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Mechanism of *Aspergillus niger* van Teigh antagonism towards *Fusarium oxysporum* Schlect f. sp. *melonis* Sny. & Hans., muskmelon wilt pathogen

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ABSTRACT: ***In vitro*** **interactions of antagonist *Aspergillus niger* isolate AN 27 and muskmelon wilt pathogen *Fusarium oxysporum* f. sp. *melonis* isolate MM 37 revealed three distinct growth phases of *A. niger* AN 27 - initial normal growth with black colony colour due to sporulation, suppressed growth and sporulation followed by revived growth and sporulation. Change in medium pigmentation from violet to pink and formation of abundant thick mycelial strands and chains of chlamydospores were observed in *F. oxysporum* f. sp. *melonis* MM 37. Change in medium pigmentation of MM 37 was attributed to the change in medium pH brought by AN 27 by constitutively producing organic acids. Mechanisms involved in AN 27 antibiosis were production of organic acids, antimicrobial substances (n-butanol extract) and extra cellular enzymes - all of which inhibited microconidial germination of MM 37. Seed coating with *A. niger* AN 27 spores or seedling dip in its spore suspension protected muskmelon seedlings from Fusarial wilt in Rapid Pathogenicity test.**

KEY WORDS: *Aspergillus niger*, biological control, *Fusarium oxysporum* f. sp. *melonis*, muskmelon wilt

INTRODUCTION

Muskmelon wilt caused by *Fusarium oxysporum* Schlect. f. sp. *melonis* Sny. & Hans. is an important disease causing up to 90 per cent yield losses (Palodhi and Sen, 1983). Being soil borne, control through chemical means is neither feasible nor desirable. Though disease management practices through biological means employing *Trichoderma* spp. have been extensively studied (Sivan and Chet, 1989), wilt problem still persists in the field. At this juncture, *Aspergillus niger* van Teigh, a soil invading fungus and most common rhizosphere occupant, appeared to be an ideal biocontrol agent to manage wilts (Sen *et al.*, 1992). To exploit an antagonist successfully for biological management of soil borne plant pathogenic fungi, the first step is to know the mechanisms involved.

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In the present investigation efforts were made to find the mechanism of *A. niger* antagonism against *F. oxysporum* f. sp. *melonis in vitro*.

MATERIALS AND METHODS

Aspergillus niger isolate AN 27 and *F. oxysporum* f. sp. *melonis* isolate MM 37 were obtained from the Vegetable Pathology Laboratory, Division of Plant Pathology, and seeds of muskmelon (*Cucumis melo* L) cv. Pusa Madhuras was obtained from Division of Vegetable Crops, Indian Agricultural Research Institute, New Delhi. *In vitro* interactions were studied using dual culture method (Morton and Stroube, 1955). Influence of AN 27 growth on medium pH was studied by inoculating AN 27 in Czepek Dox broth with an initial 6.8 pH and observations on change of medium pH were recorded after 4, 7, 10, 12 and 14 days of incubation. Effect of pH on growth of test fungal isolates was studied at a pH range of 3 to 10 using buffered Czepek Dox broth.

Estimation of organic acids from the culture filtrates of AN 27 was done by following Furth and Herman's colour reaction for citric acid (Hartford, 1962), and Mahadevan and Sridhar (1986) for oxalic acid. Effect of citric and oxalic acids on the microconidial germination of MM 37 was done in cavity slides incubated at 28±1° C. Observations were recorded after 24 hours.

Culture filtrate obtained from 10 day old AN 27 grown Czepek Dox broth (90 ml) was mixed with 35 ml of n-butanol for over night and the organic phase was separated and dried at room temperature to obtain crystals. These crystals were utilized to evaluate its effect on microconidial germination of MM 37.

Utilization of MM 37 mycelium as carbon source by AN 27 was assessed by supplementing Czepek Dox broth without sucrose with mycelial preparation of MM 37. Seven-day-old mycelial mat of MM 37 was crushed and centrifuged thrice at 4000 rpm. Czepek Dox broth without sucrose was supplemented with this mycelial preparation @ 2g per litre on dry weight basis and inoculated with AN 27. Quantitative estimation of cellulases and proteases produced by AN 27 was done based on Mahadevan and Sridhar (1986). Glycol chitin was used to estimate chitinase production following Ohtakara's Ostwald Viscometer method (1988). Partially purified enzyme extracts of AN 27 were used for their effect on microconidial germination of MM 37.

Rapid Pathogenicity Test (Wensley and Mc Keen, 1962) was used to assay the effect of AN 27 and its antibiotic principles on muskmelon seedlings and also its effect on disease development by MM 37. Six-day-old muskmelon seedlings with fully opened cotyledons were placed in 30 ml vials (one seedling per vial) containing conidial suspension (10^7 conidia/ ml) of MM 37 and observed for 11 days at 28±1° C for disease development. The biocontrol capabilities of AN 27 were assayed either by using the seedlings raised from spore coated seeds or by dipping the roots of untreated seedlings in AN 27 spores and MM 37 conidia mixed suspension. In case of efficacy of antibiotic principles of AN 27, muskmelon seedlings raised from untreated seeds were dipped in antibiotic principles for one and a half hour prior to placing them in MM 37 conidial suspension containing vials. The plants were graded for disease reaction following Radhakrishnan and Sen (1982) as follows: (0) Cotyledons turgid; (1) Cotyledons flaccid; (2) 1 + yellowing of cotyledons; (3) 2 + marginal necrosis; (4) 3 + advanced marginal necrosis and (5) total wilt.

RESULTS AND DISCUSSION

When the test antagonist *A. niger* AN 27 and test pathogen *F. oxysporum* f. sp. *melonis* MM 37 were allowed to interact in dual culture plates *in vitro*, three stages of interactions were observed.

1. Pre interaction stage

This stage was from 0 to 4 days of incubation. During this stage, test antagonist AN 27 grew normally giving black colour to the colony, and test pathogen MM 37 had violet pigmentation in the colony and medium beneath as in check plates.

2. Interaction stage

This stage was between 5 to 12 days of incubation. By 5 th day of incubation, both the test fungal colonies came close with a 'Zone of inhibition' measuring 0.25 cm between them. By 7 th day, an opaque zone was formed beneath AN 27 colony, which was followed by change in medium and colony pigmentation of MM 37 from violet to pink. At this stage, AN 27 lost its normal sporulating ability and only suppressed sporulation was observed retaining white colour of the colony. An opaque zone was observed beneath AN 27 colony in the medium. Microscopic examination of interaction zone after 10 days of incubation revealed formation of thick mycelial strands and chlamydospores in MM 37 colony. When opaque zone was observed under microscope, particulate matter apparently produced by AN 27 was accumulated around inhibition zone surrounding MM 37 peripheral growth. This indicated secretions of AN 27, which were unable to diffuse freely in to the medium due to the influence of MM 37 from the opposite side, formed that opaque zone. Hyphal coiling and appressoria formation by AN 27 were not observed.

3. Post interaction stage

This stage started after 12 days of incubation. After twelfth day, AN 27 started overgrowing on MM 37 colony with revived sporulation and black colony colour. Efforts to re-isolate MM 37 from dual culture plates after 15 days of incubation failed indicating that MM 37 was no more viable. Thus the *in vitro* interactions revealed antagonistic nature of AN 27 against MM 37.

In the present investigation, it was found that pH of the solid medium was 2.0 on 7th day of incubation. When AN 27 was grown on liquid medium, the pH was brought down from an initial 6.8 to 3.5 in 4 days and to 2.0 in 7 days. Booth (1971) observed that pigmentation in *Fusarium* cultures was most markedly affected by pH of the medium. Thus, the changes in colony and medium pigmentation of MM 37 may be attributed to the changes in medium pH during interaction apparently brought by AN 27. When observations were recorded on the mycelial dry weight of the test fungi at different pH levels, AN 27 had maximum growth at pH 3.0 (0.65g) followed by 4.0 (0.53g). MM 37 had significantly higher growth at a pH range of 5.0 to 7.0 (0.55g) followed by 3.0, 4.0 and 8.0 (0.45 g). This indicated that lower pH alone was not responsible for the death of MM 37.

Aspergillus niger is known to produce non-fatty organic acids such as citric acid and oxalic acid (Bilgrami and Verma, 1978) constitutively. In the present study, AN 27 produced citric acid to the extent of 0.9 to 3.0mg/ml culture filtrate and oxalic acid to the extent of 2.0 to 21.5 mg/ml culture filtrate with in a period of 4 to 14 days, respectively. Citric acid could not reduce the germination of MM 37 conidia effectively. Even at a concentration of 4 mg/ml of citric acid (giving a pH of 2.7), 78.9 per cent of MM 37 micro-conidia could germinate. Further, the production of citric acid by AN 27 was only 3mg/ ml even after fourteen days of incubation. Thus citric acid alone is not responsible for the changes in medium pH and death of MM 37. On the other hand, oxalic acid could inhibit the MM 37 micro-conidial germination completely (100% inhibition) even at 2mg/ ml concentration (giving a pH of 2.3), *i.e.*, the quantity which AN27 could produce after 4 days of incubation. Thus while production of citric and oxalic acids together were responsible for the changes in medium pH, oxalic acid was found to be one of the main factors responsible for the death of MM 37.

A yellow crystalline substance (1mg/ml) was obtained when 10-day-old culture filtrate of AN 27 was extracted with n-butanol. This n-butanol extract was found to inhibit MM 37 micro-conidial germination at and above 0.5mg/ml concentration with complete inhibition at 1 mg/ml. This indicated that n-butanol extract too was responsible for the death of MM 37 which accumulated @ 1mg/ml in 10 days. Asalmol (1988) reported toxicity of n-butanol extract to *Fusarium* cultures.

Sideris (1924) reported that at isometabolic point fungi tend to adjust the hydrogen ion concentration of the medium to the optimum by

Table 1. Disease development in muskmelon seedlings – rapid pathogenicity test

Suspension mixture	Treatment	Disease score on 0-5 scale after				
		3 days	5 Days	7 Days	9 Days	11 Days
AN 27 spores	Check	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
	MM 37	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
AN 27 seed coating	Check	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
	MM 37	0.00 (0.71)	0.00 (0.71)	0.29 (0.89)	0.40 (0.95)	2.10 (1.26)
Oxalic acid @ 2 mg / ml	Check	2.25 (1.66)	5.00 (2.35)	5.00 (2.35)	5.00 (2.35)	5.00 (2.35)
	MM 37	1.25 (1.32)	2.75 (1.800	4.25 (2.18)	5.00 (2.35)	5.00 (2.35)
Citric acid @ 3 mg/ml	Check	1.50 (1.41)	1.75 (1.50)	2.25 (1.66)	2.33 (1.68)	2.33 (1.68)
	MM 37	0.75 (1.12)	2.75 (1.80)	4.25 (2.18)	5.00 (2.35)	5.00 (2.35)
n-butanol extract @ 0.15mg/ml	Check	0.00 (0.71)	0.60 (1.05)	1.00 (1.22)	1.60 (1.45)	1.60 (1.45)
	MM 37	0.00 (0.71)	1.00 (1.22)	1.20 (1.30)	1.20 (1.30)	1.60 (1.45)
Check	Check	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
	MM 37	0.00 (0.71)	1.25 (1.32)	1.50 (1.41)	2.49 (1.73)	5.00 (2.35)
CD (P=0.01)		0.06	0.05	0.04	0.06	0.09

Figures in parentheses are square root transformed values.

Check indicates water alone. MM 37 indicates conidial suspension of MM 37.

secretion of metabolic products. Sebek (1952) found that *Fusarium* spp. produced most abundant pigments between 2.7 to 4.5 pH. Some of these pigments produced by *Fusarium* are found to be toxic to other microorganisms (Vesander and Hesseltine, 1981). Thus, MM 37 too exerted initial pressure on invading AN 27 and prevented its entry into its territory by secreting metabolic products that are toxic to AN 27. In this context it is important to note formation of opaque zone due to accumulation of AN 27 secretions and suppressed sporulation in AN 27. However, prolonged association resulted in MM 37 succumbing to AN 27.

When AN 27 was allowed to grow on MM 37 mycelial preparation as sole carbon source, the growth (0.1g mycelial dry weight) was 78 per cent less compared to that on sucrose (0.45g). Fungal cell wall contains mannan-protein and chitin in the outer layer, mainly glucan in the middle layer and protein glucan in the inner layer (Webster, 1980). In the present study, activity of extra cellular enzymes such as cellulases, chitinases and proteases could be detected when MM 37 was used as sole substrate. However, activity of proteases was much higher on MM 37 (259.9 protease units measured as mg glycine released per ml of enzyme extract) compared to that on casein (175protease units). Activity of AN 27 cellulases was much lower on MM 37 (5.6 mg cellulase units measured as mg glucose released per ml of enzyme extract) compared to that on carboxy methycellulose (21.6 cellulase units), *i. e.*, only 25.9 per cent. Chitinase activity measured as per cent reduction in viscosity of glycol chitin was 13.6 units which is only 42.9 per cent compared to that on glycol chitin (31.7 chitinase units). Further, the partially purified enzyme extracts of AN 27 could inhibit the micro-

conidial germination of MM 37 to the extent of 37.8, 10 and 10.6 per cent respectively with cellulase, chitinase and protease extracts.

Thus the present study revealed that organic acids (especially oxalic acid), toxic compounds such as n-butanol extract and extra cellular enzymes produced by AN 27 assisted in killing MM 37 mycelia and micro-conidia. Though AN 27 could not utilize MM 37 as sole carbon source for its growth effectively, the activity of extra cellular enzymes produced by AN 27 might have assisted in degrading MM 37 resting structures such as thick mycelial strands and chlamydospores resulted in death of MM 37 completely. Hence MM 37 could not be reisolated from dual culture plates.

Oxalic acid is known to be phytotoxic. In such a case, both *A. niger* and its toxic metabolites should have caused disease to muskmelon plants. Phytotoxic effects and disease control ability of AN 27 *per se* and its metabolites were assessed using Rapid Pathogenicity Test (Table 1). Muskmelon seedlings were viable without senescence up to 11 days when their roots were dipped in sterile water containing vials. When the disease caused by MM 37 was scored on 0-5 scale, disease reached to a score of 1.25 within 5 days of incubation. By eleventh day, the score was 5.0, *i. e.*, total wilt. Oxalic acid (2 mg/ml) and citric acid (3 mg/ml) were found to be toxic to muskmelon plants.

When roots of muskmelon seedlings were dipped in a suspension containing mixture of AN 27 spores and MM 37 micro-conidia (each at 10^7 propagule per ml), the seedlings were completely protected from MM 37 wilt. Besides, no phytotoxic symptoms were noticed when the seedlings were dipped in AN 27 spore suspension alone. This indicated that AN 27 *per se* is not phytotoxic. Muskmelon seedlings raised from AN 27 spore coated seeds had significantly lower disease development (2.1) compared to that in MM 37 check (5.00) even after 11 days of incubation. Thus, in

Mechanism of *A. niger* antagonism towards *F. oxysporum* Schlect f. sp. *melonis* muskmelon wilt pathogenthe present investigation, AN 27 protected muskmelon seedlings from MM 37 wilt when applied either as seed coating or as spore suspension. Though slightly phytotoxic, n-butanol extract (0.15mg/ml) contained the disease caused by MM 37 when muskmelon seedlings were just predipped in n-butanol extract for one and a half hour before exposing to MM 37 conidial suspension. This indicated the elicitor nature of n-butanol extract of AN 27 besides being antimicrobial.

The present investigation thus revealed that the mechanism of *A. niger* AN 27 antagonism is through direct action on the pathogenic fungus with the help of organic acids, antimicrobial substances such as n-butanol extract and extra cellular enzymes. While organic acids helped AN 27 in creating a favorable environment for its own growth and multiplication besides being toxic to the test pathogen *F. oxysporum* f. sp. *melonis* MM 37, enzymes helped in degradation of pathogen propagules, specially the more resistant chlamydospores. Additional advantage of utilizing *A. niger* AN 27 as a biocontrol agent (BCA) would be its capability to produce elicitor molecules such as n-butanol extract which by itself is toxic to the pathogen.

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