1997; Xuehong *et al.,* 2003; Xueqiang *et al.,* 2004).

To pull off this problem, we choose the natural nontoxic O- carboxymethyl chitosan as the main raw material compound with other complex components to develop a novel environmental non-hazardous environment-friendly cotton seed coating agent.

Through numerous laboratory experiments and field trials, we systematically studied the influence of this seed coating agent on the seed germination rate, the cotton pests, bacteria, and cotton production.

The results showed that the important index of the seed price and safety are better than those of conventional cotton seed coating agent, which was of great significance on cotton production, environmental protection, and promote the healthy development of ecological agriculture.

MATERIALS AND METHODS

Experimental Materials and Contents

Experimental Materials

Constant temperature and humidity incubator (WS-01, Hubei Huang Shi Hengfeng Medical Devices Co., Ltd., P.R. China). Autoclaves (YXQ-SG46-48SA, Shanghai Motion Industries, Ltd., P.R. China), Electronic constant speed blender (GS28B, Shanghai Anting Electronic Instrument Factory, P.R. China), Dish(Φ90 mm, Shanghai Yuejin Medical Instrument, P.R. China), Electronic balance (FA2004, Shanghai Yue Fung Instrument Co., Ltd, P.R. China), O- carboxymethyl chitosan (Self made), Coalescent (Polyacrylamide, Guangzhou Runxin Chemical Co., Ltd, P.R. China), Antifreeze (Butanol, glycerol, ethanol, Shanghai Chemical Reagent Co., Ltd, P.R. China), Penetrant SJ (Self made), Warning color (Violet pigment, Guangzhou Tianyangtai Natural Pigment Co., Ltd, P.R. China), Fusarium oxysporum (Chinese Academy of Agricultural Sciences, Hubei Province, P.R. China), Cotton Seed (2# of Tian Rong, Hubei Provincial Seed Group Company, P.R. China), Traditional Cotton Seed Coating Agent (2.5% Shileshi Suspension Seed Coating Agent, Switzerland importing), ZSB-CT Biological Seed Coating Agent (HSBC Zhejiang Provincial Seed Co., Ltd, P.R. China)

Experimental Contents

Preparation of O-carboxymethyl chitosan cotton seed coating agent

(1) A certain amount of O-carboxymethyl chitosan was dissolved in dilute acid, followed by stirring until dissolution completely at room temperature for 3-5 hours. The solution referred to as NP (the same below).

(2) Dubbed aqueous solution of the mass concentration of 1% to 2% by sodium hydroxide, borax, potassium dihydrogen phosphate, zinc sulfate and other additives to set aside.

(3) The NP, antifreeze glycerol, film formers aid polyacrylamide, penetrants SJ, trace element solution and violet natural pigment were mixed in a certain proportion uniformly, followed by stirring at constant speed stirrer electronic for 4-5 hours. Then we obtained the novel environmental friendly cotton seed coating agent (referred to as ES, the same below).

The determination of ES physicochemical parameters (Cong *et al.*, 2010; Qiang and Jianxian, 2009; Long, 2010).

(1) Quick-drying determination: 1g sample was diluted 10-fold, then take 1g dilution to coat seed (after 100℃drying to constant weight) in the ratio of 1:50 and place coated seeds in the oven at 40℃ to dry. Record the time when it dried to constant weight.

(2) Film determination: Put the sample on the glass with the size of 2x10cm to cast in a film with the thickness of 0.08mm and place it into the oven on the temperature of 30±1℃ drying. Then soak it in a 15cm dish equipped with water for 0.5 hours. Film-forming was studied for III grades. Film uniformity and the membrane was scraped from the glass plate fully when immersed in water glass plates as class I; Film uniformity and the membrane was scraped from the glass plate incompletely when immersed in water glass plates as class II; Film-forming agent cannot form a film in the glass plate as class III.

(3) Air permeability determination: Take 2g samples, and sprinkle on the surface of circular filter paper evenly. Then dry it and filter paper with 200g of distilled water in the beaker on the mouth, sealing. Put the beaker in water bath pot of constant temperature, and set the temperature of 80 degrees Celsius. Taken

out after 3 hours and weigh the quality surplus water in the beaker. Repeated three times and calculate the average values of evaporation.

(4) Permeability determination: Take 2g samples and sprinkle it on the surface of the circular filter paper evenly. Cover the filter paper in weighted clean beaker after drying. Take 10ml of distilled water and drop in filter paper on the surface and weigh beaker weight after 3 hours. Repeat three times and calculate the average values of quality of distilled water.

(5) Water-soluble determination: Cast the sample into film in the 2x10cm glass plate after drying and immerse it into 15cm plate with 2/3 water. Calculate the membrane dissolution area of glass plate after 18hours. Calculation of film dissolution area is the same with the calculation of the blade plaque area.

(6) Swelling determination: Cast the sample into film in the 2x10cm glass plate and weigh it in electronic balance after drying. Immerse it into 15cm dish with 2/3 water. Weigh it again after 6hours and calculate the swelling rate. The swelling rate is calculated by the following formula:

The swelling rate = soaked specimen mass / dried sample mass x 100%

Laboratory germination test (Tian and Jing, 2003; Cuili and Songbiao, 2008)

Coat the cotton seed with ES, the traditional coating agent (2.5% Shileshi permis) and ZSB-CT biological seed coating agent in accordance with the 1:60 medicine respectively. Place the cotton seed coated (40 tablets/dish) into the Petri dishes with wet filter paper by sand bed culture method and cover it with a layer of high-temperature sterilized wet sand. Each experiment set 3 parallel samples and the experimental results are from the mean of three parallel samples by comparing with the blank group (CK, cotton seeds without adding any seed coating agent). Then place the Petri dish into the constant temperature and humidity incubator with the temperature of 28±1℃ and the relative humidity of 85%. Observe germination potential (GE) after 4 days and statistics the germination rate (GP) and germination index (GI) after 7days.

$$GE = \frac{Gm}{Gn} \times 100\% \qquad \text{Formula 2-1}$$

$$Gp = \frac{Ga}{Gn} \times 100\% \qquad \text{Formula 2-2}$$

$$GI = \Sigma \frac{Gt}{Dt} \qquad \text{Formula 2-3}$$

The effect experiment of ES on the pest control

(1) Bacteriostatic test of ES (Ninghai *et al.*, 2005; Burkhanova *et al.*, 2007)

We do antibacterial experiment (Haiping *et al.*, 2000) with filter paper method to study the antimicrobial effect of ES by using the pathogens Verticillium dahliae and Fusarium Wilt as tested bacteria. Join 10ml aseptic water into the purified disease Yin Petri dishes and make spore suspension under aseptic operation (100 times the view contains 50~100 spores). Then mix them in 42～45℃medium with the proportion of 1:9 to slab, 10ml per dish. Place the filter paper soaked liquid evenly with the diameter of 5~6mm in aseptic conditions into the the plate, 5 points each plate, two pieces of filter paper each point. Observe the growth status of the peripheral disease Yin under different drugs or agents of different concentration treatment with filter paper in which one of the points (two filter paper) as control is to immerse aseptic water, otherwise flat Petri dish (spores) without filter paper disks is set as the control. Determine the

effect of each drug Yin suppression after a certain time constant temperature culture. The following formula is about the calculation of the inhibitory rate:

$$\text{The inhibitory rate}(\%) = \frac{\text{spore number of blank control} - \text{spore number of treatment}}{\text{spore number of blank control}} \times 100\%$$ Form

ula 2-4

(2) The pest antifeedant test of ES (Chinese Agricultural Standard, 2006; Shan *et al.*, 2007)

Referring to "test pesticide insecticide determination criterion of indoor biological research", the cotton bollworm is used as test plant diseases and insect pests and study on the aphid effect of non selective leaf disc method for ES aversion to cotton. Select growing well, without macular, cotton seedling leaves without pests to dry cleaning and impregnate them into different treatment agents (concentration is the same as above) for 2s.

After natural drying, the blade of treatment group and the control group were placed in the Petri dish with the diameter of 9cm of pad moistened filter paper, then the 3 in star cotton aphids starved for 4h in the same size were put into the Petri dishes, each dish one larva. The processing is repeated for 10 times, and then the Petri dishes were put into the incubator constant temperature with the temperature of 20~25℃. Then record the leaf area with coordinate paper each 24 hours. Non selective antifeedant rate is calculated by using the following formula:

$$\text{Non selective antifeedant rate}(\%) = \frac{\text{Controls intake} - \text{Treatment intake}}{\text{Controls intake}} \times 100\%$$

Formula 2-5

Field Efficacy Test

The field test carried out on the cotton test base of Hubei province Ezhou, P.R. China. Test of cotton varieties were Tianrong No. 2 (Chinese seed company of Hubei Province, P.R. China). Experiment set with four treatments: ES, 2.5% Shileshi suspension seed coating agent, ZSB-CT coating and blank control. The seed of every treatment would be coated by seed coating agent with a certain ratio while seedling in mid April and transplanting in mid May. The experiment was in a complete randomized block design, with three replicates of each treatment, by order, 2 zone, residential area of 9.9 square meters, 90 cm spacing, and the district field management, operations are completely consistent, and in the same day. Investigate the cotton emergence rate (leaf number were 2 true leaves), seedling quality and yield of cotton and some other main index after growing.

RESULTS AND DISCUSSION

Determination of Physicochemical Indicators of O-arboxymethyl Chitosan

As can be seen from Table 1, the solution used in experiment of different concentrations of O-carboxymethyl chitosan have good physicochemical indicators. The air permeability, water permeability and swelling rate reached an optimal level in the concentration of 3.5%, and the water-soluble and film-forming properties kept a stable high-level in the concentration of 3.5%, while the fast-drying failed to reach the best performance at the same time, but still had a very good effect.

Therefore, the O-carboxymethyl chitosan solution in the concentration of 3.5% can be selected as the main component film-forming for this seed coating agent.

Comparison of Germination Potential and Germination Rate with Different Treatments

As can be seen from Table 2, 3 treatments can all promote cotton seeds to germinate compared with the control group CK. The germination potential of the seed coated by ES remained at about 85% and increased by approximately 2.8% and 3.7% when compared with the cotton seed coated by 2.5% Shileshi and ZSB-CT, and increased by nearly 7.3% when compared with CK. While the ES coated seed germination rate remained at around 88% and increased by about 2.1% and 3.6% when compared with the 2.5% Shileshi and cotton ZSB-CT coating treatment. The germination rate increased nearly 7.2% compared with the CK. In the germination index, the emergence rate of ES coated seed is far higher than that of CK (8.76%), while the Shileshi and ZSB-CT is respectively higher 2.25%, 5.37%. The results show that seed coating could enhance seed germination rate. The promoting effect of ES on germination rate is obviously better than that of Shileshi.

The Effect Comparison of Inhibition Rate under Different Treatments

As can be seen from table 3, different seed coating agents for cotton played an inhibitory effect on pathogenic growth, and with the concentration increasing, the inhibition was more obvious. In the experimental group of ES, the antibacterial effect is better than that of Shileshi seed coating agent and ZSB-CT biological seed coating agent with different concentration. When the treatment concentration was 100 g/ml, inhibition rate of ES on Fusarium and Verticillium dahliae reached 95.6% and 94.7% respectively and respectively higher than that of Shileshi seed coating agent by 6.7% and 7.6%. When the concentration was 20 g/ml, inhibition rates of ES were 83.8% and 83.5%, significantly higher than that of Shileshi seed coating agent 76.3% and 76.4% and that of ZSB-CT biological seed coating agent 76.8% and 78.3%. So ES has a good inhibitory effect on pathogenic bacteria of cotton. We can see from table 4, the feeding area on leaf and feeding rate of helicoverpa armigera after treated by the ES seed coating agent of different concentrations was lower than that treated by 2.5% Celest suspension seed coating agent and ZSB-CT biological seed coating agent. And with the increasing of concentration, the antifeedant effect enhanced. Among them, the antifeedant effect of cotton bollworm after ES treatment is most obvious, while it was higher than that treated by 2.5% Celest suspension seed coating agent and ZSB-CT biological seed coating agent at different concentrations. In summary, ES effects stronger than traditional coating agent on the antifeedant of cotton bollworm.

Results and Comparison of Field Experiment of ES

It can be seen from table 5, seed coating agent not only can promote seed germination, but also can promote the growth of root. Through the absorption of nutrients, promoted the vegetative organs of vigorous growth, plant height, fruit branch number, bud number and boll number increased significantly, of which the effect of ES was most obvious. As can be seen from the results of 2012. Seed germination rate treated by ES was the highest, reaching 88.1%, while the ZSB-CT was 85%. The seed germination rates treated by 2.5% Celest and CK were both 80.8%. From the results of 2013, the seed germination rate is still higher than the Celest seed coating agent, ZSB-CT biological seed coating agent and CK, which increased by 7.6%, 2.9% and 2.2%.

From the results of field trial production, we can see that the cotton production coated with ES increased by 18.1% than CK, 10.9% than the Celest and 6.9% than ZSB-CT in 2012. The effect is more obvious in 2013 while the proportion is increased by 16.1%, 12.4% and 10.5% when compared with CK, Celest and ZSB-CT.

Through the field test results for two years, we found that the coating could significantly improve the germination rate of seed and seedling quality, thus increasing the yield of cotton. Although the traditional cotton seed coating agent treatment can improve the germination rate of cotton seedlings, but the effect is not obvious and stimulation effect is not stable. The ES treatment of cotton has the highest germination rate. After germination, the trace elements of fertilizer in coating agents continued to play a role, which can seedlings seedling, promote cotton seedling growth, improve the quality of cotton seedling, and thus significantly increase the yield of cotton. In addition, the results of two years field experiment of ES are basically consistent and the data is persuasive.

Mechanism Analysis of ES

Film-forming Property of ES

The effective ingredient of ES is O-carboxymethyl chitosan solution. It has good film-forming property and can form a dense protective layer on the surface of the seeds. Which has a fine spatial structure and contains a lot of hydroxy groups. Its porous makes it possible to have good air permeability and water permeability after covering the seed surface, which ensures not only the water and oxygen needed for seed germination stage, but also provide the channel for slow release of effective components contained in the seed coating agent.

Antimicrobial Resistance of ES

O-carboxymethyl chitosan can interact with the acidic substances of bacterial surface such as lipopolysaccharide and capsular polysaccharides to form complex polyelectrolyte, changing the permeability of the membrane and disrupting the normal physiological activity of bacteria, which plays a bacterial suppression effect. Using this environmental friendly seed coating cotton seed can avoid the invasion of bacteria from soil so as to achieve the purpose of seeds protecting, which benefits the normal growth and development of the cotton plant and finally can improve cotton production and quality. Furthermore, O-carboxymethyl chitosan is a vector of plant information consists of cell walls, cell medium, bacterial enzymatic and hydrolyzate. The cotton plants can accelerate the differentiation of plant tubers and effectively promote the germination of cotton seedlings by the inducing of O-carboxymethyl chitosan, and also can induce the plant cells to synthesis substances such as plant antibiotics and protease inhibitors which can inhibit pathogen invasion (Pengfei and Jianqiang, 2001).

Avoidance for Pest of ES

It was rarely reported about the mechanism of O-carboxymethyl chitosan solution for pest avoidance at home and abroad. Less than one hundred kinds of antifeedant activity of plant metabolites have been found. Studies on antifeedant mechanism showed that some antifeedant agents can act directly on taste receptors of insects, and then passed to the insect's central nervous system, thus preventing the feeding behavior of insects. While some antifeedant directly acted on the nervous system of insects, causing neurological disorder discharges, thereby interfering with insect gustatory access to information, resulting in insect feeding behavior inappropriately (Picart *et al.*, 2005; Kuroiwa *et al.*, 2005; Zhiheng, 2002;

Xiaofang *et al.*, 2010). Through laboratory tests prove that O-carboxymethyl chitosan solution have antifeedant effect on the bollworm significantly, which may be due to the O-carboxymethyl chitosan solution have the effect of stimulation or inhibition of receptors on bollworm receptor, which caused antifeedant behavior of cottonn bollworm.

Production Increasing Mechanism of ES

The main stimulation mechanism of ES mainly consists of the following five part:

The first reason is that ES can improve root vigor of crops, which is good for plant roots to absorb and utilize water and mineral, thereby promoting the growth of seedlings; Secondly, ES has a good film-forming, hydrophilia and porous permeability. This film is beneficial to suck nutrients, reduce the loss of nutrients, and accelerate metabolites exclude, thereby increasing vigor and germination rate of seeds, and thus lay the foundation for improving crop yields; Thirdly, ES can significantly improve the activity of the enzyme, effectively remove the peroxide oxidation of cells, protects the cells from the toxic hydroxyl radical (OH-), and improve the antioxidant ability of the plant, thus effectively increase the number of tillers and fruit fullness degree, achieve increase production purposes; Fourthly, ES can improve crop chlorophyll content in the leaves of the corp, accelerate endosperm starch hydrolysis process of seed germination, provide sufficient nutrients and carbon source for the seed germination and crop production, so as to achieve yield purpose; Fifthly, ES contains large amounts of amino and hydroxyl ligands groups, which have good dissolution and complexing ability of metal ions and trace elements in the seed agent coating, ES can help seeds and crops absorbing trace element, thus achieve the purpose of collaborative production.

Conclusion

By using natural polysaccharide polymer NP as the main raw material, supplemented by micronutrient fertilizer and trace elements and other additives to prepare a novel and efficient eco-friendly cotton seed coating agent. It improves the germination rate and quality of cotton seedlings through the promotion of seed germination, promote root growth and suppress the occurrence of pests and diseases, which improved the cotton production. It results in an increase of cotton production by 10.5% -12.4% and a decrease of seed costs by 18% compared with traditional seed coating agent. And an increase of cotton production by 16.1% -18.1 % compared with untreated cotton seeds CK. It is safe and non-toxic and non-pollution, has significant economic and environmental benefits and important application value in the main cotton producing areas.

Table 1: Physicochemical index of ES

Concentration(%)	**2**	**2.5**	**3**	**3.5**	**4**
Quick-drying (min)	18	16	11	12	13
Film-forming	Ⅱ	Ⅰ	Ⅰ	Ⅰ	Ⅰ
Air permeability(g)	11.4	13.0	14.4	14.8	10.2
Water permeability(g)	7.5	8.3	8.9	9.1	7.1
Water solubility(%)	90	95	95	95	95
Swelling rate(%)	108	128	187	256	245

Table 2: Comparison of germination potential and germination rate with different treatments

Treatments	Germination potential/%	Germination rate/%	Germination index/%
CK	78.1	80.5	35.48
2.5% Shileshi	81.7	84.1	41.87
ZSB-CT	82.6	85.8	44.99
ES	85.4	87.7	47.24

Table 3: The effect comparison of inhibition rate under different treatments

Repeated test No.	2.5% Shileshi		ZSB-CT		ES	
	Cotton Fusarium Wilt	Cotton Verticillium Wilt	Cotton Fusarium Wilt	Cotton Verticillium Wilt	Cotton Fusarium Wilt	Cotton Verticillium Wilt
C1	88.9	87.1	89.2	89.9	95.6	94.7
C2	86.1	86.3	87.3	87.1	90.3	90.8
C3	80.2	81.2	81.8	84.7	88.6	87.7
C4	78.0	79.9	78.3	82.2	85.4	84.6
C5	76.3	76.4	76.8	78.3	83.8	83.5

Table 4: Test results of helicoverpa armigera antifeeding under different treatments

Repeated test No.	2.5% Shileshi Feeding area (mm^2)	antifeedant rate (%)	ZSB-CT Feeding area (mm^2)	antifeedant rate (%)	ES Feeding area (mm^2)	antifeedant rate (%)
C_1	175.4 ± 11.45 e	88.82	238.3 ± 14.56 f	84.05	143.5± 13.47b	92.77
C_2	310.9 ± 27.34 e	80.19	312.6±24.16 e	79.1	232.7 ± 24.54 b	86.19
C_3	523.9 ± 51.12 d	66.62	634.2 ± 43.90 d	57.56	345.4 ± 54.11 d	71.68
C_4	734.5 ± 59.45 c	53.20	863.2 ± 61.23 c	42.21	513.5 ± 59.45 c	66.25
C_5	901.3 ± 65.72 b	42.57	976.3 ± 60.81 b	34.67	712.3 ± 47.66 e	59.37
C_6	1494.4 ± 90.10 a	-	1494.4 ± 90.10 a	-	1494.4 ± 90.10 a	-

Note: the same column letters tested at 0.05 level by Duncan's new multiple range, where C6 is the blank treatment

Table 5: Results and comparison of field experiment

year	treatment	Germination percentage (%)	Plant height (cm)	boll number (A/plant)	fruit branches(A/plant)	Bud number (A/plant)	yield per mu/kg	Cost (yuan·kg^{-1})
2012	ES	88.1±0.06*	82.5±0.55**	11.7±0.33**	12.3±0.33**	9. 7±0.33	268.4±6.01*	16.4
	Celest	80.8±1.40*	70.7±0.35**	9.0±0.00**	8.3±0.33**	9.3±0.33	242.0±5.00*	20.0
	ZSB-CT	85±0.37	78.7±0.39*	9.5±0.08**	8.3±0.33**	9.4±0.56	251.0±4.08*	23.6
	CK	80.8±1.01*	63.3±0.46**	8.3±0.33**	6.7±0.33**	7.3±0.00	227.2±4.81*	0
2013	ES	90.9±0.35	81.8±0.43**	13.7±0.33*	13.0±0.00**	10.7±0.33**	315.8±3.11**	16.4
	Celest	88±0.42	68.7±0.39*	9.5±0.08**	8.3±0.33**	9.4±0.56	281.0±4.08*	20.0
	ZSB-CT	88.7±0.47	69.3±0.27**	11.7±0.33*	9.3±0.33**	9.0±0.58**	285.7±3.12**	23.6
	CK	83.3±1.14	68.8±0.16**	8.9±0.33*	8.7±0.33**	8.8±0.33**	272.8±6.79**	0

*Note: * P<0.05，** P<0.01*

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