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Research Article

ISOLATION OF FUNGI ANTAGONISTIC TO *FUSARIUM OXYSPORUM* AND COMPARISON OF THEIR LYTIC ENZYME PROFILE

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ABSTRACT

Microbial biological control agents are more useful in various ways and use a great variety of mechanisms to protect plants from pathogens and production of lytic enzyme is one of them. The present study shows the isolation, identification and lytic enzyme profiles of fungi antagonistic to a well-known phytopathogen *Fusarium oxysproum* MTCC 4162, from the rhizosphere. Out of 22 isolates, 9 showed antagonistic activity against *F. oxysporum* MTCC 4162 in dual culture method *in vitro*. *Penicillium notatum* and *Rhizopus oryzae* were the promising antagonists, followed by three species of Aspergillus. The glucanase, chitinase and protease activities were identified using *in vitro* tests. Highest specific glucanase activity was shown by *P. notatum*(118.65±10.5 µg glucose produced $min^{-1}mg^{-1}$ protein), followed by *R. oryzae* (107.20±11.2 µg glucose produced $min^{-1}mg^{-1}$ protein).*Penicillium notatum* showed highest specific chitinase activity (85.17±10.5 µg NAGA produced $min^{-1}mg^{-1}$ protein) while highest specific protease activity was detected in *R. oryzae* (133.28±15.8 µg tyrosine produced $min^{-1}mg^{-1}$ protein). The study found correlation between antagonistic activity and lytic enzymes activity.

Keywords: Biological control, Antagonism, Fusarium, Glucanse, Chitinsae, Protease

1. INTRODUCTION

Every year heavy economic losses to agricultural crops occur due to infections by phytopathgens, mainly fungi. Apart from chemical fungicides, biological control of plant pathogens is now being considered as a viable and effective method. Biological control system uses one organism to inhibit the growth, rate of infection or reproduction of other organism, usually pathogen. This method is not only environmentally safe, but also proved to be the sole method in some cases [1].

Antagonism among fungi has been observed for a long time and is now used as a tool for biocontrol of the pathogenic fungi. Plant pathogens, *i.e. Fusarium oxysporum* are among the most important biotic agents causing serious losses and damages to agricultural products. A number of different strategies are currently being employed to manage and control plant pathogenic fungi [2, 3]. Agriculturists and farmers rely more on chemical fungicides world-wide. However, the increasing load of chemical pollution on environment, as well as the development of resistance against these fungicides has made the choice of biological control more popular in agriculture [4].

Since the biological control of phytopathogen may be the outcome of interactions taking place at many levels between the two organisms, the role of more potent mechanism of action is what the scientists looking for [5]. Production of biologically active metabolites is considered one of those mode of actions, potent for biological control. Various lytic enzymes are among these metabolites, as they have a capability to break down structural molecules of the pathogen, which include chitin, proteins, cellulose, hemicellulose and DNA [6]. Studies have shown that these lytic enzymes have direct or indirect potential to inhibit the phytopathogens [7].

The present study is planned to isolate the fungi that show antagonism against a well-known plant pathogen, *Fusarium oxysporum* from the local area of Jabalpur and to study their lytic enzymes profile that may be responsible for the antagonistic activity.

2. MATERIAL AND METHODS

2.1. Procurement of reference culture

A reference phytopathogenic strain of *Fusarium oxysporum* MTCC 4162 was procured from Microbial Type

Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on potato dextrose agar media slants during the study.

2.2. Isolation and identification of fungi

The fungi antagonistic to *F. oxysporum* MTCC 4162 were isolated from various soil samples. For this, 1 g of soil sample was taken and suspended in 10 ml of sterile distilled water. The sample was shaken for few minutes. The supernatant was further diluted up to the 10^{-4} dilution using sterile distilled water and 1 ml of it was poured onto potato dextrose agar media containing 0.01% chloramphenicol. The plates were incubated at 28±2°C for 3-7 days. Each distinct fungal colony was isolated, purified and maintained on potato dextrose agar [8].

2.3. Identification of fungal isolates

After incubation macroscopic features of all the colonies were recorded by studying following colony characters - colony growth (diameter), colony colour, texture, presence or absence of aerial mycelium, and Shape of the colony. Morphological identification was done according to Ellis *et al*. [9], and Zakaria and Aziz [10]. For further identification, microscopic characters using cover slip culture technique was studied.

2.4. Antagonistic activities of soil mycoflora against *Fusarium oxysporum* MTCC 4162

The antagonistic potential of different fungal isolates from soil were evaluated against *F. oxysporum* MTCC 4162 (reference culture) by using the dual culture technique. Mycelial disc (5mm dia.) of each test antagonist taken from 7 day old culture was placed at equal distances from the periphery of the Petriplate against same sized mycelial disc of *Fusarium oxysporum* MTCC 4162 at opposite end on PDA (20 ml) contained in 90 mm diameter Petriplates. The PDA plates inoculated only with phytopathogen served as control. The plates were incubated at 25±2°C. The results were recorded by measuring growth of the pathogen in both test and control experiments and the percent inhibition of radial growth was calculated as [11]:

$$\% \text{ inhibition} = (R_1 - R_2)/R_1 \times 100$$

Where R_1 = radial growth of pathogen in control, R_2 = radial growth of pathogen with antagonists.

2.5. Screening of antagonistic mycoflora for enzyme coproduction

The fungal species with antagonistic activity were screened for their ability to produce extracellular glucanolytic, chitinolytic and proteolytic enzymes. For this 100 ml of potato dextrose broth (pH 7.5) was inoculated with fungal spore suspension containing 10^4-10^6 spores and incubated at 25±2°C with shaking at 100 rpm for 7 days. After incubation, cell-free culture supernatant was prepared by centrifugation of fungal growth at 8000 rpm for 10 minutes. The culture filtrates were filtered through Whatman No.1 filter paper and the filtrate thus obtained was partially purified. For this, cell free supernatant was slowly saturated with solid ammonium sulphate to achieve 70% saturation levels and kept at 4°C overnight to extract the enzymes. The suspension was centrifuged at 5000 rpm for 15 min at 4°C, and the precipitates were dissolved in 50 mM phosphate buffer (pH 7.0) and dialysed against distilled water at 4°C overnight through a dialysis membrane (Hi Media, India) with a molecular weight cut off at 12000 Da for 48 hr with three changes of the distilled water. The stocks thus obtained were preserved at 0-4°C in PVC bottles and used as enzyme source for further quantitative analysis of glucanase activity, cellulase activity and protease activity. Total protein was analyzed by Folin reaction described by Lowry *et al*. [12] and using a standard curve of bovine serum albumin.

Glucanase activity was determined in all the above mentioned culture supernatants by following the method of Gautam*et al*. [13] with a modification. Briefly, to 0.5 ml Carboxy Methyl Cellulose (CMC solution, 1%) 0.5 ml of partially purified enzyme extract was added and were incubated at 37°C for 30 minutes in water bath. Controls devoid of enzyme extract were also run simultaneously. To it 3 ml of 3, 5-Dinitro salicylic acid (DNS) reagent was added and to stabilize the colour further 1.0 ml of sodium potassium tartrate (40% w/v) was added. Tubes were then placed in a boiling water bath (50°C) for 5 minutes for colour development and then cooled on an ice bath, followed by diluting each solution appropriately by addition of 15 ml distilled water to each tube. Contents of the tubes were mixed and absorbance was noted at 550 nm against a reagent blank (distilled water) in a UV-VIS spectrophotometer. Rate of enzyme activity was determined in terms of amount of glucose produced per minute, calculated by using a standard curve of glucose.

For estimation of chitinase activity, the assay system estimating reducing sugars released by enzyme action was adapted for the study [14]. Briefly, to 2.5 ml buffer (pH 7.0), 2.5 ml 1% substrate (colloidal chitin) was added followed by 0.5 ml partially purified enzyme. Controls devoid of enzyme extract were also run simultaneously. The tubes were incubated at 37°C for 1 hr. after which the reaction was stopped by adding 3.0 ml 10% dinitrosalicylic acid (DNS) reagent and 1.0 ml of (w/v) sodium potassium tartrate 40% (colour stabilizing solution), followed by heating for 5 minutes in a boiling water bath. The coloured solution was then centrifuged at 8000×g for 5 minutes and the absorption of supernatant was measured at 540 nm wavelength in a spectrophotometer. The amount of reducing sugar produced was estimated from a standard curve of reducing sugar N-acetyl-D- glucosamine (NAGA).

Protease activity was determined by using Hammerstein casein as substrate [15]. One gram casein was dissolved in 100 ml phosphate buffer (0.1M, pH 8). Substrate (1 ml) was added to 1 ml partially purified enzyme and incubated at 37 °C in a water bath for 10 min. The reaction was terminated by adding chilled 2 ml trichloro acetic acid (TCA) (10%, w/v) and mixture was allowed to stand at 4°C for 30 min. the reaction mixture was centrifuged (8,000 rpm, 15 min) to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm against a blank. Blank was prepared by adding TCA prior to the addition of casein solution. Rate of enzyme reaction is defined as amount of tyrosine produced per ml per minute under assay condition, using a standard curve of tyrosine.

3. RESULTS

To isolate fungal species rhizospheric soil samples were collected from different sites of Jabalpur (M.P) region during the year 2018-2019. The isolated fungal species were identified using available reference literature and monographs on the basis of their microscopic and macroscopic characters. IN total more than 22 fungal species, belonging to 10 genera were isolated during the study.

Dual culture technique was utilized to determine the *in vitro* antagonistic potential of isolated fungal species (test fungus) to suppress the growth of phytopathogen *F. oxysporum* MTCC 4162 on PDA (Fig 1).

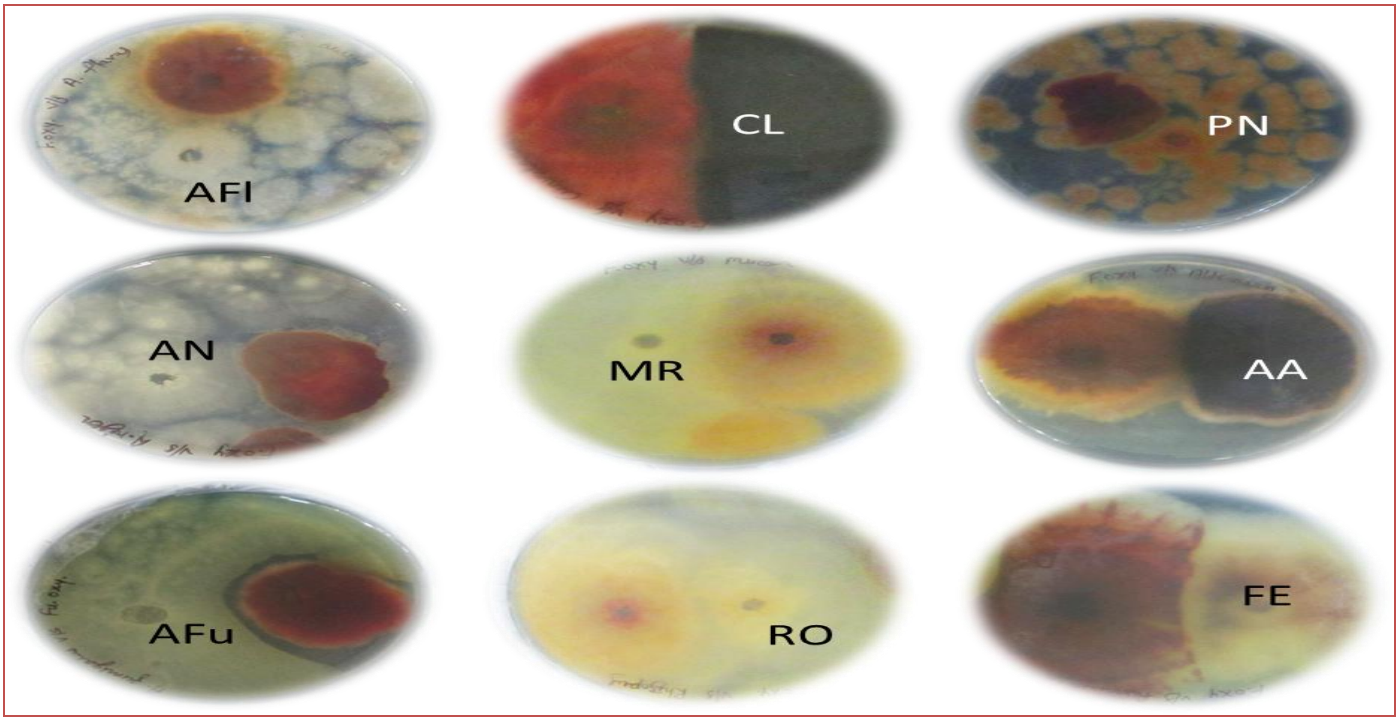

Fig. 1: Screening of antagonistic fungi against *Fusarium oxysproum* MTT 4162 by suppression of radial growth using dual culture method

AFl-Aspergillus flavus, AN- Aspergillus niger, AFu- A. fumigatus, CL-Curvularia lunata, MR- Mucor racemosus, RO- Rhizopus oryzae, PN- Penicillium notatum, AA- Alternaria alternata, FE- Fusarium equiseti

Out of 22 tested fungi, only 9 could show some inhibition of the *F. oxysporum* MTCC 4162. After seven days of incubation radial growth in diameter (mm) of test fungus and *F. oxysporum* MTCC 4162 were measured and compared with the radial growth of the *F. oxysporum* MTCC 4162which was allowed to grow alone (Table 1). Two strains viz *R. oryzae* and *P. notatum* showed significant suppression of growth of *F. oxysporum* MTCC 4162(15 mm) when compared to control (78 mm) showing 58.7% inhibition. Strains of Aspergillus viz *A. fumigatus* followed by *A. flavus* and *A. niger* along with *M. racemosus* were the promising strains as they restricted the radial growth of the phytopathogen to 24, 25 and 26 mm, respectively. Further on elaborating the results, it could be observed that the growth of the phytopathogen was restricted to 44 mm by *C. lunata* and *A. alternata*. *Fusarium equiseti* was also found to inhibit the growth of *F. oxysporum* MTCC 4162 (49 mm). All 9 antagonistic fungi were further screened for the production of different lytic enzymes.

Table 1: Radial mycelial growth of *Fusarium oxysporum* alone and with the isolated fungi

Test Fungi used with *F. oxysporum* MTCC 4162	Radial mycelial growth (in mm)														Inhibition of pathogen (%)
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7		
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	
Control	-	-	10		14		25		46		66		78		-
A. flavus	-	-	10	18	12	22	14	26	18	38	22	42	25	44	45.95
A. niger	-	-	10	16	13	21	15	24	19	40	23	43	26	43	44.67
A. fumigatus	-	-	11	17	13	23	16	24	18	39	22	42	24	44	47.23
M. racemosus	-	-	12	20	15	25	18	30	20	36	23	48	26	52	44.67
C. lunata	-	-	12	13	22	21	27	29	34	36	42	42	44	46	21.59
F. equiseti	-	-	11	12	18	19	26	25	32	30	36	32	49	41	15.18
P. notatum	-	-	10	12	12	16	13	22	14	32	15	38	15	46	58.77
A. alternata	-	-	12	11	16	18	28	27	34	36	42	43	44	46	21.59
R. oryzae	-	-	11	12	14	24	14	37	14	42	15	48	15	52	58.77

Reduced mycelial growth of F. oxysporum MTCC 4162 with test fungi in comparison to control shows the antagonistic activity.

The fungal species with antagonistic activity viz *R. oryzae*, *P. notatum, A. fumigatus*, *A. flavus*, *A. niger, M. racemosus, C. lunata, A. alternate* and *F. equiseti* were also screened for their ability to produce extracellular glucanolytic, chitinolytic and proteolytic enzymes. Maximum amount of specific glucanase activity was shown by *P. notatum*(118.65±10.5 µg glucose produced $min^{-1}mg^{-1}$ protein), followed by *R. oryzae* (107.20±11.2 µg glucose produced $min^{-1}mg^{-1}$ protein), While minimum was secreted by *A. niger* (46.72±2.8 µg glucose produced $min^{-1}mg^{-1}$ protein) (Fig. 2).

Penicillium notatum showed highest specific chitinase activity (85.17±10.5 µg NAGA produced $min^{-1}mg^{-1}$ protein) while minimum amount was produced by *A. niger* (60.65±4.8 µg NAGA produced $min^{-1}mg^{-1}$ protein) (Fig. 3).

Highest specific protease activity was detected in *R. oryzae* (133.28±15.8 µg tyrosine produced $min^{-1}mg^{-1}$ protein) while minimum was secreted by *C. lunata* (61.29±6.1 µg tyrosine produced $min^{-1}mg^{-1}$ protein) (Fig. 4).

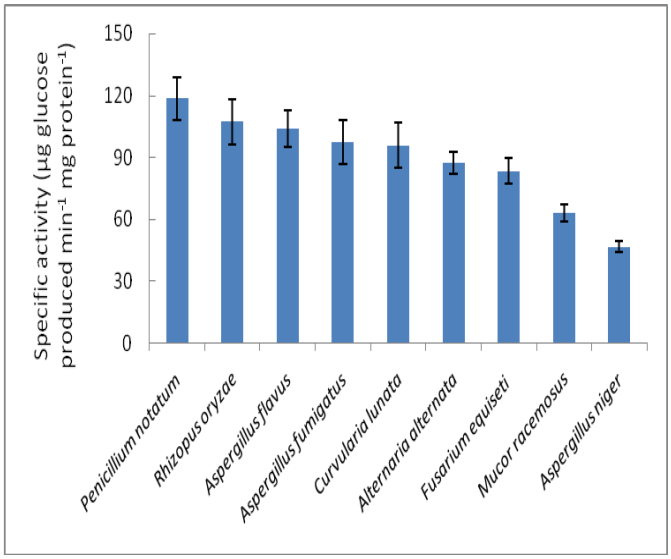

Fig. 2: Specific gluconase activity of the test fungi antagonistic to *Fusarium oxysporum* MTCC 4162 in decreasing order

Data are presented as mean ± SD (n=3).

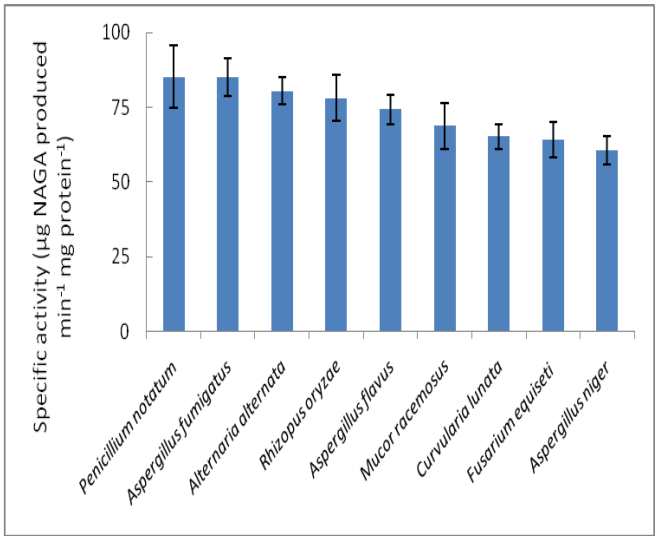

Fig. 3: Specific chitinase activity of the test fungi antagonistic to *Fusarium oxysporum* MTCC 4162 in decreasing order

Data are presented as mean ± SD (n=3)

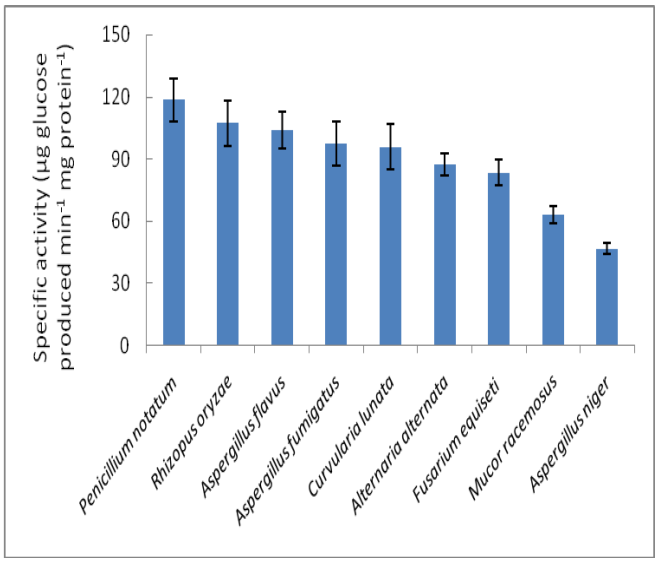

Fig. 4: Specific protease activity of the test fungi antagonistic to *Fusarium oxysporum* MTCC 4162 in decreasing order

Data are presented as mean ± SD (n=3).

4. DISCUSSION

Plant diseases cause severe losses of agricultural and horticultural crops every year. Increased incidents are being reported for the crop loss due to phytopathogenic fungi, i.e. *Pythium, Botrytis, Rhizoctonia* and *Fusarium*. Due to ill effects of chemical fungicides viz., environmental pollution, health hazards, phytotoxicity, development of resistance by the pathogen and also exorbitant cost necessitates great commercial interest for modern agriculture in substituting the use of chemical fungicides with biological control agents (biopesticides) against the pathogens. The use of antagonistic microflora, is becoming an inevitable component in the integrated management strategy of plant diseases. The rhizosphere is the first line of defence for roots, against attack by pathogenic fungi. Therefore, there is an excellent opportunity to find rhizosphere competent microorganisms that can act as potential biopesticides [16].

Recently, there were many reports about the application of antagonistic fungi in controlling plant disease such as the use *Penicillium, Cheatomium* and *Trichoderma* species [17, 18]. Microbial biological control agents use a great variety of mechanisms to protect plants from pathogens and production of extra cellular lytic enzymes is one of them. Lytic enzymes can break down polymeric compounds, including chitin, proteins, cellulose, hemicellulose and DNA of other fungi in the vicinity, thereby controlling the growth of other fungi [7, 19].

R. oryzae and *P. notatum* was found potent against selected phytopathogenic fungi when tested through dual culture assay and showed more than 80% antagonistic activity. Dong and Cohen [20] reported that *P. chrysogenum* protects cotton plants against wilt disease caused by *F. oxysporum* f. sp. *vasinfetum*. *Aspergillus* sp. are also well known bio-control agent of plant pathogen. In the present study around 70% growth of *F. oxysporum* var. redolens MTCC 4162 was inhibited by *A. niger, A. flavus, A. fumigatus*. Anil Kumar and Rajkumar [21] performed test of the antagonism, and reported that *A. niger, A. flavus, A. fumigatus* and *A. tamarii* possess best bioactivity against *Fusarium oxysporum* f. sp. *lycopersici*. Boughalleb-M'Hamdi *et al.* [22] also reported that the three *Aspergillus* species (*A. flavus*, *A. niger* and *A. terreus*) exhibited an important growth inhibition against the colonies of *F. oxysporum* f. sp. *melonis*. El-Sheshtawi *et al.* [23] demonstrated that *T. harzianum* and *Penicillium oxalicum* can act as biological control agents for Fusarium wilt.

Some of these enzymes also play antifungal activities, applied *in vitro* and in plant rhizosphere, either alone or combined application. A basic principle in mycoparasitism has been attributed to chitinase, glucanases and proteases. However, fungal proteases may be significantly involved in antagonistic activity, not

only in the breakdown of the host cell wall (composed of chitin and glucan polymers), but also by acting as proteolytic inactivators of pathogen enzymes involved in the plant infection process [24]. Cell wall lytic enzymes, however, are not only involved in mycoparasitism but are also important for cell wall recycling during ageing and autolysis as well as cell wall remodelling during active growth (e.g. hyphal branching) [25].

The extensive use of chitinase and glucanase producing microorganism as biological control agents against many fungal pathogens has been reported. These lytic enzymes break down cell wall polysaccharides into short oligomers and by this way facilitate the hyperparasite to penetrate into the cytoplasm of the target fungi. The inhibition of soil borne fungi, *F. oxysporum* f. sp. ciceri by *Trichoderma* species, due to production of extracellular cell wall degrading enzyme such as chitinase, β-1, 3-glucanase, β-1, 6-glucanase, protease, cellulase and lectin, which help *Trichoderma* in colonizing the host, has been reported [26]. Another speculation can well be that antagonistic activities are the reflection of faster use of available carbon and nitrogen resources by the antagonist (using the various extracellular lytic enzymes) as compared to the pathogen, leaving the later with suppressed growth.

5. ACKNOWLEDGEMENT

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Conflict of interests

The authors have no conflict of interests.

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