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INHIBITORY EFFECT OF CYANOBACTERIAL EXTRACT ON FUNGAL PATHOGENS OF TOMATO

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ABSTRACT

Among the various pathogenic fungi Fusarium sp. are saprophytes in the soil and organic matter with a cosmopolitan distribution. While some strains have been known to cause vascular wilt disease in crops including vegetables, bananas and date palms some other strains also protect plants from wilt. Among the genus Fusarium, F. oxysporum has been known to affect tomato plants significantly decreasing its production as they damage roots during their growth stages. Today, the focus on the treatment of plant decreases is mainly oriented towards the use of extracts of natural products as alternatives to synthetic fungicides for their safety and negligible environment impact. The present study involves the use of cyanobacterial extract in the control of F. oxysporum infecting tomato plants.

KEY WORDS

Fusarium species, Tomato plants, Cyanobacterial extract of Anabaena

1. INTRODUCTION

Pathogenic fungi are one of the most common organisms responsible for considerable plant yield losses than other microorganisms (Sexton and Howlett, 2006). Among the various pathogenic fungi, *Fusarium* sp. are saprophytes in the soil and organic matter with a cosmopolitan distribution. While some strains have been known to cause vascular wilt disease in crops including vegetables, bananas and date palms some other strains also protect plants from wilt (Kim and Kim, 2008). Among the genus Fusarium*, F. oxysporum* has been known to affect tomato plants (Suarez-Estrelia *et al.*, 2007) significantly decreasing its production as they damage roots during their growth stages (Kim and Kim, 2008). Today, the focus on the treatment of plant diseases is mainly oriented towards the use of extracts of natural products as alternatives to synthetic fungicides for their safety and negligible environment impact (Brimmor and Boland, 2003).

Literature reveals that algae have been one of the commonly used biological agents that have been used to control plant pathogenic fungi (Abdel-Kader, 1997; Hewedy *et al.*, 2000; Kiviranta *et al.*, 2006; Kim and Kim, 2008). Hence the present study involved the use of cyanobacterial extract in the control of *F. oxysporum* infecting tomato plants.

2. MATERIALS AND METHODS

Microorganisms: The N_2-fixing blue-green algae (cyanobacterium), *Anabaena flos-aquae* species was cultured at 25° C in the N- free BG-11 (basal medium) liquid medium (Kim, 2006; Kim and Lee, 2006) under the illumination of cool-white fluorescent lamps at a photon flux density of 40 µE/m^2/s. Cyanobacterial growth was determined by measuring the cell concentration and chlorophyll-a concentration according to the method of Parsons and Strickland (1963). The culture was filtered by GF/C (Whatman) and extracted with 90% methanol at 60°C for 10 min. After extraction, the solid suspension was removed by centrifugation. Absorbance of extracts was measured at 665, 645, and 635 nm using a

spectrophotometer. The concentration of chlorophyll-a (Chl-a) was calculated using the following equation.

Chl-a (mg/l) = 11.6 A_{665} − 1.31 A_{645}−0.14 A_{515}

Optimization of antifungal compound production: To optimize culture conditions for production of antifungal compound, approximately 1.0 × 10^4 cells/ml of algae in the exponential growth phase was inoculated into 500 ml Erlenmeyer flasks containing 250 ml of medium. The flasks were incubated at 28°C with agitation (150 rpm in a rotary shake incubator) under the continuous illumination of cool-white fluorescent lamps with light intensity of 40 µE/m^2/s. In this study, the culture media such as N-free BG-11 (Kim and Lee, 2006), BG-11 (Rippka *et al.*, 1979), BG11+N, and Allen's medium (Allen, 1952) were applied to algal cell culture for algal cell growth, antifungal activity and production of antifungal compounds. Micronutrients were added at 1 ml per liter (Rippka *et al.*, 1979).

After selection of the optimal medium for production of antifungal compounds in Erlenmeyer flasks, the combined effects obtained from the optimization were consequently applied to antifungal compound production (Lee *et al.*, 2006). A pre-culture of 250 ml was prepared in a 500-ml Erlenmeyer flask. The cells were transferred to 10 1BCPs containing the optimal medium with 0.2 vvm flow-rate-aeration and 5% CO_2 gas and 95% air under a constant continuous light intensity of 40 µE/m^2/s. The temperature and pH were kept constant at 25°C and 7.0±0.5, respectively, during the period of cultivation.

Preparation of cyanobacterial extracts: The freeze-dried cyanobacterial cells (40 g) were extracted twice with 80 ml of methanol for 24 h at room temperature. The extract was separated from the cell residue by filtration (Whatman filter paper No. 3). The filtrate was concentrated at 40°C under reduced pressure in a rotary evaporator. The 285 mg of pale brown extract was collected, suspended in 80% ethanol, and kept at 4°C.

Determination of cyanobacterial cell growth and antifungal compound production: Cell growth of *Anabaena flos-aquae* was estimated using cell dry weight. Cells were harvested by centrifugation, washed twice with distilled water, and dried to constant weight at 85°C for 12 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the disk. The experiment was performed in triplicate, and the mean of the diameter of the inhibition zones was calculated. Quantitative bioassay of the antifungal compound was confirmed by comparing with a standard curve of commercial antifungal compound concentration (Loo *et al.*, 1945). Cyanobacterial methanol extract (500 µg) was applied to sterile paper disks (8 mm in diameter) for antifungal assay. Paper disks containing only methanol were used as control. *Fusarium oxysporium* (1.0 × 10^6 spores/ml) were added to molten potato dextrose agar at 40°C. The seeded agar was poured into 9-cm Petri dishes. The algal extract treated paper disks were placed in the center of the seeded agar plates. Plates were incubated for 3 days at 28°C, and then inhibition zones of mycelial growth around the paper disks were measured. The commercial fungicide Mancozeb, a polymeric complex of zinc and manganese salts of ethylene bisthiocarbamate (EBDC) was used as reference standard of antifungal activity. The inhibition zone produced by the crude extract was converted to the concentration of Mancozeb with the standard curve. Antifungal activity and antifungal compound production are presented as mg equivalent to Mancozeb/g of cyanobacterial dry weight and mg equivalent to Mancozeb/l of medium, respectively.

Effects of cyanobacterial extracts on the inhibition of *F. oxysporum* infection in tomato seeds: Experiments to determine the effect of cyanobacterial extract concentrations on the inhibition of infection of tomato seeds were carried out in Petri dishes. The surface of tomato seeds was sterilized with 10% sodium hypochloride (v/v) then coated with cyanobacterial extract. The coated seed were incubated at 25°C for 24 h, and then the fungal spores were spread on the seeds. The inoculated seeds were laid on moist seed-cultivating paper in petridishes and were incubated at 30°C in dark conditions. After 7 days-incubation, seeds or seedling were observed.

The influence of cyanobacterial extract concentration on *F. oxysporum* cell growth in tomato seeds was investigated at several different concentrations, ranging from 100 to 500 µg/seed. The cyanobacterial extract was coated with methanol base, zeolite mixing, and dissolving in Tween 20 (10%, v/v) to enhance the antifungal activity. The same vehicles without the addition of the cyanobacterial extract were used as controls. The fungal inoculum concentrations used in these experiments were 10, 100, and 500 spores/seed. Each experiment was performed in 10 replicates. The anti-fungal activity of cyanobacterial extract in tomato

plant was evaluated in a growth room. Tomato seeds (*Lycopersicon esculentum*) were prepared as previously described. Two different inoculation methods: (i) seeds were directly inoculated by fungal spores with the concentration of 500 spores/seed before they were sown in plastic pots (5×15×10 cm) containing steam-sterilized soil mixture (peat moss, perlite and vermiculite, 5:3:2, v/v/v) sand, and (ii) loam soil (1:1:1, v/v/v). Soil mixture was inoculated with the fungal spores (1.0×10^3 spores/g of steam-sterilized soil mixture) before the seeds were sown. Tomato plants were raised in a growth room at 30°C with 80 µE/m^2/s and 60~90% relative humidity. Plants were watered regularly. After 14 days of sowing, infection was determined.

3. RESULTS AND DISCUSSION

Optimization of culture medium to produce antifungal compound reveals that the medium BG-11 containing 1 g/l $NaNO_3$ recorded the best results (Table-1). The effect of crude cyanobacterial extract concentration on the inhibition of *F. oxysporum* in tomato seeds are presented in Table-2.

As evident from the table, infection in seedlings were found to decrease with the increase in dose of cyanobacterial extract and cyanobacterial extract concentrations of 400 mg/seed and more showed no signs of infections. Literature reveals that similar results, i.e., algal extract inhibiting the growth of fungi have also been reported by other workers. Thus, de Cano *et al.* (1990) recorded the inhibition of *Candida albicans* and *Staphylococcus aureus* from terestial bacterium *Nostoc*, while Caire *et al.* (1993) screened cyanobacteria compounds against human fungal pathogens and Fish and Codd (1994) analysed the bioactive compounds produced by thermophilic and thermotolerant bacteria. Borowitzka (1995) reported that the extracts of *Nostoc muscorum* significantly inhibited the growth of *Candida albicans* and *Sclerotinia sclerotiorum* and recently Kim and Kim (2008) recorded the inhibitory effects of algal extract on the growth of tomato pathogen *Fusarium oxysporum*. Thus, these observations are in line with those observed in the present study.

The effect of cyanobacterial extract on the inhibition of *F. oxysporum* in tomato seeds after 15 days of treatment are recorded in Table-3. As evident from the table, with regard to the length of the root, the experimental setup-III (containing seed, cyanobacterial extract (zeohite) and pathogen) recorded the highest growth (16 cm) followed by the setup-II (containing seed, cyanobacterial extract, (CH_3OH) and pathogen) which recorded 12 cm of growth. However, with regard to hairy roots, the experimental setup-IV (containing seed + cyanobacterial extract (Tween 20) and pathogen) recorded the highest growth followed by experimental setup-II. With regard to the length of the stem, the experimental setup-IV recorded the highest growth (142 cm) followed by experimental setup-III (13.2 cm). However, a comparison of the average number of infected seedlings in the various experimental setups reveals that the experimental setup-III recorded the highest rate of infection (2.0%) and the lowest by the experimental setup-IV (0.3%). Thus, it appears that experimental setup-IV is the best among the various setups analyzed in the present study.

The effects of cyanobacterial extract on tomato wilt pathogen control in tomato seeds cultivated in soil are presented in Table-4 and Table-5. It appears that the degree of infection appears to be the same when the seeds were inoculated indirectly by a mixture of fungal inoculum and steam sterilized soil mixture. In the present study cyanobacterial extract concentrations of 400 mg/seed and above did not show any signs of infection suggesting that it can act as a natural fungicide which can effectively control the fungal pathogen. Abo-Shady *et al.* (2007) who analyzed the effect of cyanobacterial extracts reported that it strongly inhibited the growth of pathogenic fungi that were isolated from leaves, stems and roots of Fabu bean while Biondi *et al.* (2004) reported the inhibitory effects of *Nostoc* extracts on various pathogenic fungi.

Rizk (2006) while doing *in vivo* studies reported that *F. oxysporum* is very sensitive to cyanobacterial extracts while Moussa and Shanab (2001) recorded the strongly inhibitory effect of cyanobacterial extracts on *F. oxysporum* and Kim and Kim (2008) reported the inhibitory effect of algal extracts on *F. oxysporum*. Thus, it is not suprising that the cyanobacterial extracts showed inhibitory effects on the growth *F. oxysporum* species in the present study. However, the algal concentrations required to inhibit the growth *F. oxysporum* appeared to be on the higher side. According to Kim and Kim (2008) the sensitivity of cyanobacteria metabolism not only depends on the fungal genus, but also on the species and on the mode of growth. This could be the reason for the higher amount of

cyanobacterial extracts required to neutralize the effect of fungi in the present study. Nevertheless, this study shows the need to study the chemical structure of the antifungal compounds produced by the cyanobacteria and their mode of action in inhibiting the fungal growth.

Table 1: Growth, antifungal activity, and antifungal compound production by Cyanobacteria *Anabaena flos-aquae* in different media

Medium	N source	Medium $NaNO_3$ (g/l)	Composition K_2HPO_4 (g/l)	$CaCl_2$	pH	Dry weight of algae (g/l)	Antifungal	
							Activity (mg/g)	Compound production (mg/l)
N-free BG-II	–	–	0.05	0.04	7.5	0.90	2.6	0.32
BG-II	$NaNO_3$	1.0	0.05	0.04	7.5	1.20	3.0	0.66

Table 2: Effect of crude cyanobacterial extract concentration on the inhibition of *Fusarium oxysporum* in tomato seeds

S. No.	Treatment	Average no. of infected seedling
1.	Seed + pathogen	25.0
2.	Seed + cyanobacterial extract (50 µg/seed) + pathogen	11.5
3.	Seed + cyanobacterial extract (100 µg/seed) + pathogen	6.0
4.	Seed + cyanobacterial extract (200 µg/seed) + pathogen	0.8
5.	Seed + cyanobacterial extract (300 µg/seed) + pathogen	0.4
6.	Seed + cyanobacterial extract (400 µg/seed) + pathogen	0
7.	Seed + cyanobacterial extract (500 µg/seed) + pathogen	0
8.	Seed + cyanobacterial extract (600 µg/seed) + pathogen	0
9.	Seed + cyanobacterial extract (700 µg/seed) + pathogen	0

Table 3: Effect of Cyanobacteria extract formulation on the inhibition of *F. oxysporum in* tomato seeds 15 days after treatment

Treatment	Length of root (cm)	No. hairy roots	Length of stem (cm)	Average no. of infected seedling
Seed + pathogen	0	0	0	25.0
Seed + cyanobacterial extract (CH_3OH) + pathogen	12.0	3.4	12.0	0.7
Seed + cyanobacterial extract (zeolite) + pathogen	16.0	2.9	13.2	2.0
Seed + cyanobacterial extract (0.1% Tween 20) + pathogen	11.0	4.8	14.2	0.3
Seed + mencozeb + pathogen	12.0	3.8	10.2	0.4

Table 4: Effect of fungal pathogen inoculum and algal extract concentration on the inhibition of *Fusarium oxysporum* in tomato seeds

Treatment	Average no. of infected seedling
Seed + pathogen (10 spores/seed)	14.4
Seed + pathogen (100 spores/seed)	18.0
Seed + pathogen (500 spores/seed)	25.0
Seed + cyanobacterial extract (100 µg/seed) + pathogen (10 spores/seed)	3.2
Seed + cyanobacterial extract (100 µg/seed) + pathogen (100 spores/seed)	6.4
Seed + cyanobacterial extract (100 µg/seed) + pathogen (200 spores/seed)	8.7
Seed + cyanobacterial extract (200 µg/seed) + pathogen (10 spores/seed)	0.2
Seed + cyanobacterial extract (200 µg/seed) + pathogen (100 spores/seed)	0.3
Seed + cyanobacterial extract (200 µg/seed) + pathogen (200 spores/seed)	0.1

Table 5: Effect of algal extract on tomato wilt pathogen control in tomato seeds cultivated in soil

Fungal pathogen inoculation	Treatment	Average no. of infected seedling
Seed inoculation	Seed + pathogen (500 spores/seed)	16.5
	Seed + algal extract (200 µg/seed) + pathogen (500 spores/seed)	2.6
	Seed + algal extract (300 µg/seed) + pathogen (500 spores/seed)	0.17
Soil inoculation	Seed + pathogen (1×10^3 spores/g of soil)	15.8
	Seed + algal extract (200 µg/seed) + pathogen (1×10^3 spores/g of soil)	1.2
	Seed + mancozeb (200 µg/seed) + pathogen (1×10^3 spores/g of soil)	0.80

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