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Table: 1, Figs : 5

Bio-degradation of Precocene II by *Novosphingobium capsulatum*

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ABSTRACT

Precocene II is a major allelochemical produced by the weed *Ageratum conyzoides.* Its degradation is important in the recovery of *A. conyzoides* invaded plant communities. Using a strain of *N. capsulatum* isolated from the *A. conyzoides* invaded/infested soil; we examined the effects of various factors viz., incubation time, temperature, initial pH, and carbon and nitrogen source on the degradation of precocene II. We found that under optimal conditions with supplementation with a carbon and nitrogen source, up to 79.8% precocene II is degraded by *N. capsulatum.*

Keywords: *Ageratum conyzoides,* Allelochemical, *Novosphingobium capsulatum,* Precocene II degradation.

INTRODUCTION

Allelopathy plays an important role in the successful invasion and establishment of invasive plants (15). The production of large amounts of allelochemicals facilitates the invasion and this restricts the growth of local plants. This presents a great challenge to the development and restoration of degraded ecosystems. Current methods used to reduce the impact of allelopathy are: use of activated carbon, soil dilution (6,9), soil enzymes (13) and microbial biodegradation (1,12). Due to lack of lasting impact and high cost, the use of activated carbon for adsorption of allelochemicals or dilution of allelochmicals in soil by irrigation, have limited application. Therefore, the use of soil enzymes or microorganisms to degrade the allelochemicals has received attention.

A. conyzoides (*Ageratum conyzoides*, *Vernonia patula*) is an annual herbaceous plant that has strong allelopathic activity. It produces the precocene (precocene I: 30.9% and precocene II: 51.6%) allelochemicals. The latter has stronger allelopathic activity than the former (5). Rhizosphere soils of plants that secrete allelochemicals also contain micro organisms that use the allelochemicals as a carbon source and degrade these chemicals (1, 10-12). However, so far no such report is available about the degradation of precocene II by any rhizospheric bacteria. To explore this possibility, a strain of *N capsulatum* isolated from the soil on which *A. conyzoides* had grown was selected and examined for its potential to degrade precocene II. The effects of various cultural factors on the degradation of precocene II by this bacterium have been examined.

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MATERIALS AND METHODS

N. capsulatum was isolated from *A. conyzoides* invaded soil. For isolation, 40 spots (according to an S-shaped curve) from every *A. conyzoides* invades site were chosen. After removing the surface vegetation, soil samples were collected using an Auger (5 cm dia) from a depth of 0 to 20 cm. The 40 soil samples were then fully mixed and screened through a sieve to remove roots etc. One kg of the well mixed soil sample was brought to laboratory for isolation of the precocene II degrading bacteria.

To isolate the precocene II degrading bacteria, 10 g of the soil of *A. conyzoides* invaded area was mixed with 90 ml sterile water in 250 ml Erlenmeyer flasks, shaken for 20 min, then diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} with sterile water . One hundred ul of each dilution was transferred to sterile inorganic salt culture medium (ISM, g/L, $MgSO_4 \cdot 7H_2O$, 0.2, $CaCl_2 \cdot 2H_2O$, 0.02, $FeSO_4 \cdot 7H_2O$, 0.01, KH_2PO_4, 0.4, Na_2HPO_4, 0.6, $MnSO_4 \cdot H_2O$, 0.02, NH_4NO_3, 2.0) with Precocene II (0.1g/L) as carbon source. The flasks were incubated at 25 ℃. FOR 2 days after which the amount of precocene degraded in each flask was determined. The flask in which maximum precoccene was degraded was used to isolate the precoccne degrading bacteria by further plating on Potato sucrose agar((PSA,Peeled potato 200 g/L, sucrose 20 g/L,agar 20g/L, sterilized for 30 min at 121℃)). The dominant bacteria on these plates were selected and was identified as *N. capsulatum* by examining the physiological and biochemical characteristics and 16s rDNA sequence analysis in the Detection Center of Microbiology, Guangdong. This culture of *N.capsulatum* was maintained on PSA medium by regular transfers.

Preparation of *N. capsulatum* suspension

The bacterium was cultured on PSA medium for 24h at 25℃. A loopful of the bacterial lawn from a fresh PSA agar slant culture was transferred to sterile potato sucrose broth (50 ml) in 100 ml conical flasks and incubated at 25℃ for 24 h on an incubator shaker (120 rpm). The culture was then diluted with sterile water or normal saline to an OD 0.5 $_{600}$. It was centrifuged at 4000 rpm and re-suspend in fresh sterile water to an OD of $0.5_{600.}$

To examine the effect of various factors (inoculum size, incubation temperature, aeration), 50 ml inorganic salt medium in 100 mL conical flasks,was sterilized,cooled, inoculated with the bacterial suspension at 2% (v/v) and incubated on a rotary shaker at 150 rpm. Each treatment was in triplicate. When analysis had to be done at intervals, one flask was withdrawn at the appropriate interval and the residual precocene determined. The culture suspension was centrifuged and the clear supernatant was used to determine the residual precocene content. Precocene II (Boyle Chemical Co., Shanghai) from soil or from culture broths was extracted by liquid-liquid extraction methods and then determined by HPLC (Dionex, USA) as described by Zhang *et al.* (14).

The standard precoccene, peptone was purchased from Land Bridge Technology Co. from Beijing, China, yeast powder from Bio-technology Co., Kunming Jiehui, China and other chemicals from Chemical Reagent Factory, Guangzhou, China.

Effects of various factors on degradation of precocene II

(i). **Incubation time:** Precocene II (OD_{600}=0.5) inoculated flasks were incubated at 25°C on a shaker (150 rpm) and samples were withdrawn at 4, 8, 12, 20, 24, 26, 28 and 32 h for determining the residual precocene II. Thirty 100 ml flasks containing 50 ml of ISM and precocene were inoculated with the bacterial suspension (OD_{600}=0.5) at 2 % level and the culture was incubated at 25℃ and 150 rpm . Three flasks were withdrawn after 4, 8, 12, 20, 24, 26 , 28 and 32 h to measure the degradation of Precocene II.

(ii). **Inoculums size:** Fifty ml of ISM containing precocene II was inoculated with 1.0, 1.5, 2.0, 2.5 and 3.0% (v/v) bacterial suspension and incubated as above and the amount of precocene degraded was determined after 24 h.

(iii). **Aeration:** To determine the effects of aeration, flasks with 50 mL inoculated ISM were incubated on a rotary shaker at 50, 100, 150, 200 and 250 rpm at 25°C. Samples were analyzed for residual precocene after 24 h.

(iv). **Incubation temperature:** To determine the effects of incubation temperature, inoculated ISM containing precocene II was incubated at 20, 25, 30, 35 and 40°C in a incubator shaker (150 rpm) for 26 h, then the residual precocene was determined.

(v). **Initial pH:** To determine the effects of initial pH, 50 ml of ISM containing precoene was inoculated at 2% level and then the pH of the medium was adjusted before sterilization to 6.0, 6.5, 7.0, 7.5 and 8.0 using 1% HCl or 1% NaOH. The flasks were incubated at 25°C on a shaker (150 rpm) for 24 h, thereafter, the residual precocene content was determined.

(vi). **External carbon source:** To determine the effects of external carbon sources (sucrose, lactose, glucose, sugarless starch and maltose) the carbohydrate source was added to 50 ml ISM before sterilization at 0.2% with Precocene II as unique carbon source. The flasks were was incubated at 25°C on a shaker (150 rpm) for 26 h, thereafter, the residual precocene II was determined.

(vii). **Nitrogen Source:** To determine the influence of nitrogen source, 50 ml ISM with precocene and containing different nitrogen sources (ammonium nitrate, ammonium chloride, peptone, urea or yeast powder added at 1g/L before sterilization, was inoculated with bacterial suspension and incubated at 25°C and 150 rpm for 26 h after which the residual precocene II in the medium was determined.

(viii). **Combined effects of Carbon and Nitrogen:** To determine the combined effects of additional carbon and nitrogen sources, 50 ml of IMS containing combinations of carbon and nitrogen source were inoculated with the bacterial suspension and incubated at 25°C on rotary shaker 150 rpm for 24 h, afterwarda extent of precocene degraded was determined.

Statistical Analysis of Data: All experiments were done in triplicate. The SAS and Duncan method (4) was used for analyzing the data for statistical significance.

RESULTS AND DISCUSSION

When cultured on PSA the ***N.** capsulatum* colonies were opaque, round, yellow with a smooth surface and a neat edge. Cells were Gram negative, short rod-shaped, non-spore- forming.

Precocene II degradation: The time course of precocene II degradation by *N. capsulatum* is shown in Fig. 1. During the first 8 h, degradation was slow but increased rapidly up to 20 h, and thereafter there was no decrease in its content.

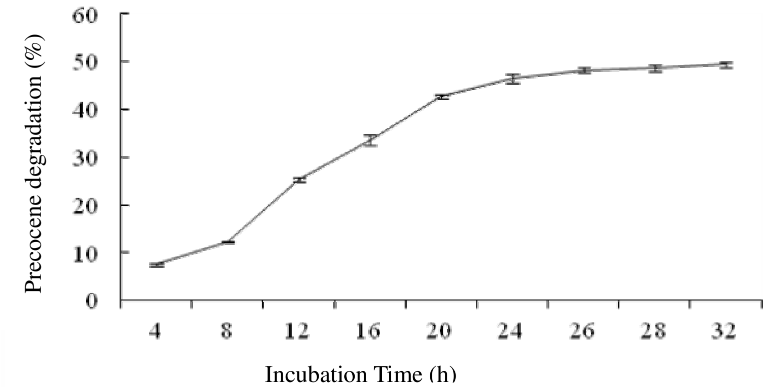

Figure 1. Effects of Incubation time on Precocene degradation by *N. capsulatum*

Although, there was no effect of either the size of inoculum or aeration on precocene degradation, but there was no degradation without inoculation... Degradation occurred almost equally at all levels suggesting that these factors (1-3% inoculums and 50-250 rpm) had no effect on the ability of the organism to degrade Precoccene II (Table 1).

Table 1. Effects of Inoculum dose and Incubation rotation speed on degradation of Precocene II by *N. capsulatum*

Inoculum Dose (%)	% Degradation	Incubation speed (rpm)	% Degradation
1.0	50.9a	50	50.8a
1.5	51.1a	100	51.0a
2.0	50.9a	150	50.9a
2.5	51.2a	200	51.1a
3.0	50.1a	250	51.2a

The Incubation temprature directly affected the precoccene degradation ability of *N. capsulatum* (Fig. 2) and maximum degradation was noticed at 25 °C. The degradation of precoccene II increased with the initial pH and reached an initial pH of 7.5 was optimum (Fig. 3).

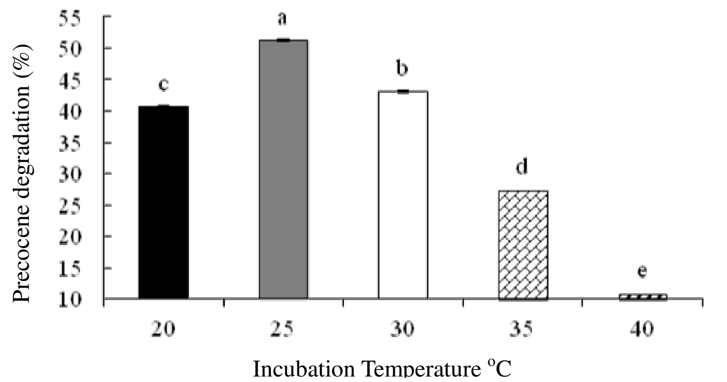

Figure 2. Effects of Incubation temperature on Precocene degradation by *N. capsulatum.* The different small letters on Bars indicate the significant difference at $P < 0.05$ (Duncan)

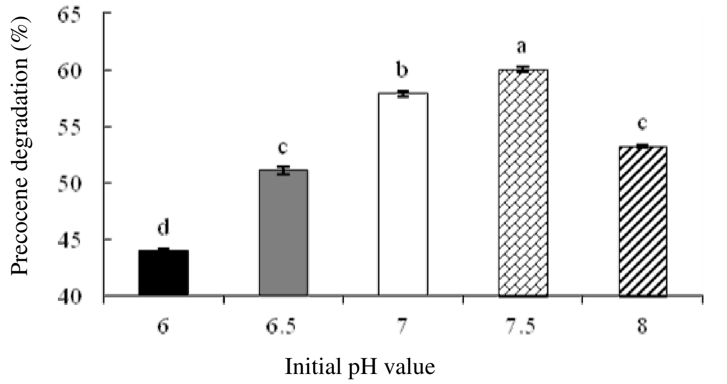

Figure 3. Effects of initial pH on precocene degradation by *N. capsulatum.* The different small letters on Bars indicate the significant difference at $P < 0.05$ (Duncan)

Addition of external carbon source affected degradation of precocene by *N. capsulatum* and it varied with the carbon sources. The addition of a carbon source increased degradation and lactose was the best carbon source (69.7% degradation). Degradation without any additional carbon source was only 51.2% suggesting that precoccine is also used as a carbon source by this organism.

ISM contained sodium nitrate and was replaced by ammonium nitrate, ammonium chloride, peptone, urea or yeast powder at 1g/L in 50 mL with Precocene II as the carbon source. Replacing $NaNO_3$ with other nitrogen sources improved the precocene degradation (Fig. 4) and the extent of degradation varied with the nitrogen source. When urea, ammonium nitrate or ammonium chloride was the nitrogen source, degradation was much lower than with either yeast powder or peptone. It therefore appears that both yeast extract powder and peptone facilitate better degradation of precocene II.

To determine the combined effects of selected carbon and nitrogen sources on precocene degradation, the medium was supplemented with lactose and either yeast extract or peptone and precocene degradation was determined after 24 h. The degradation was highest (78.5 %) when yeast powder was the nitrogen source with lactose as the carbon source (Fig. 5).

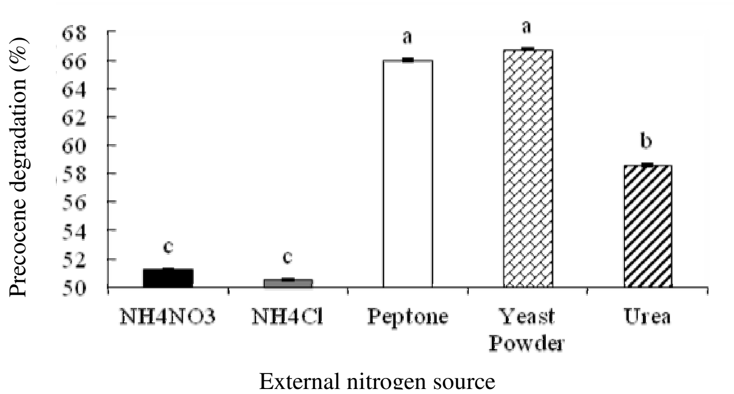

Figure 4. Effects of nitrogen source on precocene degradation by *N.capsulatum.* The different small letters on Bars indicate the significant difference at P <0.05 (Duncan)

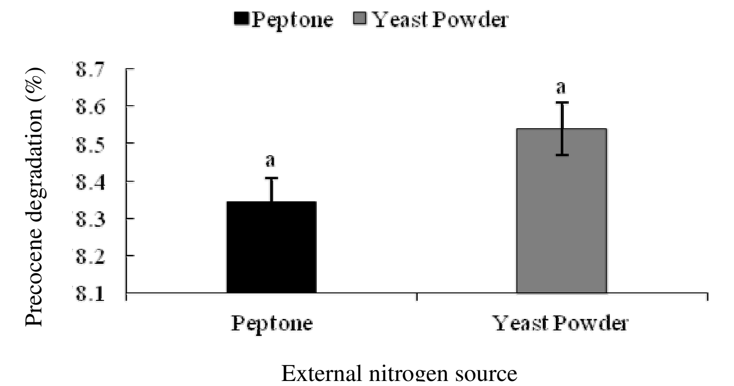

Figure 5. Effects of external carbon (lactose) and nitrogen sources on precocene degration by *N. capsulatum.* The different small letters on Bars indicate the significant difference at P <0.05 (Duncan)

Since the first report of *N. capsulatum* in 1962 (7),studies have shown that it helps in controlling the potato leaf disease caused by *Phytophthora infestans* (2, 3). So far however, there is no report on any of its other properties. We selected *N capsulatum* from the soil of *A conyzoides* invaded area presuming that it may have the ability to degrade the main allelochemical produced by *A conyzoides* namely precocene II. This is a new attempt at breaking the allelopathic impact of *A. conyzoides* and offers a new way to weaken its allelopathy. Similar studies on the ability of microorganisms to degrade allelochemicals have been reported**.** A strain of *Pseudomonas putida* J1 isolated from the rhzioshpere soil of black walnut was found to use juglone (5-hydroxy-1,4-naphthoquinone) as the sole carbon source (10). Microbial communities that degrade phenolic allelochemicals produced by Cladina *stellaris or Acomastylis Rossii* have been found in their rizosphere soils (1,12). Bacteria such as *Ageratum conyzoides, Matsuebacter* sp FB25, *Agrobacterium vitis* EB26 and *Pseudomonas* sp. FB22 with ability to degrade and utilize phenolic allelochemicals as the major carbon source have been reported from the surrounding water column of *Myriophyllum spicatum* L (8). Our studies reported here show that *N. capsulatum* has the ability to degrade precocene II and with supplementation with a carbon source such as lactose and a nitrogen source such as yeast extract or peptone, the degradation can be significantly improved. However, it is interesting to know that this organism can also grow in minimal medium at 25°C without aeration and degrades precocene although the extent of degradation is less. This has an advantage, if it is to be used in reclaiming precocene contaminated soils. Further detailed studies on using this organism in the soil and its ability to recover the soils from the allelopathic effect of precocene need to be carried out.

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