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RESEARCH ARTICLE

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Mechanism of herbicidal action of the component I from *Pythium aphanidermatum*

Abstract The herbicidal mechanism of the components extracted from *Pythium aphanidermatum* was examined in this study. Component I was isolated using the HPD500 macroporous adsorption resin and HPLC. Its impact on seed germination and plant growth of weeds was determined and the contents of MDA, superoxide anion radical, and the activities of hills and roots were examined. The root length of weed plants was inhibited under illumination while the stem height was inhibited evidently under darkness. The relative electric conductivity of *Digitaria sanguinalis* and *Amaranthus retroflexus* under illumination was 94.55 $\mu S \cdot cm^{-1}$ and 58.75 $\mu S \cdot cm^{-1}$, respectively, whereas that under darkness was 85.25 $\mu S \cdot cm^{-1}$ and 36.25 $\mu S \cdot cm^{-1}$, respectively. The MDA contents of *Digitaria sanguinalis* and *Chlorella pyrenoidosa* were 0.08385 $\mu mol \cdot L^{-1}$ and 0.1742 $\mu mol \cdot L^{-1}$ under illumination, respectively, while those were 0.0129 $\mu mol \cdot L^{-1}$ and 0.01935 $\mu mol \cdot L^{-1}$ under darkness, respectively. Simultaneously, superoxide anion radical content was higher under illumination than under darkness. These results showed the photosynthesis was affected by component I extracted from *Pythium aphanidermatum.*

Keywords *Pythium aphanidermatum*, herbicidal component, herbicidal mechanism

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1 Introduction

In order to find out new lead compounds and ensure the safety of new pesticides to the environment, the natural active products have become one of the bases of pesticide innovation (Chen and Xue, 1995). The discovery of natural herbicidal substances has great potential for exploration of new pesticides. Seeking for pesticides with particular action mechanism is always one of the main goals of manufacturers. The reason is that the component with particular action mechanism is active and shows no cross-resistance to conventional pesticides, and is safe to the environment and more suitable for the integrated pest management strategy and the development of sustainable agriculture (Wu et al., 2001). Exploring the mechanism of natural products can not only find new targets and biochemical theory, but also identify toxicity group, which can provide a new way for searching herbicides (Zhou et al., 2002; Peng and He, 2003). Previous results showed that the toxin from *Pythium aphanidermatum* (Eds.) Fitzp. had a high herbicidal activity and was photoactivated (Zhang, 2005). With the increase in light intensity, the herbicidal activity of toxin was strengthened. *Digitaria sanguinalis* (L.) Scop., *Amaranthus retroflexus* L. and *Chlorella pyrenoidosa* L. were taken as the materials in our experiment. After treatment with component I, the changes of chlorophyll content, Hill activity, MDA content, superoxide anion radical content were measured to find out the herbicidal mechanism of the component I, which may lay a foundation for the development of new herbicides.

2 Methods

2.1 Experimental materials

The plants of *Pythium aphanidermatum* (Eds.) Fitzp. (PA1), *Digitaria sanguinalis* (L.) Scop., *Amaranthus retroflexus* L. and *Chlorella Pyrenoidosa* L. planted at

the Agricultural University of Hebei were taken as the materials in this study.

2.2 Preparation of culture filtrate of PA1 and isolation of herbicidal components

Two thousand and seven hundred mL culture filtrate of PA1 was adsorbed using the method of HPD500 macroporous resin adsorption chromatography (80 g) and then the adsorbent resin was eluted with 500 mL of 10% ethanol solvent. The eluted solvent was extracted with ethyl acetate concentrated in vacuum and re-dissolved in 2 mL of methanol. Then the methanol solution was detected by the HPLC with C_{18} column (4.6 mm × 150 mm × 0.45 μm), using CH_3OH-H_2O as flowing phase. Linear gradient elution was 2% methanol to 100% methanol in 25 min, with 1 mL·min^{-1} flow rate at 254 nm under 25°C, and semiproeparative chromatography was used in purification at 254 nm after examination. The semiproeparative chromatography was Nove-pak-C_{18} column (300 mm × 7.8 mm × 6 μm), using CH_3OH-H_2O (92∶8) as flowing phase, at 1 mL·min^{-1} flow rate. The eluting solution was concentrated in vacuum and the herbicidal component was obtained and then stored at 4°C in a refrigerator before use.

2.3 Planting method

D. sanguinalis and *A. retroflexus* were planted according to the method of Zhang et al. (2005).

2.4 Pre-culture of *C. pyrenoidosa*

C. pyrenoidosa was inoculated into 100 mL SE culture medium in a 250 mL flask (Huang, 2007), and then cultivated for three generations under the condition of 25°C and light intensity of 4000 lx, when the density reached 2.3 × 10^7·mL^{-1}, *C. pyrenoidosa* was stored till use.

2.5 Herbicidal activity of component I under different light conditions

2.5.1 Effect of component I on inhibiting seed germination and plant growth of weeds

According to the method of Zhang et al. (2005), the effect of component I on inhibiting seed germination and plant growth of weeds was examined.

2.5.2 Effect of component I on the membrane permeability of *D. sanguinalis* and *A. retroflexus*

0.3 mL methanol solution with 100 mg·L^{-1} herbicidal component was put into a centrifugal pipe. After the solvent volatized, 3 mL de-ionized water was added into the centrifugal pipe, then 0.05 g of *D. sanguinalis* and 0.05 g of *A. retroflexus* were put into the solution, taking 3 mL-water-treatment as control. All the treatments were done three times. Each treatment was conducted from 09:00 to 15:00 under natural light and dark conditions, respectively. Temperature and light intensity were measured once every hour. After the treatments, electric conductivity was measured by conductance instrument and the relative electric conductivity was calculated (Dong et al., 1999). Relative electric conductivity (μS·cm^{-1}) = $D_1 - D_2 - (D_3 - D_4)$, where D_1, D_2, D_3 and D_4 represent the electric conductivities of treatment with component I, water solution with herbicidal component, treatment with water and water only, respectively.

2.6 Effect of component I on photosynthesis

2.6.1 Design of treatment of *D. sanguinalis* and *C. pyrenoidosa*

Twenty mL *C. pyrenoidosa* extract cultivated for three generations was put into a 50 mL centrifugal pipe and centrifuged. The 0.3 mL methanol solution with 100 mg·L^{-1} herbicidal component was put into another centrifugal pipe and 3 mL de-ionized water was added into the pipe after the solvent volatized. Then the water solution with the herbicidal component was added into the centrifugal pipe with *C. pyrenoidosa*. The treatment was conducted from 10:00 to 11:00 under natural light and darkness conditions, respectively. The placebo treatment with water without herbicidal component was taken as control. The method described above was noted as "A".

When *D. sanguinalis* and *A. retroflexus* grew to 2 cm, 20 leaves from the two plants were treated with 50 μL methanol solution which contained a herbicidal component, and the methanol solvent was used as control. All the treatments were conducted from nine to fifteen under natural light and darkness conditions, respectively. The method described above was noted as "B".

2.6.2 Effects of component I on the chlorophyll content of *C. pyrenoidosa* under the condition of light

C. pyrenoidosa obtained by method "A" was put into 5 mL of extraction solvent (acetone:ethanol:water = 4.5∶4.5∶1 (V/V/V)) for 24 h under darkness. After the absorbency of supernatant liquid was measured at 663 nm and 645 nm, taking the extraction solvent as control, the chlorophyll content and relative loss of chlorophyll were calculated (Jiang et al., 2006; Arnon, 1949) with the following formulae:

$$C\ (\mu g \cdot mL^{-1}) = 8.02 \times OD_{663} + 20.20 \times OD_{645},$$

$$\text{Relative loss of chlorophyll} = \frac{\text{chlorophyll content in control} - \text{chlorophyll content in treatment}}{\text{chlorophyll content in control}} \times 100\%.$$

2.6.3 Effect of component I on the β-carotene content of *C. pyrenoidosa* under the condition of light

The *C. pyrenoidosa* obtained by method "A" was treated in 5 mL of extraction solvent (90% acetone) for 24 h under darkness. After the absorbency of supernatant liquid was measured at 450 nm, taking the extraction solvent as control, the β-carotene content of *C. pyrenoidosa* was detected.

2.6.4 Effects of component I on the hill activity of *C. pyrenoidosa* and *D. sanguinalis* under the condition of light

The chloroplasts that were isolated from *C. pyrenoidosa* treated by method "A" and the *D. sanguinalis* treated by method "B", respectively, were used for measuring inhibition of component I on hill activity by colorimetry (Tang et al., 2004).

2.6.5 Effects of component I on the MDA content of *C. pyrenoidosa* and *D. sanguinalis*

The MDA contents of *C. pyrenoidosa* treated by method "A" and *D. sanguinalis* treated by "B" were determined by colorimetry. The absorbency was measured at 532 nm, 600 nm, and 450 nm (Zhao, 1999), respectively. And the concentration was calculated according to the following formula:

$$C\,(\mu mol \cdot L^{-1}) = 6.45(A_{532} - A_{600}) - 0.56A_{450}.$$

2.6.6 Effects of component I on the superoxide anion radical content of *C. pyrenoidosa* and *D. sanguinalis*

After *C. pyrenoidosa* and *D. sanguinalis* were respectively treated by methods "A" and "B", the superoxide anion radical contents were determined using the hydroxylamine method (Wang and Luo, 1990).

3 Results

3.1 Examination of herbicidal component by HPLC

After the culture filtrate of PA1 was adsorbed by macroporous resin adsorption chromatography, the adsorbent resin was eluted in 10% ethanol solvent, which was concentrated and examined by HPLC. It was found that there were three main absorption peaks, and their retention times were 2.685 min, 4.550 min, and 24.282 min, respectively (Fig. 1). However, only two main peaks were found in the culture medium examined, and the other peak with retention time of 4.550 min did not exist. Therefore, the component of the peak with retention time of 4.550 min was prepared by the semi-preparative chromatography and component I was obtained. Then the herbicidal activity of component I was tested and the results showed that the inhibition rate could reach up to 85% for inhibiting plants when the concentration of 100 $mg \cdot L^{-1}$ was used in the experiment.

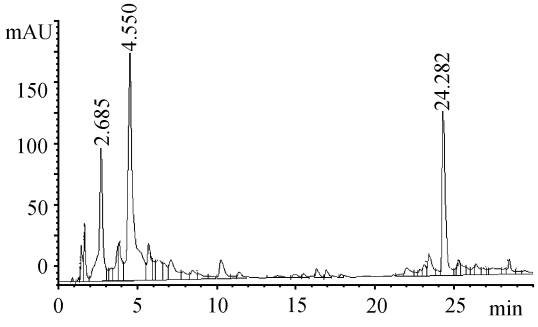

Fig. 1 The HPLC chart of 10% ethanol elution HPD500

3.2 Effects of component I on the inhibition of seed germination and plant growth under darkness and light conditions

After treatment by component I, the length of root and stem of the seeds were measured. And the results showed that the seed germination rates of both *D. sanguinalis* and *A. retroflexus* were 100%, after treatment with component I at the concentration of 100 $mg \cdot L^{-1}$. That implied component I had no inhibition to seed germination of these plants at this concentration. However, the root restraint rate of component I to *A. retroflexus* was higher than that of *D. sanguinalis*, the root restraint rate to *A. retroflexus* were 83.22% and 73.86% under light and darkness, respectively, while the root restraint rate to *D. sanguinalis* was only 9.80% under light condition, and component I had no effect to the length of the root of *D. sanguinalis* under darkness condition. The root restraint rate of component I to *D. sanguinalis* and *A. retroflexus* under 3700 lx was higher than that under darkness, but the stem restraint rate under 3700 lx was lower than that under darkness (Table 1).

3.3 Growth responses of *D. sanguinalis* and *A. retroflexus* after treated with component I under different light conditions

The growth responses of *D. sanguinalis* and *A. retroflexus* were examined with the conductivity method after

Table 1 Effect of component I on the inhibition of seed germination and plant growth

item		3700 lx			darkness		
		component I treatment	water treatment	inhibition rate/%	component I treatment	water treatment	inhibition rate/%
D. sanguinalis	root length/mm	44.26	49.07	9.80	35.15	35.03	0
	stem length/mm	13.52	14.50	6.78	30.50	42.70	28.57
A. retroflexus	root length/mm	2.50	14.88	83.22	2.51	9.63	73.86
	stem length/mm	8.64	8.64	0	10.79	18.37	41.27

Note: inhibition rate = (the length of component I treatment – the length of water treatment) / the length of water treatment × 100%.

treatment by component I under different light conditions. The results showed that their electric conductivity when treated under light was higher than that under darkness (Table 2), which indicated that the effect of component I on themembranes of *D. sanguinalis* and *A. retroflexus* was stronger under light than under darkness. At the same time, it was found that the relative electric conductivity of *D. sanguinalis* was higher than that of *A. retroflexus* after treatment by component I. And the relative electric conductivities of *D. sanguinalis* and *A. retroflexus* treated under light were 94.55 $\mu S \cdot cm^{-1}$ and 58.75 $\mu S \cdot cm^{-1}$, respectively, while those were 85.75 $\mu S \cdot cm^{-1}$ and 36.25 $\mu S \cdot cm^{-1}$ under darkness, respectively. These results indicated that the influence of component I on the membrane permeability of *D. sanguinalis* was stronger than that of *A. retroflexus*.

3.4 Effects of component I on the chlorophyll content of *C. pyrenoidosa* under different light intensities

The chlorophyll of *C. pyrenoidosa* was extracted by extraction solvent after treatment with component I under light and darkness. The results showed that the chlorophyll content of *C. pyrenoidosa* decreased after treatment, and the content under light condition was much lower than that under darkness. The relative loss of chlorophyll was 90.59% under light, but only 8.78% under darkness (Table 3). It was evident that the light condition could accelerate the effects of component I on the chlorophyll content of *C. pyrenoidosa.*

3.5 Effects of component I on the β-carotene content *C. pyrenoidosa* under different light intensities

The β-carotene of *C. pyrenoidosa* was extracted by extraction solvent after treatment with component I under light and darkness conditions. Then its absorbency was measured at 450 nm under the condition of light. The results showed that the absorbency of the treatment with water was 0.829, while that with component I was 0.051, under light condition. However, under darkness, the absorbency with water was 0.869 and that with component I was 0.807. It indicated that the effect of component I on the β-carotene content of *C. pyrenoidosa* under light was stronger than that under darkness.

3.6 Effects of component I on the Hill activity of *C. pyrenoidosa* and *D. sanguinalis* under different light intensities

Hill activity of *C. pyrenoidosa* and *D. sanguinalis* was measured after treatment with component I under darkness and light. The results showed that the relative loss of *C. pyrenoidosa* in Hill activity under light and darkness was 88.19% and 26.83%, respectively. And the relative loss of *D. sanguinalis* under light and darkness was

Table 2 The effect of component I on the electric conductivity of *D. sanguinalis* and *A. retroflexus* under different light intensities

item		component I treatment	water treatment	component I contrast	water contrast	relative electric conductivity
the electric conductivity of *D. sanguinalis*/($\mu S \cdot cm^{-1}$)	natural light (50300–103500 lx)	248.00	51.20	112.00	9.75	94.55
	darkness	236.00	48.50	112.00	9.75	85.25
the electric conductivity of *A. retroflexus* /($\mu S \cdot cm^{-1}$)	natural light (50300–103500 lx)	189.00	28.00	112.00	9.75	58.75
	darkness	164.00	24.50	112.00	9.75	36.25

Table 3 Effects of component I on the chlorophyll content of *C. pyrenoidosa* under different light intensities

item		component I treatment	water treatment	relative loss/%
the chlorophyll content of *C. pyrenoidosa*/($\mu g \cdot mL^{-1}$)	natural light (59000–89500 lx)	0.63	6.67	90.59
	darkness	7.68	8.42	8.78

57.33% and 24.34%, respectively. It indicated that the effect of component I on Hill activity of *C. pyrenoidosa* was greater than that of *D. sanguinalis* due to the possible cause that the structure of *C. pyrenoidosa* was simple in comparison with *D. sanguinalis* (Table 4).

3.7 Effects of component I on the lipid peroxidation of *C. pyrenoidosa* and *D. sanguinalis*

The MDA content of *C. pyrenoidosa* and *D. sanguinalis* were measured after treatment with component I. The results showed that the photosynthesis membranes were damaged (Table 5), and the MDA contents of *D. sanguinalis* under light and darkness were 0.1742 μmol·L^{-1} and 0.0193 μmol·L^{-1}, respectively, while that of control was 0. The MDA content of *C. pyrenoidosa* treated under light was higher than that under darkness, the contents under light and darkness were 0.0838 μmol·L^{-1} and 0.0451 μmol·L^{-1}, respectively, while the contents of control under light and darkness were 0.0129 μmol·L^{-1} and 0.006 μmol·L^{-1}, respectively. It indicated that the effect of component I under light condition on lipid peroxidation was more obvious in comparison with that under darkness condition.

3.8 Effects of component I on superoxide anion radical content of *C. pyrenoidosa* and *D. sanguinalis*

The superoxide anion radical content of *C. pyrenoidosa* and *D. sanguinalis* were measured after treatment with component I. The results showed that the superoxide anion radical content of *D. sanguinalis* treated under light was higher than that treated under darkness (Table 6), and that treated under darkness and light were 0.33 g·min^{-1} and 0.71 g·min^{-1}, respectively, but that of control was 0. These results indicated that methanol couldn't harm *D. sanguinalis*. The increase of superoxide anion radical content might be owing to component I. Besides, the effect of component I on superoxide anion radical content under light was stronger than under darkness.

Same as the *D. sanguinalis*, the superoxide anion radical contents of *C. pyrenoidosa* in treatment and control under light were 0.15 g·min^{-1} and 0.04 g·min^{-1}, respectively, while those in treatment and control under darkness were 0.05 g·min^{-1}and 0.01 g·min^{-1}, respectively. The results indicated that *C. pyrenoidosa* was also harmed to different degrees.

Table 4 Effects of component I on Hill activity under different light intensities

item		component I treatment /(μmolO_2·mg^{-1}chl·h^{-1})	water treatment /(μmolO_2·mg^{-1}chl·h^{-1})	relative loss/%
Hill activity of *C. pyrenoidosa*	natural light (59000–89500 lx)	7.54	59.83	88.19
	darkness	56.96	77.68	26.83
Hill activity of *D. sanguinalis*	natural light (50300–103500 lx)	262.03	614.18	57.33
	darkness	444.62	587.66	24.34

Table 5 Effects of component I on the lipid peroxidation under different light intensities

item		component I treatment	contrast
the MDA content of *C. pyrenoid*osa/(μmol·L^{-1})	natural light (59000–89500 lx)	0.0838	0.0451
	darkness	0.0129	0.006
the MDA content of *D. sanguinalis*/(μmol·L^{-1})	natural light (50300–103500 lx)	0.1742	0
	darkness	0.0193	0

Table 6 Effects of component I on the superoxide anion radical content under different light intensities

item		component I treatment	contrast
the superoxide anion radical content of *C. pyrenoid*osa/(g·min^{-1})	natural light (59000–89500 lx)	0.15	0.04
	darkness	0.04	0.01
the superoxide anion radical content of *D. sanguinalis*/(g·min^{-1})	natural light (50300–103500 lx)	0.71	0
	darkness	0.33	0

4 Discussion

To date, traditional random-screening of herbicides is the primary way to create new herbicides. For the development of herbicides, the mechanisms which inhibit the growth of weed plants provide some of the key steps in plant physiology and biochemistry, in despite of different chemical structures. The possible mechanisms have been explored and can be divided into eight categories with about nineteen hypotheses, such as inhibition of photosynthesis, aminophenol synthesis, lipid synthesis, pigment synthesis, folic acid synthesis, cell development, and interfering with hormone synthesis, or destroying the integrity of membranes (Su, 1998; Zhang, 1999; Wakabayashi and Boger, 2002; Mallory and Retzinger, 2003). But on the whole, the research progress of herbicidal mechanism is still slow because the target of commercial herbicides is very limited, and many researches focus on a small number of targets, which cause cross-resistance. Therefore, finding some new targets of herbicides becomes more and more important. Many kinds of natural microbial pesticides have different chemical properties in comparison with synthetic pesticide, and their sites are not covered by the existing herbicides, so studying the mechanisms become very important, which can contribute to finding new targets and providing a theoretical basis for the development of new pesticides. For these reasons, the mechanism of herbicidal substance of *P. aphanidermatum* was investigated in this research.

In order to understand the mechanism of component I from culture filtrate of *P. aphanidermatum*, *C. pyrenoidosa* with a simple structure of chloroplast and the nucleus was selected as the test material, in addition to the two common weeds. At present, potted plants are usually chosen for researching the herbicidal mechanism, but some plants may not be sensitive enough to compounds inhibiting photosynthesis. Using *C. pyrenoidosa* as the test material was just to make up for the deficiencies (Crossmann et al., 1992; Shen et al., 1999). In addition, a large number of studies about the toxic effects of pesticides on algae have been reported, but the poisoning mechanism of pesticides on the algae was seldom studied (Delorenzo et al., 2001).

Considering the material that was not treated with component I but only with water could exist differences under darkness and light, in the design of experiments, component I solution treatment and water treatment were conducted simultaneously, for setting up water treatment to remove the influence. In the course of the experiment, if *C. pyrenoidosa* was treated too long under light, the chlorophyll of *C. pyrenoidosa* treated with water or component I would all be destroyed. It was known that strong light was not suitable for growing *C. pyrenoidosa* in our experiments, so the *C. pyrenoidosa* was treated from 10:00 to 11:00, which condition is suitable for this experiment.

The toxin from *Pythium aphanidermatum* (Eds.) Fitzp. was photoactivated, so its effect on photosynthesis was researched. Other studies on the chloroplast ultrastructure, light electron transport system activity, and pigment as well as the reaction on the aspects of enzymes were conducted. In this study, only MDA, membrane permeability, chlorophyll, and superoxid anion radical, Hill activity were tested.

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