Comparison of Staining Methods for the Detection of Xylanase and Pectinase Activities in *Phytophthora meadii*

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Abstract

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year. Plant cell wall-degrading enzymes (PCWDEs) play significant roles in phytopathogenic fungi during their growth and infection process and pathogenesis. Fruit rot of arecanut caused by *Phytophthora meadii* is a major limiting factor for arecanut production. Although vast amount of information is available on the secretion of extracellular plant cell wall degrading enzymes by phytopathogenic fungi, no information on the production of PCWDE's is available in *Phytophthora meadii*. In the present work, we explored the *in vitro* production of plant cell wall degrading enzymes (xylanases and pectinases) using Congo red and Gram's iodine as chromogenic dyes by cup plate assay. *Phytophthora meadii* secretes maximum amount xylanase and pectinase on 8^{th} and 5^{th} days respectively at pH 4.5. Several methods are in use for the selective screening of PCWDE's. But we found that both Congo red and Gram's iodine are equally effective in the detection of xylanase whereas Congo red gives best results for pectinase activity. This is first report on the use of Congo red and Gram's iodine for the detection of PCWDE's in *Phytophthora meadii.*

Key words: *Phytophthora meadii*, plant cell wall-degrading enzymes, chromogenic media, fruit rot, plate assay, comparative study, arecanut

I Introduction

Cells of higher plants are surrounded by the wall, a resilient and heterogeneous network made up of different classes of polymers, mainly cellulose, xyloglucan, pectin and structural proteins. In addition to providing structural support, the cell wall constitutes an efficient line of defense against microbial invaders. Phytopathogenic fungi secrete a battery of plant cell wall depolymerases which play significant roles in infection process, plant pathogenesis and nutrient acquisition [1, 2]. Major classes of extracellular cell wall degrading enzymes (CWDEs) are pectinases, xylanases, cellulases, glucanases, proteases, cutinases and lipases. Pectins are high

molecular weight polysaccharides found in higher plants. They form the primary cell wall and the main components of the middle lamella R[3]. The involvement of fungal pectinases and other hydrolases in the degradation of pectic constituents of cell walls and middle lamella of plant tissues has been reported for diverse types of diseases such as soft rot, dry rot, wilts, blights and leaf spots which are caused by pathogenic agents such as fungi, bacteria and nematodes [4].The role of pectin degrading enzymes in causing cell wall degradation is so important that it determines the virulence of many pathogens [5]. Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8) is a hydrolytic enzyme involved in depolymerization of xylan, the major renewable hemicellulosic polysaccharide of plant cell wall. It is produced by bacteria [6, 7, 8],bacteria and fungi, fungi[9,10], actinomycetes [11] and yeast[12]. Recently, interest in xylanases and pectinases has markedly increased due its wide variety of biotechnological applications and their implication in fungal pathogenesis [13,14,15].

Phytophthora meadii is a phytopathogenic fungus causing fruit rot or koleroga of arecanut. Fruit rot is a serious disease that could lead to great economic losses. This disease is so rampant in all the arecanut growing regions receiving heavy rainfall during southwest monsoon period. In Karnataka, arecanut is cultivated in various districts of Central Western Ghats region and the production is severely affected due to the incidence of the fruit rot[16,17,18]. Several previous reports have shown that many phytopathogenic fungi secrete a high number of enzymes to the extracellular medium during the infection process to degrade the various plant tissues and defensive barriers. These include cell-wall-degrading enzymes (CWDE), such as pectinases, cellulases, and xylanases, as well as cutinases, lipases, and proteases [19]. Extracellular enzymes have the presumably important role of both facilitating the growth of the hyphae by softening the plant tissues and converting complex plant material into readily assimilable nutrients[20].

In virtue of the shortage of information about extracellular enzymatic activity of this pathogenic fungi and later to understand their role in infection process and pathogenesis, this present study evaluated the potential of *Phytophthora meadii* for xylanase and pectinase enzyme secretion using a simple and effective cup plate method with different staining methods.

II Materials and Methods

All the chemicals and reagents used in the study were of AR grade and were procured from Hi-media labs, Mumbai, India.

2.1 Fungal Culture

Culture of *Phytophthora meadii*, previously isolated from infected arecanut was kindly provided by Dr. P. Chowdappa, Division of Plant Pathology, Indian Institute of Horticulture Research (IIHR), Hesaraghatta, Bangalore and maintained on Carrot Agar medium (CA) at 25^0C.

2.2 Detection and comparison of screening methods for xylanase activity

The organism was grown on agar plates composed of 0.1%(w/v) K_2HPO_4, 0.01%(w/v) $MgSO_4$, 0.01%(w/v) NaCl, 0.5%(w/v) $(NH_4)_2SO_4$, 0.01% (w/v) $FeSO_4$, 3.5% (w/v) agar agar and 1% (w/v) xylan from birchwood as sole source of carbon. Prior to sterilization, the pH of the media was adjusted to 4.5. The plates were inoculated with a fungal mycelium disc (5 mm diameter) cut from the edge of a 7-day old culture grown on CA and plates were incubated at 37^0C for 10 days[21]. On 3rd, 5th, 8thand 10th days the plates were removed and enzyme production was monitored by staining the plates either with Gram's iodine or Congo red. The first set of plates was flooded with 0.1% Congo red for 15-20 minutes and then with 1M NaCl for 15-20 minutes[22] and the second set of plates was flooded with Gram's iodine (0.1

gm iodine, 3.33 gm potassium iodide in 150 ml distilled water) for 3 to 5 minutes. After incubation both set of plates were observed for zone of clearance around the colony [23]. Zone formed around the colony is measured by using Antibiotic zone scale.

Further, to confirm the xylanase activity 8th day old culture supernatant (30 µl and 60 µl) was poured into the wells in 1% (w/v) birchwoood xylan amended agar plates, which were made with a gel cutter, and the plates were incubated for 24 hours at 37^{0}C and flooded individually with 00.1% Congo red for 15-20 minutes, followed by 1M NaCl for 15-20 minutes, and Gram's iodine for 3-6 minutes.

2.3 Detection and comparison of screening methods for pectinase activity

The organism was grown on agar plates composed of 0.1% (w/v) K_2HPO_4,0.1% (w/v) $MgSO_4$,0.01% (w/v) Nacl,0.5% (w/v) $(NH_4)_2SO_4$,0.01% (w/v) $FeSO_4$,3.5% (w/v) agar agar and 1% (w/v) pectin from citrus fruits as a sole source of carbon. Prior to sterilization, the pH of the media was adjusted to 4.5. The plates were inoculated with a fungal mycelium disc (5 mm diameter) cut from the edge of a 7-day old culture grown on CA and plates were incubated at 37^{0}C for 10 days [21].On 3rd, 5th, 8thand 10th days the plates were removed and enzyme production was monitored by staining the plates either with Gram's iodine or Congo red. The first set of plates was flooded with 0.1% Congo red for 15-20 minutes and then with 1M NaCl for 15-20 minutes[22] and the second set of plates was flooded with Gram's iodine (0.1 gm iodine, 3.33 gm potassium iodide in 150 ml distilled water) for 3 to 5 minutes. After incubation both set of plates were observed for zone of clearance around the colony[23] Zone formed around the colony is measured by using Antibiotic zone scale.

Further, to confirm the pectinase activity 5th day old culture supernatant (30 µl and 60 µl) was poured into the wells in 1% (w/v) pectin from citrus fruits amended agar plates, which were made with a gel cutter, and the plates were incubated for 24 hours at 37^{0}C and flooded individually with 00.1% Congo red for 15-20 minutes, followed by 1M NaCl for 15-20 minutes, and Gram's iodine for 3-6 minutes.

III Results and Discussion

The plant cell wall is comprised of cellulose, hemicellulose, glycoproteins, pectin and lignin which makes up the structural framework of the wall [24]. Enzymes are involved in the initial entry of the pathogen, its spread within the plant tissue and the degradation of host tissue into metabolites, thus playing a fundamental role in host-parasite interaction [25]. The phytopathogenic organisms produce a variety of enzymes to degrade the complex polysaccharides of the plants. Cell wall degrading enzymes released by pathogens are known to be responsible for pathogenesis. The Therefore, we used birchwood xylan and pectin from citrus fruits as sole source of carbon substrates to assess the ability of *Phytophthora meadii* to secrete enzymes and compared which staining method is better for a plate assay of detecting fungal extracellular enzymes. Evaluation of enzyme activity was conducted by measuring clear zone (plaque) peripherally formed around the fungal colony resulting from reaction between chromogenic dyes and the unhydrolyzed polysaccharides. All the detection results of xylanolytic and pectinolytic activities based on the formation of clear zone were given Figure 1, 2, 3 & 4 respectively. In case of birchwood xylan substrate, clear zone that showing the presence of xylanase activity could be detected in both the staining methods. Further there was no much difference in the zone of hydrolysis values when stained with Congo red and Gram's iodine. Based on this we are suggesting that both staining methods equally effective for the detection of xylanse activity in *Phytophthora meadii.* Maximum xylanolytic activity was observed on 8th day as indicated by clear zone size (Table 1). All measurement values were obtained from 3 replicate plates of each staining method. In contrast to xylanase, pectinolytic activity was maximum at 5th day (Table 2) and distinct prominent clear zone was seen in plates stained with Congo red. Hence Congo red

staining is a better method for the detection of pectinase activity in *Phytophthora meadii*. In conclusion, our results will be useful to the researchers who are screening large number of microorganisms for these cell wall degrading enzymes having potential applications in various industries. This work generated basic information on the secretion of plant cell wall degrading enzymes (PCWDE's) by *Phytophthora meadii* which causes fruit rot of arecanut. Like other phytopathogenic fungi *Phytophthora meadii* might use these depolymerases during its infection. To the best of our knowledge, this is the first report on the use of Congo red and Gram's iodine staining methods for the detection of xylanase and pectinase secretion by *Phytophthora meadii* using plate assay.

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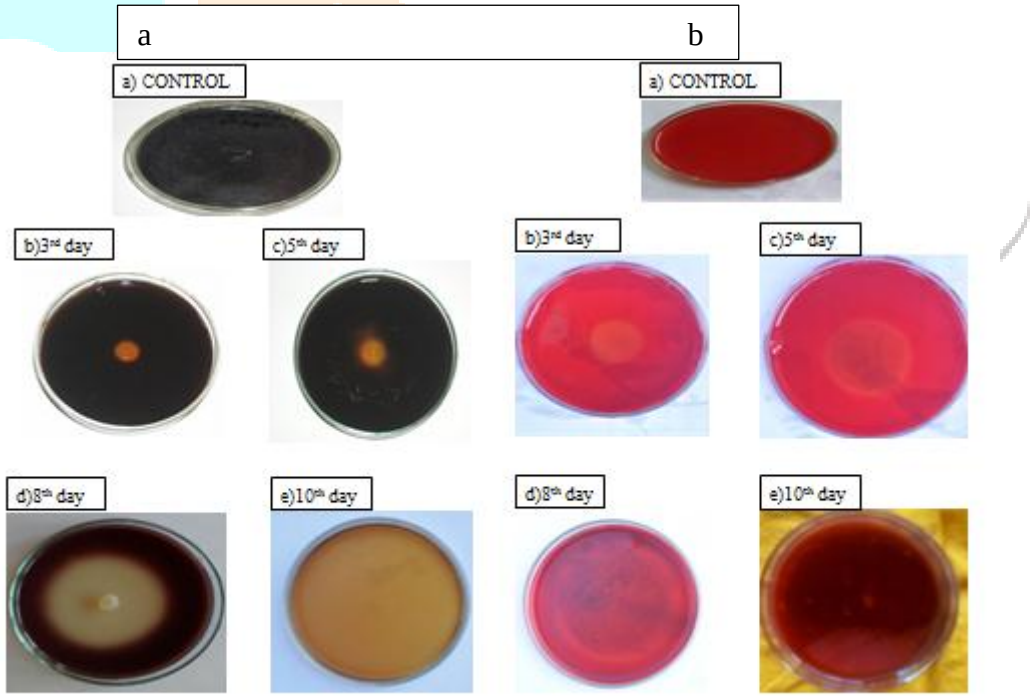

Fig 1. Detection of xylanase activity (a) petri dishes with substrate (1% birchwood xylan) and without (control) flooded with gram's iodine; (b) with and without substrate flooded with congo red.

(Ca) (Cb)

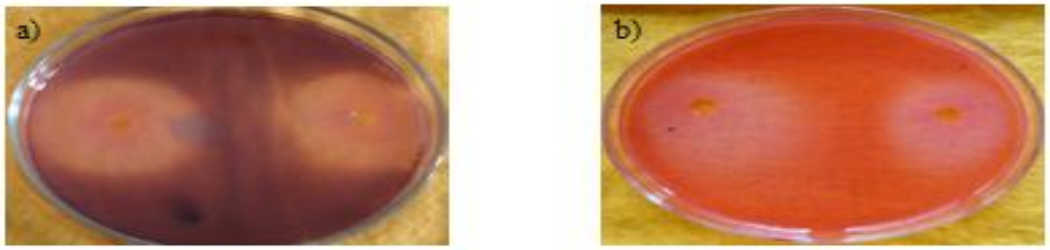

Fig 2. Detection of xylanase activity in 8-day old culture supernatant. Culture supernatant (30 & 60 µl) poured into the wells in xylan amended plates and incubated for 24 hours at 37^0C. (Ca) petri dish flooded with Gram's iodine; (Cb) with Congo red.

Table 1. Clear zone / Halo size for xylanolytic activity

	Clear zone / Halo size (mm) at pH 4.5			
Days of incubation	**3rd**	**5th**	**8th**	**10th**
Gram's iodine stain	±0.2	±0.3	±2.52	±1.78
Congo red dye	±0.8	±0.8	±2.51	±1.65

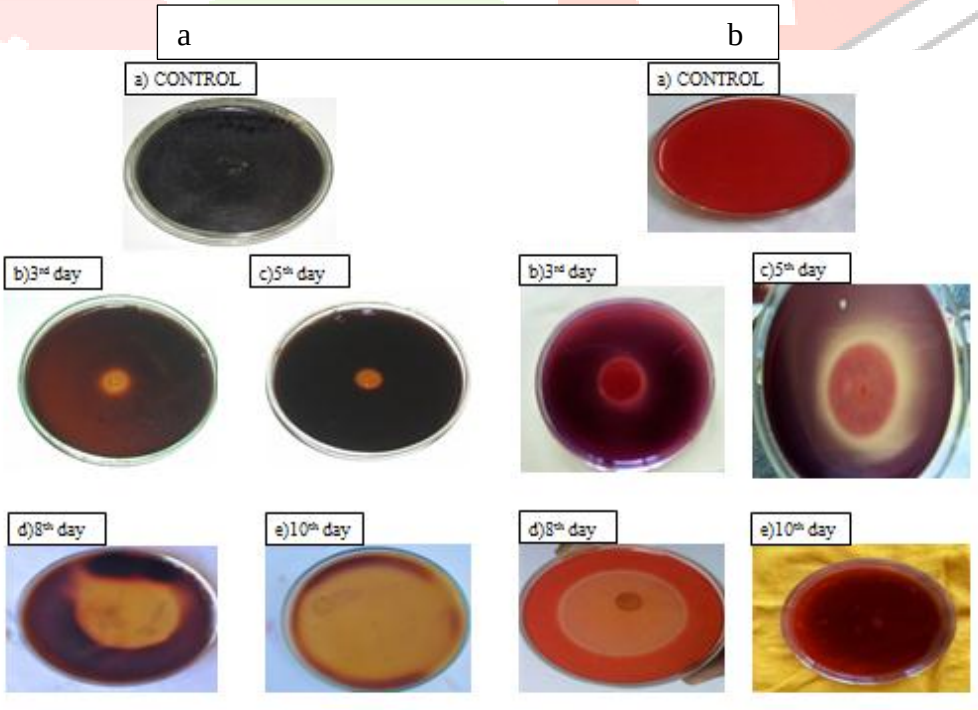

Fig 3. Detection of pectinase activity (A) Petri dishes with substrate (1% pectin from citrus fruits) and without (control) flooded with Gram's iodine; (B) with and without substrate flooded with Congo red.

(Ca) (Cb)

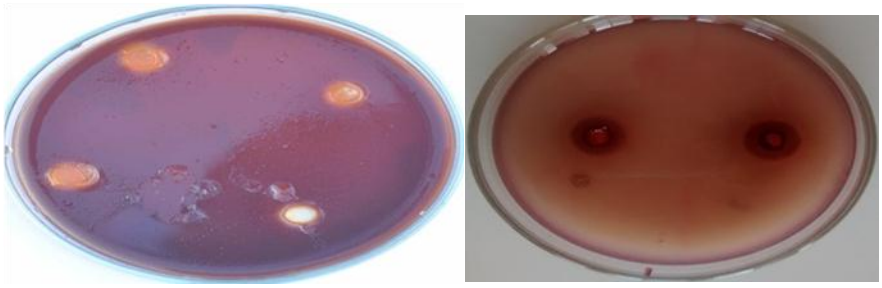

Fig 4. Detection of pectinase activity in 8-day old culture supernatant. Culture supernatant (30 & 60 µl) poured into the wells in pectin amended plates and incubated for 24 hours at 37^0C. (Ca) petri dish flooded with Gram's iodine; (Cb) with Congo red.

Table 2. Clear zone / Halo size for pectinolytic activity

	Clear zone / Halo size (mm) at pH 4.5			
Days of incubation	**3rd**	**5th**	**8th**	**10th**
Gram's iodine stain	±0.2	±0.3	±1.3	±1.42
Congo red dye	±0.8	±2.1	±1.89	±1.67

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