STUDY OF VIRULENCE FACTORS IN PHYTOPATHOGENIC FUNGI FROM POMEGRANATE PLANT

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Abstract

Virulence factors are molecules expressed and secreted by pathogens that enable them to achieve colonization of a niche in the host, immunoevasion, immunosuppression, entry into and exit out of cells and obtain nutrition from the host. Virulence factors are very often responsible for causing disease in the host because they are often responsible for converting non-pathogenic bacteria into dangerous pathogens.

Fungi are eukaryotic, carbon-heterotrophic microorganisms. To satisfy their need for organic nutrients, most fungal species live a saprophytic lifestyle. The interaction between plant and phytopathogenic fungi are complex. Virulence is a complex interrelationship between the infecting organism and the host. Pathogenesis involves interaction (and sometimes modification) of factors on both sides.

Fungi are important pathogens of plants that cause significant yield losses.. Numerous fungi are devastating and are serious threat to agricultural industry. A number of fungal mechanisms and molecules have been shown to contribute to fungal pathogenicity or virulence, understood as the capacity to cause damage in a host. Among them, cell wall degrading proteins, inhibitory proteins and enzymes involved in the synthesis of toxins are included.

In the present study plant pathogenic fungal cultures were obtained from National Research Centre on Pomegranate (NRC) Solapur.A detailed morphological study of these fungi was done by growing them as slide culture and microscopic observation of the same All the eight fungal cultures were tested for production of various enzymes like cellulase, chitinase, amylase, lactase and others. The study will be helpful in developing biocontrol agents against these fungi.

Index Terms: Virulence factors,Enzymes,Fungal pathogens, Biocontrol

Introduction

Virulence is viewed to be a relative term and defined as the relative capacity of a microbe to cause damage in a host. Phytopathogens do not have to contend with an adaptive immune system but instead must possess powerful physical and enzymatic mechanisms for piercing the plant cell wall.(1) Fungal plant pathogen comprises an important group of microorganisms that cause significant economic losses in agriculture around the world. They are able to infect any tissue at any stage of plant.

These factors include an ability to adhere to hosts' tissues, production of enzymes that cause tissue damage and direct interference with host defence.(2)

Common strategies phytopathogenic fungi include forming specialized infection structures

synthesis and secretion of different enzymes (*i.e.*, cuticle and cell wall degradation enzymes) production of secondary metabolites with antimicrobial properties (*i.e.*, phytoalexins, glycosides, glucosinolates, *etc*.) or even producing several families of toxins.(3)

The fungi attack the plants to obtain nutrition. For this they have to overcome the physical barrier of the cell wall. Fungi first take up the nutrition in their immediate vicinity and then extend towards other areas of nutrition availability. The continuous need to reach new food supplies for growth and reproduction is the most driving force for fungal virulence. Growing through the plant cell wall allows the fungus to get access to simple sugars ,amino acids, minerals and nucleotides which are abundant in the cytoplasm.(4)

Pomegranate is a major cash crop .Development of fungal infections results in decrease in productivity, change in appearance and lesser acceptance by consumers in general. Many types of fungi have been known to infect Pomegranate. The ability of these fungi to produce a wide array of enzymes would be a guideline in developing newer and safer natural antifungals against these fungi.

Traditionally, disease control is carried out using proven chemical treatments. These treatments, however, have many drawbacks, due to their negative impact on the environment and public healthFor some diseases, chemical control is very effective, but it is often non-specific in its effects, killing beneficial organisms as well as pathogens, and it may have

undesirable health, safety, and environmental risksFor example, the emergence of fungicide-resistant strains, de-registration of fungicides, and public concerns regarding the health and environmental impacts of agrochemicals, may all act to limit their application in the future. (5)Though there is incredible amount of biological information about fungal plant pathogens, there is no commercial fungicide developed from a molecular approach

Lactic Acid Bacteria (LAB) are known to produce a wide range of antifungal agents which can be used against these phytopatogenic fungi.These bacteria, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Acidification inhibits the growth of spoilage agents. Proteinaceousbacteriocins are produced by several LAB strains the industrial importance of the LAB is further evidenced by their reputed safe **(GRAS)** status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces.

LAB are known to produce different acids mainly lactic and acetic ,caproic ,propionic acid,Diacetyl,Reuterin,cyclomethylhydantoin ,mevalonolactone,and other unidentified low molecular weight compounds.(7-12).The LAB isolated from Pomegranate have been used to inhibit the phytopathogenic fungi and hence can be successfully used to prevent agricultural and economic losses.

Materials and Methods

The fungal cultures used in this study were obtained from National Research Centre on Pomegranate (NRC),Solapur,India.All media ingredients used are of Hi Media and chemicals used are of research grade.

The fungal cultures used in the study were as follows:

1) *Aspergillus sp.*
2) *Cercospora sp.*
3) *Colletotrichum 1338*
4) *Colletotrichum 1335*
5) *Fusarium oxysporium(M)*
6) *Fusarium oxysporium(O)*
7) *Ceratocystis fimbriata N4*
8) *Ceratocystis fimbriata W3*

All fungal cultures were cultured on C Dox agar and then observed for their morphology by slide culture method.

Production of extracellular enzymes by the phytopathogenic fungi was assessed by digestion of suspended or dissolved substrate in agar plates afterinoculation with 3 mm mycelial plugs and incubation for 3-5 days at R.T. (13)

The zone of enzyme activity surrounding the fungalcolony was measured.

Amylase activity was assessed by growing the fungi onglucose yeast extract peptone (GYP) agar medium (glucose, 1 g; yeast extract,0.1g; peptone, 0.5g; agar, 16 g; distilled water, 1000 mL; pH 6) with 2%soluble starch. After incubation, the plates were flooded with 1% iodine in 2%potassium iodide. The clear zone formed surrounding the colony was considered positive for amylase activity.

For cellulose activity, the fungi were cultured on yeast extract peptone agar medium (yeast extract, 0.1g; peptone 0 .5 g; agar,16 g and distilled water, 1000 mL) supplemented with 0.5% Na-carboxymethylcellulose (CMC). After incubation, the plates were flooded with 0.2 aqueous Congo Red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the cellulase activity.

For chitinaseactivity, the fungi were grown on colloidal chitin agar medium (colloidal chitin, 2 g; agar, 16 g; distilled water, 1000 mL). The clear zone surrounding the colony after incubation was considered positive for chitinase activity

Laccase activity was assessed by growing the fungi on GYP agar medium amended with 1-naphthol, 0.005% (pH, 6) and incubated. On oxidation of 1-naphthol bylaccase, the medium changes from clear to blue.

For lipase activity, the fungi were grown on peptone agar medium (peptone, 10 g; NaCl 5 g; $CaCl_2$ $2H_2O$0.1 g, agar, 16 g, distilled water, 1000 mL; pH 6) supplemented with Tween 20(separately sterilized and added 1 mL to 100 mL medium). A clear zone around the colony indicated lipase-positive fungi

Protease assay was performed by growing the fungi on GYP agar medium amended with 0.4%gelatin (gelatin, 8 g/100 mL distilled water, sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitated with ammonium sulphate and digested area around the colony was clear.

For tyrosinase activity, fungi were grown on GYP agar medium. After incubation a mixture of 0.11% *p*-cresol and 0.05 % glycine was overlaid on the surface of the fungal colony. Culture plates were observed after24 hours for the appearance of red brown colour around the colony which indicated tyrosinase activity.

For pectinase activity the fungi were grown on pectin agar medium.(pectin 1g,diammonium –O-PO_4,0.3g,KH_2PO_4,$MgSO_4$,Agar 3g, distilled water, 100mL,pH 6.6) After incubation, the plates were flooded with 0.2 aqueous Congo Red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the pectinase activity.

To see the inhibition of the fungi,biocontrol in form of Lactic Acid Bacteria was used. The LAB produce a range of different acids with lactic acid and acetic acid being the most prominent. These acids can lower the pH in the vicinity of the fungi and can affect the activity of the different enzymes which are produced by fungi in order to penetrate the plant system.

The activity of acids Lactic,Acetic,Succinic,Formic and Ethanol was tested against two phytopathogenic fungi *Penicillium sp.*and*Fusarium sp.*Suitable dilutions of these acids were made and antifungal activity was recorded against the said fungi on C-Dox agar.

Five different isolates of LAB from aerial surfaces of pomegranate plants were tested for their antifungal activity against all the fungi for which virulence studies were done.This was done by agar overlay method. On basis of morphological, biochemical and nature of fermentation these isolates were identified up to genus level.

LAB were grown on the Lactobacillus MRS medium It was overlaid by Czapekdox Agar ***Penicillium*** **and** ***Fusarium*** **were** spread on the Czapekdox Agar layer and incubated for 24-48 Hrs. A zone of inhibition was observed for and the diameter of zone of inhibition by each LAB culture against the fungi was noted down.

Table1 : Production of enzymes by fungal isolates from Pomegranate

Fungal isolate	Amylase	Laccase	Lipase	Protease	Cellulase	Tyrosinase	Chitinase	Pectinase
Cercospora	+	-	+	+	+	+	+	-
Ceratocystis N4	+	+	+	-	+	+	+	+
Fusarium(o)	+	-	-	-	+	+	+++	+
Colletotrichum 1335	+	-	+	+	-	-	++	+
Aspergillus	++	-	+	-	+	-	+	+
Ceratocystis W3	-	+	+	-	+	-	+	+
Colletotrichum 1338	+	-	+	+	+	-	+++	+
Fusarium(M)	+	-	-	+	+	+	++	+

+ = zone diameter of approximately 2.9-3.2 cm

++ = zone diameter of approximately 3.5-4.2cm

+++ = zone diameter of approximately 5.6-7.2cm

Table2:Inhibition zones observed for dilutions of various acids against *Penicillium sp* and *Fusarium sp* on CzapekDox agar after 24 Hrs.incubation

		Lactic Acid		**Acetic Acid**		**Succinic Acid**		**Formic Acid**		**Ethanol**	
No	Dilution	*Penicillium* (cm)	*Fusarium* (cm)	*Penicillium* (cm)	*Fusarium* (cm)	*Penicillium* (cm)	*Fusarium* (cm)	*Penicillium* (cm)	*Fusarium* (cm)	*Penicillium* (cm)	*Fusarium* (cm)
1	1:2	3.7	3.5	2.4	2.5	-	-	3.5	3.0	1.5	1.5
2	1:4	3.7	2.7	2	2.0	-	-	3.0	3.5	1.1	1.0
3	1:8	3.5	2.3	1.7	1.0	-	-	3.0	3.5	-	-
4	1:16	3.0	1.7	-	-	-	-	3.0	2.0	-	-
5	1:32	2.2	2.5	-	-	-	-	3.0	1.5	-	-
6	1:64	1.5	1.8	-	-	-	-	1.5	1.3	-	-
7	1:128	1.5	1.0	-	-	-	-	-	1.0	-	-
8	1:256	-	0.9	-	-	-	-	-	1.0	-	-
9	1:512	-	0.9	-	-	-	-	-	-	-	-
10	1:1024	-	0.7	-	-	-	-	-	-	-	-

- = No Inhibition

Table3: Antifungal activity of selected LAB isolates against fungi by Agar Overlay Method

LAB Isolate	Aspergillus	Fusarium(M)	Cercospora	Colletotrichum 1335	Colletotrichum 1338	CeratocystisN 4	Ceratocystis W3	Fusarium(O)
FL3(2)	+	+	+	+	-	-	-	+
FL2(3)	+	+	+	+	+	-	-	+
FL2(5)	-	+	+	-	-	+	+	+
FL3(3)	+	+	+	-	-	-	+	+
F3415	-	+	+	+	-	+	-	+

+ = Inhibition - = No Inhibition

FL3(2)- *Enterococcus sp.*

FL2(3)-*Leuconostoc sp.*

FL2(5)- *Enterococcus sp.*

FL3(3)-*Leuconostoc sp.*

F3415- *Enterococcus sp.*

Results and Discussion:

As seen from the results (Table 1) the different phytupathogenic fungi from Pomegranate are seen to produce different enzymes which serve as important virulence factors in causing damage to the host. All enzymes are not produced by all the fungi .Also all enzymes may not be expressed at one time.Different enzymes play different roles in establishing the pathogen in the host.

Amylases act on starch and polysaccharides to hydrolyze the glycoside bonds This results in degradation of wide variety of natural polysaccharides.(16) Laccases belong to the small group of enzymes called blue multi copper oxidases.Laccase is abundantly produced by fungi that degrade lignin,The different degrees of lignin degradation depend on the environmental conditions and the fungal species involved The enzymaticmachinery of the various fungi differ. Fungal laccases have beenimplicated in many cellular processes, including delignification, sporulation, pigment production, fruiting body formation and plant pathogenesis.(17)Lipases and proteases are important enzymes in pathogenesis which attack the plasma lemma after the degradation of cell wall by proteases along withpectolytic and cellulolytic enzymes (18)
Melanin and laccase affect the virulence capacity of pathogenic microorganisms. Melanins are a challenge to the defense mechanisms of the hostsMelanins also protect fungi from hydrolytic enzymes UV, solar or gamma radiation extreme temperatures], and heavymetals and several other toxic compounds.(19) The breakdown of cellulose to soluble sugars by phytopathogens involves the action of a multienzyme system aiding the phytopathogen to gain access into the internal tissue components.(20)

It is logical to assume that the inhibition of enzymes by targeted strategies should produce new antifungal agents In this context, the use of natural products or related compounds as specific enzyme inhibitors would prove to be of great value as they would be species specific and the environmental impact would be reduced to a minimum. (14,15)

It was observed that presence of acids (Table 2) i.e exposure to low pH greatly inhibited the growth of fungi.These enzymes are most active when a pH of 4 -6 is presentThe molecular structures of enzymes are affected by pH. Changes in pH can denature an enzyme by changing the protein's ionic charge. Ionic bonds create the functional shape of the enzyme; when the ion charge changes, the shape of the enzyme changes, which changes its function in turn.(21,22,23) and hence their activity can be minimized by exposure to lowered pH values.

LAB are known to produce acids and hence can be used to counteract these enzymes.This study reports the antifungal activity of *Enterococcus sp.* and *Leuconostoc sp*. of plant origin .against a variety of phytopathogenic fungi like *Aspergillus ,Penicillium, Fusarium,Cercospora ,Colletotrichum and Ceratocystis* isolated from infected pomegranate plant.(Table 3)

Young and Clausen (2005) have found that *Lactobacillus casei* and *Lactobacillus acidophilus* inhibit mould growth in liquid culture.Livermicocca et al (2000) found antifungal activity by *Lactobacillus plantarum*.Magnusson et al (2001) have identified a broad spectrum antifungal compound produced by *Lactobacillus plantarum*.and*Lactobacillus coryniformis*.Rouse et al (20080 have reported antifungal peptides produced by *Pediococcuspentosaceus*against *Penicillium expansum.*Roy et al (1996) showed the activity of *Lactobacillus lactis* producing a proteinaceous compound against species of *Fusarium* and *Aspergillus.*Gourama et al (1997) have shown production of proteinaceous substance by *Lactobacillus casei* against *Penicillium sp.*

Hence lowering of pH and production of other antifungal compounds by LAB can be used to overcome phytopathogenic fungi.

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