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Intraspecific Variation in Production of Pectinases by Three Species of Seimatosporium Causing Stem Rot and Dieback of Eucalyptus Tereticornis

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ABSTRACT: Pectin degrading enzymes are essential for the invasion and colonization of host tissues by many phytopathogenic fungi. In this study, we look at the production of the three main pectinolytic enzymes in three species of *Seimatosporium*: *S. falcatum*, *S. fusisporum*, and *S. discosioides*. Pectin containing media, particularly Czapeks medium + 0.1% pectin and Czapeks medium + 0.5% pectin, persistently elicited greater enzyme secretion which suggests an adaptive enzymatic response to aid in overcoming the growth challenges posed as a result of the carbon sources. Polygalacturonase (PG) and pectin methyl galacturonase (PMG) activity patterns exhibited a temporal control and species specific preference which indicates some level of regulation where *S. discosioides* displayed markedly higher PMG activity comparatively to other two species. From these results, we hypothesize that the different *Seimatosporium* species possess distinct strategies to adapt to the surrounding environment by differing responses to constituent materials of the substratum.

KEYWORDS: pectinases, polygalacturonase, pectin methylesterase, pathogenicity.

I. INTRODUCTION

Pectic substances are a group of closely related complex polysaccharides that are major components of the primary cell wall and middle lamella of higher plants, playing a crucial role in maintaining the integrity and cohesion of plant tissues. The main structural components include homogalacturonan and rhamnogalacturonan, which are part of the larger pectin matrix (Mohnen, 2008; Zhang et al., 2022). Protopectin, an insoluble form found in immature plant tissues, is converted into soluble pectin by the enzyme protopectinase. Rhamnogalacturonan, a type of pectin domain, contributes to the structural complexity of the pectic network.

Pectinic acid refers to partially de-esterified pectin containing more than 75% uronic acid, while pectin has a high degree of methyl esterification, and pectic acid is the fully de-esterified form. The enzymes that act on these pectic substances are collectively termed pectinases (Satapathy et al., 2020). These enzymes are categorized into hydrolases, lyases, and esterases, based on their mode of action on glycosidic or ester bonds. Notably, polygalacturonase (PG) and

pectin methylesterase (PME) play key roles in the depolymerization and modification of pectin through hydrolysis, trans-elimination, and de-esterification reactions (Haile et al., 2022).

Pectic enzymes play a major role in host-pathogen interactions (Ramaraju et al., 2017), symbiosis, plant litter decomposition, and nutrient recycling in the biosphere. These enzymes have many applications in the food industry, including increasing color extraction in wine and yield of carotene in carrot juice and clarification of fruit juices (Raveendran et al., 2018). Pectic enzymes are indispensable for colonization of host tissue by facultative pathogens as well as biotrophs like rusts. Fungal pathogens are known to vary widely in their capacity to produce these enzymes not only among different genera (Collmer & Keen, 2003) but also among species of the same genus (Oeser et al., 2017). Now, an attempt has been made to correlate the pathogenicity of fungus to its pectinase-producing ability. Therefore, in the present investigation, production of pectic enzyme by three species of *Seimatosporium* was investigated and discussed.

II. MATERIAL AND METHODS

A). Isolation and identification of pathogens

The diseased specimens collected from standing trees were used for isolations of the pathogen. The infected region was surface sterilized with the help of ethyl alcohol and placed on Asthana and Hawkers medium-A slants and incubated at room temperature. The mycelial growth emerging from the tissue bits was transferred to fresh agar slants and allowed to grow monosporic cultures obtained were identified with the help of standard manuals (Ellis, 1985). The identifications were further confirmed by Dr. B.C. Sutton, IMI, Kew, England. Monosporic cultures were raised from the identified species of *Seimatosporium* (*S. falcatum* IMI No. 360858; *S. fusisporum* IMI No. 360861; *S. discosioides* IMI No. 357390) and were selected for these studies.

B). Growth of fungal pathogens on different synthetic media

Three species of *Seimatosporium* (*S. falcatum*, *S. fusisporum*, and *S. discosioides*) were selected for the study of the production of pectic enzymes. These fungi were grown in the following synthetic media for assessing the potential of producing pectic enzymes.

1. Asthana and Hawkers medium (glucose 5.0g, potassium nitrate 3.5g, potassium dihydrogen phosphate 1.75g, magnesium sulphate 0.75g, agar 5 g, and 1 liter of distilled water)
2. Czapek's medium (sodium nitrate 2g, potassium phosphate 1g, potassium chloride 0.5g, agar 15 g, and 1 liter of distilled water)
3. Glucose-Peptone medium (peptone 10 g, dipotassium hydrogen phosphate 5g, glucose 4g, and 1 liter of distilled water).
4. Czapek's medium + 0.1% pectin
5. Czapek's medium + 0.5% pectin.

25 ml of media was dispensed in 250 ml Erlenmeyer conical flasks and sterilized at 21 lbs pressure for 30 min. The pH was adjusted to 5.5 with the help of 6N HCl/NaOH. The flasks thus prepared were inoculated using uniform agar discs containing fungus and incubated at 25 ± 2 for 12 days. At the end of 4, 8, and 12 days of incubation, cultures were harvested on pre-weighed Whatman no. 42 filter paper. The filter paper along with fungal biomass was dried at 65°C for 48 hours in an oven. The filter papers along with mycelium were weighed to constant weight after cooling in desiccators. Simultaneously the pH of the culture filtrate was determined with the help of an Elico pH meter. The culture filtrates were employed for assaying pectic enzymes after dialysis against distilled water overnight. The experiments were run in triplicate and repeated twice. The data obtained was statistically analyzed.

C). Assay of pectic enzymes

Pectin methyl esterase was determined by the method of Anthon and Barrett (2012). 10 ml of enzyme and 5 ml of distilled water were added to 25 ml of pectin solution in 250 ml Erlenmeyer conical flasks. One or two drops of methyl red were added to it, and the pH was adjusted to 6.2 with 0.1N NaOH and incubated for 3 hours. During the digestion period, enough 0.1N NaOH was added to the reaction mixture to prevent it from turning pink every 10 min. The amount of methoxyl groups (in mg) that got split off during reaction time by 1 ml of enzyme was calculated with the formula.

$$\text{Number of methoxyl groups split off by 1ml of enzyme} = \frac{\text{total ml of 0.1 NaOH consumed}}{\text{Total volume of Enzyme used}} \times 3.1$$

i. Exo-PG and Exo-PMG

Exo-Polygalacturonase (exo-PG) and exo-Polymethyl galacturonase (exo-PMG) activity were assayed by the DNS method as described by Hancock and Millar (1965). The substrate for exo-PG was 0.5/sodium polypectate (NaPP), while for exo-PMG it was 1/pectin. The reaction mixture consisted of 3.5 ml of substratum, 1 ml of citrate buffer (5.5), and 0.5 ml of enzyme and was incubated at 30+/-10°C for 6 hours. At the end of the incubation period, 1 ml of an aliquot of the reaction mixture was withdrawn into a test tube, 3 ml of DNS reagent was added, and it was heated in boiling water for 15 min. Two ml of Rochelle salt (potassium sodium tartrate) was added while the contents were hot and then cooled under running tap water. 1 ml of distilled water in place of enzyme extract served as a blank. Intensity of color developed was read at 575 nm against the blank, and the enzyme activity was expressed as the increase in mg of reducing groups (as glucose/ml) liberated in 6 hours.

ii. Endo-PG and Endo-PMG

The activity of endo-polygalacturonase (endo-PG) and endo-polymethyl galacturonase (endo-PMG) was assayed viscometrically using an Oswald viscometer. The substrate for endo-PG was 0.6% sodium polypectate (NaPP), while for endo-PMG it was 15% pectin. The reaction mixture consists of 10 ml substratum, 3.3 ml enzyme, and 0.67 ml citrate buffer (pH 5.5). 4 ml of buffer in place of substrate, 2 ml of enzyme, and 1 ml of Tris-HCl buffer (pH 8.1) were incubated at 30+1 for 4 hours. The reaction mixture was incubated at 30±1°C. The change in the viscosity was measured at 5-minute intervals in an Ostwald-Fenske viscometer for 30 minutes. The percentage loss of viscosity was calculated by the formula.

$$\text{An min} = \frac{t_1 - t_a}{t_1 - t_0} \times 100$$

Where

An min = percentage of loss in viscosity

t₁ = flow time of reaction mixture + inactive enzyme

t_a = flow time of reaction mixture + active enzyme

t₀ = flow time of water + active enzyme at 0 time

The activity of endo-PG and endo-PMG was expressed in relative viscometric units (RVU)

$$\text{RVU} = 1000 / t_v 50$$

t_v50 = time required in minutes to reduced the viscosity of CMC to 50% of the initial viscosity.

III. RESULTS AND DISCUSSION

A) Pectin methylesterase (PME) activity

PME appears to be widespread among plants as well as microorganisms. However, fungal PME was reported to be different from that of higher plants. Production of PME by fungi appears to be a common role in tissue maceration responsible for converting pectin to pectic acid which is the preferred substratum for chain splitting enzymes.

The increased PME activity in infected tissues was attributed to its involvement in establishment of pathogen in the host tissue (Fan et al., 2017). The production of PME in vitro has been reported by Wormit & Usadel in 2018.

Table 1 reveals that all the fungi under study secreted PME in significant amounts in all the media tried. However, the degree of enzyme production varied with the fungus and the medium. *S. falcatum* opted for medium E followed by D and C. Medium A was the least preferred substratum. PME secretion increased with the progress of the incubation period during its growth in medium A and B. While the rest of the media showed the PME secretion decreased after the fourth day of the incubation period. Mycelial growth of this fungus was maximum during its growth in medium D and E, followed by B. While in the rest of the media, growth decreased after eight days of incubation.

Table.1. Production of Pectin lyase (Exo-PL* and Endo-PL**) and Pectic acid Lyase (Exo-PAL* and Endo-PAL**) by three species of *Seimatosporium* on different synthetic medium.

| Name of the Medium | Days of Incubation | <i>S. falcatum</i> | | | | <i>S. fusisporium</i> | | | | <i>S. discosioides</i> | | | |
|---------------------------------|--------------------|--------------------|---------|---------|----------|-----------------------|---------|---------|----------|------------------------|---------|---------|----------|
| | | Exo-PL | Endo-PL | exo-PAL | Endo-PAL | Exo-PL | Endo-PL | Exo-PAL | Endo-PAL | Exo-PL | Endo-PL | Exo-PAL | Endo-PAL |
| A. Asthana and Hawkers Medium | 4 | 0.2 | 5 | 5.8 | 8.4 | 6.8 | 6 | 0.9 | 6 | 1.8 | 8.4 | 3.3 | 13.5 |
| | 8 | 2.6 | 12 | 3.9 | 16.5 | 4.1 | 16.5 | 3.7 | 13.2 | 3.4 | 15.6 | 6.1 | 15.6 |
| | 12 | 2.4 | 18 | 1.4 | 19.2 | 1.8 | 20.4 | 6.1 | 22.8 | 2.3 | 13 | 3.9 | 20 |
| B. Czapeks medium | 4 | 1.0 | 4.8 | 1.6 | 16 | 6.9 | 6 | 3.7 | 8 | 7 | 6 | 6.1 | 14.4 |
| | 8 | 3.7 | 16.5 | 2.6 | 18 | 8.9 | 15.6 | 9.1 | 14.4 | 4.6 | 19 | 5.9 | 15 |
| | 12 | 3.7 | 13 | 11.6 | 20.4 | 5.4 | 30 | 10.1 | 26 | 4 | 22.5 | 2.4 | 24 |
| C. Glucose-peptone medium | 4 | 4.4 | 9 | 6.2 | 10.8 | 9.8 | 9 | 4.1 | 20 | 6.2 | 15 | 5.2 | 19.5 |
| | 8 | 1.8 | 15 | 10.5 | 28.5 | 5 | 20.4 | 8.7 | 25.2 | 9.8 | 22.5 | 7.3 | 16.8 |
| | 12 | 0.7 | 43.5 | 2.4 | 27.6 | 2.1 | 32.2 | 9.9 | 34 | 11.3 | 22.5 | 8.9 | 29 |
| D. Czapeks medium + 0.1% pectin | 4 | 7.7 | 18 | 3.1 | 16.5 | 8.9 | 18 | 5.6 | 19.5 | 8.7 | 19.5 | 5.7 | 18 |
| | 8 | 9.6 | 20.4 | 7.8 | 31.5 | 13.1 | 22.8 | 9.6 | 34.5 | 10.1 | 27 | 8.9 | 32.4 |
| | 12 | 9.9 | 42 | 3.7 | 40.5 | 9.2 | 32.4 | 11.7 | 46 | 14.9 | 22.5 | 4.6 | 21.7 |
| E. Czapeks medium + 0.5% pectin | 4 | 6.1 | 22.5 | 4.1 | 26 | 7.1 | 22.5 | 5.6 | 8.4 | 10.9 | 22.5 | 12.1 | 34 |
| | 8 | 11.8 | 36 | 8.9 | 44 | 12.7 | 36 | 11.9 | 36 | 12.3 | 38 | 6.1 | 33.6 |
| | 12 | 12.1 | 56 | 10.9 | 42 | 18.9 | 54 | 9.1 | 28.2 | 6.3 | 43.5 | 2.7 | 43.5 |

* Expressed in units (0.01 OD change was taken as 1 unit of enzyme activity)

** Expressed in relative viscometric units (RVU)

S. fusisporium was a good producer of PME. It produced maximum PME during its growth in medium E, followed by D. Eight days of incubation period was optimum for production of PME in all the media tried except in medium A. *S. fusisporium* attained maximum mycelial growth in medium E, and continuous increase in vegetative growth could be recorded in media D and E, while the rest of the media decreased after 8 days of incubation. The pH changes varied with the medium. PH changes were significant in medium C, while in medium E the final pH remained near 5.

S. discosioides also secreted a significant amount of PME. As expected, PME activity was more in medium D and E. The enzyme activity attained a peak by the fourth day of incubation itself and decreased during the subsequent incubation period. On the other hand, in media A, B, and C, enzyme activity was higher on the 8th day. Mediums D and E supported maximum vegetative growth, while medium A was a poor substratum. An increasing trend of vegetative growth of *S. discosioides* could be recorded during its growth in media B, D, and E. Eight days of incubation was optimum for the production of enzyme in media A, B, or C, while in media D and E, the enzyme activity was more during the fourth day of incubation.

Critical perusal of table 1 reveals that mediums E and D induced maximum enzyme production, which contained pectin, suggesting the adaptive nature of PME produced by all three species of *Seimatosporium*.

B) Polygalacturonase (PG) enzyme activity

Polygalacturonases, which act on pectic acid, are reported to play a crucial role in the pathogenesis of several pathogenic fungi (Wei et al., 2022). Host repression of the synthesis of these enzymes by fungi had been proposed as a possible mechanism of disease resistance in plants (Andersen et al., 2018). In general, the end products of endo-PG action on pectic acid are mono-, di-, and tri-galacturonic acids. Phytopathogenic microorganisms utilize endo-

polygalacturonases as a component of their offensive arsenal to penetrate and colonize plant tissues during infection (Federici et al., 2001). Endo-PGs are the first cell wall-degrading enzymes produced by pathogens when cultured on isolated plant cell walls or during infection (Vuong et al., 2023). Several features of PG indicate its multiple roles in pathogenesis by cleaving the internal glucosidic bonds of the plant cell wall homogalacturonans, releasing oligogalacturonides, which function as elicitors of plant defense responses (Delmer et al., 2024). The production of PG in vitro by different fungi has been reported by many workers, including Liu et al. (2017) and Siddiqui et al. (2012).

Three species of *Seimatosporium* under study secreted PG, which, however, varied with the medium and the species (table 1). Medium E supported the maximum amount of Exo-PG, while medium C supported the least activity. Exo-PG activity was higher during the first four days and decreased during the subsequent incubation period. Mediums B and E induced a good amount of endo-PG in *S. falcatum*, while mediums A, C, and D were poor substrates. The endo-PG production increased with the progress of the incubation period in all the media tried except in medium D, where it recorded maximum endo-PG activity on the eighth day of incubation. In *S. fusisporum*, the production of exo-PG was comparatively poor; its production was significant only in medium D and E, which may be due to the presence of pectin. Eight days of incubation was optimum for the production of exo-PG by *S. fusisporum* during its growth in media A, B, and E, while in media C and D, a continuous increase in secretion of exo-PG was recorded. Endo-PG production by *S. fusisporum* increased with the progress of the incubation period. *S. discosioides* was also a poor producer of exo-PG, as it secreted a considerable amount only in media D and E. Similarly, endo-PG production was also limited. Its production was maximum in medium D and E, suggesting its adoptive nature. However, a continuous increase in endo-PG activity was recorded in all the media tried.

C) polymethyl galacturonase (PMG) activity

All three species of *Seimatosporium* produced PMG in one or another medium. Exo-PG production was more in media containing pectin, suggesting its adoptive nature. Almost the same trend was observed with endo-PMG also. Exo-PMG production by *S. falcatum* was more during the first four days and declined subsequently. It secreted the maximum amount of exo-PMG during its growth in medium E, while medium C failed to support a significant amount of exo-PMG during its growth in media B, D, and E. Similarly, endo-PMG production by *S. fusisporum* was more during its growth in medium D and E. Endo-PMG production increased with the progress of the incubation period. *S. discosioides* was comparatively more efficient as it produced more exo-PMG in medium D and E. The rest of the substrates were poor for the induction of exo-PMG. Endo-PMG production was maximum on the eighth day of incubation in medium A and B, while in medium D and E, enzyme production reached its peak by the fourth day itself. These findings are consistent with earlier reports that pectin-rich media serve as strong inducers of PG enzymes in phytopathogenic fungi (Roman-Benn et al., 2023). The differential expression patterns observed among *Seimatosporium* species highlight the potential adaptation of each strain to specific host-derived substrates.

IV. CONCLUSION

The current research underscores the ability of *Seimatosporium* species to synthesize key pectinolytic enzymes such as PME, PG, and PMG in vitro, as well as the significant influence the composition of the growth medium had on enzyme production. The adaptation of these fungi to pectin-rich environments offers evidence of their ability to utilize plant-derived compounds. The timing of enzyme secretion (of which there was an initial surge in exo-enzymes followed by an endo-enzyme surge) showed some coordination, suggesting a regulated system accompanying fungal growth and substrate inflow. In terms of efficiency, *S. discosioides* outperformed the other species in harvested PMG, with *S. falcatum* and *S. fusisporum* demonstrating the highest PME and PG activities, respectively. These findings indicate more than just interspecies divergence in enzyme profiles—they point towards enzymatic contributions to tissue breakdown and host pathogenicity. Collectively, the results offer further appreciation of the role pectinase plays in the interaction of fungi and plants while calling attention to research on the enzymatic processes behind virulence and host selectivity.

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