Screening and assay of extracellular enzymes in Phomopsis azadirachtae causing die-back disease of neem

Article · June 2013

DOI: 10.13140/2.1.4814.9128

5 authors, including:

Vedashree Sobagaiah
Bangalore University
6 PUBLICATIONS **23** CITATIONS
SEE PROFILE

Mysore Krishnaiah Sateesh
Bangalore University
28 PUBLICATIONS **111** CITATIONS
SEE PROFILE

Dr. Lakshmeesha T. R.
University of Mysore
7 PUBLICATIONS **38** CITATIONS
SEE PROFILE

Mohammad Shafi Sofi Shafi Shafi
Bangalore University
9 PUBLICATIONS **16** CITATIONS
SEE PROFILE

Journal of Agricultural Technology 2013 Vol. 9(4): 915-927
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Screening and assay of extracellular enzymes in *Phomopsis azadirachtae* causing die-back disease of neem

S. Vedashree[1], M.K. Sateesh[1*], T.R. Lakshmeesha[1], Shafi Sofi Mohammed[1] and A.K. Vedamurthy[2]

[1]Molecular diagnostic laboratory, Department of Microbiology and Biotechnology, Bangalore University, Bangalore, Karnataka, India, [2]Department of Biotechnology, Oxford College of Science, HSR Layout, Bangalore, Karnataka, India

S. Vedashree, M.K. Sateesh, T.R. Lakshmeesha, Shafi Sofi Mohammed and A.K. Vedamurthy (2013). Screening and assay of extracellular enzymes in *Phomopsis azadirachtae* causing die-back disease of neem. Journal of Agricultural Technology 9(4):915-927.

Phomopsis azadirachtae is the causal agent of destructive die-back disease of neem. The molecular mechanism of pathogenicity is not clear. Pectinases, cellulases, hemicellulases and ligninases have been extensively studied because of their plant cell wall degrading nature. Hence, this study describes the screening and assay of extracellular enzymes produced by the pathogen. The pathogenicity enzymes screened were: laccase, polygalacturonase, xylanase, amylase, cellulase, protease and lipase. A total of fifteen isolates of *Phomopsis azadirachtae* were from different agroclimatic regions of Karnataka, Tamil Nadu and Andhra Pradesh from diseased samples. The isolates were subjected to screening of the above mentioned enzymes on solid media supplemented with respective substrates. All the isolates were found to produce polygalacturonase, laccase, protease and xylanase. Polygalacturonase and xylanase were further assayed in order to quantify the enzyme produced in all the isolates. In addition to pathogenicity enzymes, isolates were also found to produce some industrially important enzymes such as L-asparaginase and urease.

Key words: *Phomopsis azadirachtae*, die-back, enzymes, assay, neem

Introduction

Neem or margosa (*Azadirachta indica* A. Juss.) is an attractive broad-leaved, evergreen legendary medicinal tree which belongs to the family Meliaceae. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex (Subapriya and Nagini, 2005; Ogbuewu *et al*., 2011). From the practical side, these compounds also exhibit biological activities such as antiallergenic, antidermatic, antifeedent, antifungal, anti-inflammatory, antipyorrhoeic, antiscabic, diuretic, insecticidal, larvicidal,

*Corresponding author: M.K. Sateesh; e-mail: sateeshmkgreen@gmail.com

nematicidal, pesticidal, spermicidal and cytotoxic properties (NRC, 1992; Rembold, 1996; Abbasi *et al*., 2003). Even with all its bioactive active ingredients and multifarious biological activities, neem is not free from disease caused by microorganisms. This ecofriendly, native tree of India is affected by mitosporic species of *Phomopsis* viz., *Phomopsis azadirachtae* (Sateesh *et al*. 1997) causing die-back disease (Sateesh *et al*., 1997; ShankaraBhat *et al*., 1998; King *et al*., 2011; Sateesh, 2009;). The fungus is known to produce conidia which are suspected to be transmitted by air, raindrops, insects and seeds (Sateesh and ShankaraBhat, 1999). It has caused almost always 100% loss of fruit production. The annual loss due to this disease could be several million of rupees (Sateesh 1998).

The enzymes are essential proteins secreted by the pathogen in host infection. Even though much research has been carried out on the pathogen and disease, the molecular mechanism through which the organism enters the host is not yet clear. During literature survey, it was found that there is a major role of some cellwall degrading enzymes such as polygalacturonase, cellulase, xylanase and laccase in degradation of the host cell wall and colonizing the host tissue (Cairney *et al*., 1994; Goodenough *et al*., 1991). The enzyme tests may help to understand the role of enzymes in establishing infection; hence the isolates were screened and assayed for different enzymes.

Plant biomass is abundantly made up of lignin, cellulose and hemicelluloses (Junior *et al*., 2010; Houben *et al*., 2011). The enzymes ligninases, hemicellulases, cellulases, pectinases, xylanases, amylases and proteases are required for degrading plant biomass and tissue establishment (Saparrat *et al*., 2002; Sohail *et al*., 2009). Plant cellwall is heterogeneous and has complex structure consisting of polysaccharides, proteins and aromatic polymers. It is complex because it is made up of three layers namely: middle lamella, primary and secondary wall. The rimary wall is rich in pectin (Marquez *et al*., 2011) and secondary wall contains a wide range of additional compounds i.e., cellulose (35-50%), xylan (20-35%) and lignin 10 -25% (King *et al*., 2011). Laccases, manganese peroxidases and lignin peroxidases are involved in the degradation of lignin, a major constituent of plants (Papinutti *et al*., 2007). Polygalacturonase is involved in the degradation of pectin, an important constituent of the middle lamella (Marquez *et al*., 2011). Cellulases are the enzymes that degrade cellulose, the most abundant polymer of plant cell wall and xylanases are involved in degrading xylan (King *et al*., 2011). Microbial enzymes are being exploited commercially and have been successfully used by different industries in various applications. These enzymes are cheaper and ecofriendly and hence, it has become a multi-billion dollar business (Bhat, 2000; Sohail *et al*., 2009).

Keeping in view the importance of fungal enzymes, the current study was undertaken to screen different isolates of *Phomopsis azadirachtae* for enzymes such as amylase, cellulase, protease, lipase, laccase, polygalacturonase, L-asparaginase, glutaminase, xylanase and urease. The enzymes were screened on solid media supplemented with respective substrates to know itheir role in establishing pathogenicity and also to explore their hydrolytic potential for their possible future applications. Two important pathogenicity enzymes *viz.*, polygalacturonase, xylanase and an antitumor enzyme L-asparaginase were assayed using pectin, xylan and asparagine as substrates respectively. Hence, this paper describes the first report on screening and assay of extracellular enzymes produced by the mitosporic fungi *Phomopsis azadirachtae.*

Materials and methods

Collection of samples and isolation of pathogen

Twenty districts of Karnataka and few districts of Tamil Nadu and Andhra Pradesh were visited in order to collect the samples (seeds and twigs) of severely infected neem trees. Diseased seeds and twigs were handpicked from the trees and were sealed in paper bags. Ten trees were sampled in each district and 250 to 500 grams of seeds were collected from each tree. The samples were brought to the laboratory, stored under proper conditions and were used for isolation of the pathogen.

Fungi were isolated from neem seeds and twigs collected from infected trees from different regions. Surface sterilization of neem seeds and twigs was done by treating the seeds with 0.1% mercuric chloride solution and then rinsed five times with distilled water. Surface sterilized samples were plated onto potato dextrose agar medium amended with 100 µg/ml chloromphenicol. All strains were maintained on potato dextrose agar (PDA) slants at 4^0C. The fungi were identified on the basis of routine cultural, morphological and microscopic observation (Sateesh *et al*., 1997; Sateesh and Shankara Bhat, 1999).

Seven days old axenic cultures of different isolates of *Phomopsis azadirachtae* on PDA plates were used for preparing inoculum by retrieving 5mm diameter mycelial disc with the help of cork borer. The inoculums was placed in the middle of the fresh test substrate plates (3 replicates/ isolate were maintained) and incubated at 28^0 C for 7 days. The control plates were also maintained for each of the enzymes screened. Enzyme activities were calculated as enzymatic index (Mustafa and Kaur, 2010).

In-vitro plate screening study for different enzymes

Different strains of *Phomopsis azadirachtae* were screened for amylase, cellulase, protease, lipase, laccase, polygalacturonase, L-asparaginase, glutaminase, xylanase and urease as follow:

Amylase: Glucose yeast extract peptone (GYP) agar medium containing glucose (1g), yeast extract (0.1g), peptone (0.5g), agar (16g) and distilled water supplemented with 2% starch was prepared. Five mm diameter fungal disc was inoculated to the medium and incubated for 7 days at 25^0 C. After which, the plates were flooded with iodine solution. Clear zone of hydrolysis around the colonies indicated positive result.

Cellulase: Cellulase was screened by inoculating 5mm diameter fungal disc to the GYP agar medium supplemented with 0.5% carboxy methyl cellulose. After incubation, the plates were flooded with 0.2% aqueous Congo red and destained with 1M NaCl for 15 min. Clear zone formed surrounding the colony indicated cellulase activity.

***Laccase*:** Laccase activity was assessed by growing the fungi on GYP agar medium amended with 0.05% 1-naphthol at pH 6. Change in color of the medium from clear to blue indicated laccase activity.

***L-Asparaginase and Glutaminae*:** Modified CzapekDox medium containing (gl^{-1} of distilled water): glucose (2.0), L-asparagine (10.0), K_2HPO_4 (1.52), KCl (0.52), $MgSO_4.7H_2O$ (0.52) and agar (2.0) at pH 6.2 was used for screening of L-asparaginase enzyme. Change in color of the medium from yellow to pink indicated positive result.The same medium was used for screening of glutaminase which was supplemented with 10g of L-glutamine per liter of medium in place of asparagine.

Lipase: Glucose yeast extract peptone agar medium containing 1% tween 80 (separately autoclaved and added to media) was used and observation of clear zone formed surrounding the colony after inoculation and incubation indicates lipase activity. This is due to hydrolysis of tween 80 present in the media.

Polygalacturonase: Polygalacturonase activity was detected by growing fungi in agar medium containing yeast extract (1g), pectin (5g) and agar (15g) at pH 6 in 1L distilled water. After 7 days of colony growth, the plates were flooded with 1% CTAB. Clear zone formed around the fungal colony indicates pectinolytic acitivity.

Protease: Gelatin medium was prepared by adding 5% solution A (470 mM $NaNO_3$, 135mM KCl and 0.8mM $MgSO_4.7H_2O$) to solution B (115mM K_2HPO_4, 0.035mM $ZnSO_4.7H_2O$), 1% sucrose and 12% gelatin. Exactly 10 ml of the medium was dispensed into each test tube and autoclaved at 121^0 C for 10min. The tubes were inoculated with 5mm mycelial disc and incubated at 25^0

C for 15 days (Kitancharoen *et al.,* 1998). One uninoculated tube served as control. The tubes were chilled for 1hr at 4^0C. Liquefaction of the medium even after refrigeration indicates positive result.

Xylanase: Xylanase production was tested according to Sohail *et al*,. (2009). Mineral salt medium containing (w/v) 0.2% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, 0.03% urea, 0.03% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.1% peptone, 0.0005% $FeSO_4.7H_2O$, 0.0016% $MnSO_4.H_2O$, 0.0029% $CoCl_2.6H_2O$, 0.0014% $ZnSO_4.7H_2O$ and 1.5% agar-agar supplemented with Birchwood xylan (0.5% w/v), at pH 6 was used. A halo around the colonies after 7 days of incubation at 30^0C indicates xylanase activity.

Urease: Mineral salt medium containing 2.7mM potassium chloride, 8.4mM ammonium dihydrogen phosphate, 0.8mM magnesium sulfate, 0.035 mM zinc sulfate, 0.002 mM copper sulfate, 1% glucose, 1.5% agar, 1% urea (separately sterilized by using 0.45µm filter and added to autoclaved media), 0.02% phenol red, pH 6 was used and inoculated with 5mm mycelial disc. Change in color of the medium to pink after incubation indicates urease activity.

Assay for Enzyme activity

The polygalacturonase and xylanase activity were determined by measuring the reducing sugars produced after hydrolysis in the substrate by DNS method (Miller, 1959) against standard curves of galacturonic acid and xylose. Measurements were made in 0.1 M sodium acetate buffer during 30 min of incubation using the following substrates: pectin (0.1%) for polygalacturonase and Birchwood xylan (0.2%) for xylanase at 50^0 C and at pH 4.2 and 4.8 respectively.

The polygalacturonase activity was assayed by measuring the content of the galacturonic acid formed during 30 min incubation of 0.1% pectin in the presence of the enzyme at 50°C and pH 4.2. Xylanase activity was also detected by DNS method by estimating the content of xylose released during 30 min of incubation of the enzyme in the presence of 0.2% xylan at 50°C and pH 4.8.

L-asparaginase was assayed by estimating the ammonia produced during its activity using Nesseler's reagent (Wriston and Yellin, 1973; Imada *et al.,* 1973). Reaction mixture contained enzyme, 0.01 M L-asparagine and 0.05 M Tris-HCl buffer (pH 8.6) at 37^0 C for 30 min. Reaction was stopped by adding 100 µl of 10% TCA and the liberated ammonia was quantitatively determined by using ammonium sulfate as standard.

Enzyme activity has been expressed in International Units (U) *viz.*, the amount of the enzyme that liberated 1 µmol of substrate equivalent per minute under the assay conditions. Values given are mean ± standard error (SE),

derived from the triplicate samples. Total protein was estimated using bovine serum albumin as standard (Lowry *et al.*, 1951).

Results and discussions

Pathogen isolation

Fifteen isolates of *Phomopsis azadirachtae* were isolated from neem seeds and twigs collected from various districts of Karnataka, Tamilnadu and Andhrapradesh (Table 1, Figs. 1A and 1B). *Phomopsis* species are large Coelomycetes members including over 1000 species named primarily based on the host specificity (Uecker, 1988; Rehner and Uecker, 1994; Farr *et al.*, 2002). *Phomopsis* species identification is done by observing the shape and size of conidia, conidiogenesis, mycelial nature and mycelial pigmentation. It produces two types of conidia alpha and beta conidia. Among the different isolates used, significant variation was observed in colony color, colony diameter, texture, margin, elevation and other characteristics on potato dextrose agar medium (Fig 1C) which are tabulated in Table 2. Out of the ten enzymes screened, the pathogen was found to produce six enzymes i.e., polygalacturonase, laccase, xylanase, gelatinase, urease and L-asparaginase. The level of enzyme production was expressed in enzymatic index (Mustafa and Kaur, 2010).

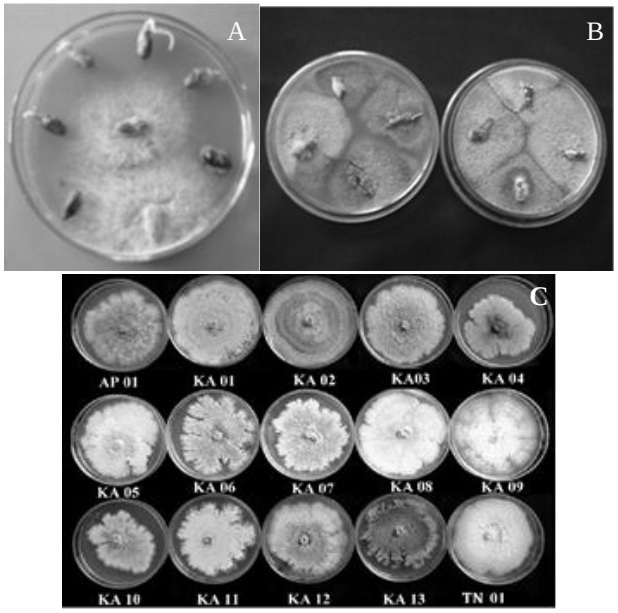
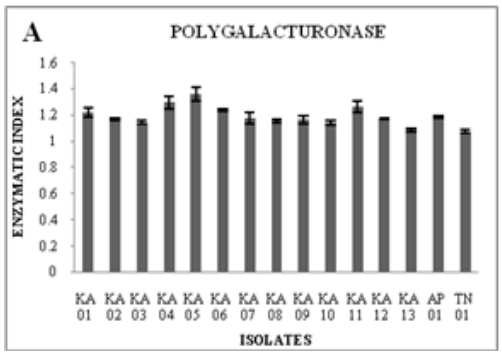
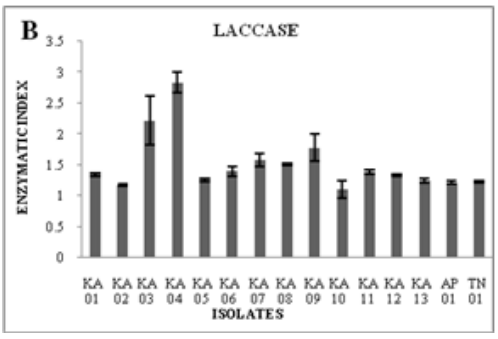


Fig. 1. (A) Isolation of P. Azadirachtae from neem seed, **(B)** Isolation of P. Azadirachtae from neem twig, **(C)** Fifteen isolates of *Phomopsis azadirachtae* isolated from different agroclimatic regions on PDA.

In-vitro plate screening study for different enzymes

By measuring the colony diameter and zone of clearance obtained after incubation, it was found that highest polygalacturonase activity was exhibited by isolate KA 05 with enzymatic index of 1.36, followed by KA 04 (1.29) and KA 01 (1.26) (Fig. 2A). The formation of clear zone indicated pectinase activity, which was due to degradation of pectin to its monosaccharides. When CTAB was added, it precipitated intact leaving halo near the degraded pectin. The highest laccase activity was exhibited by isolate KA 04 (2.83) followed by isolate KA 03 (2.22) and KA 09 (1.77) (Fig. 2B). Laccase activity was indicated by conversion of media from clear to blue due to oxidation of α-naphthol in the media. The highest xylanase production was observed in isolates KA 08 01, KA 09 and KA 04 with enzymatic index of 1.27, 1.17 and 1.16 respectively. Xylanase activity was denoted by the formation of clear halo around the colony which was due to utilization of xylan by the organism. Isolates KA 12 (4.311) KA 07 (4.071), AP 01(3.866) were highest producers of L-asparaginase enzyme and isolates KA 07 (7.307), KA 06 (5.245) AP 01(4.843) were highest producers of urease enzyme. L-asparaginase and urease activity was indicated by the formation of pink zone which was due to increase in pH by the release of ammonia by the activity of L-asparaginase and urease on asparagine and urea respectively. All isolates of *Phomopsis azadirachtae* were positive for gelatinase enzyme by complete liquefaction of the gelatin even after chilling for 2 hours at 4^0 C. None of the isolates were found too produce amylase, cellulase and glutaminase. Fig. 3 shows the activity of different enzymes.





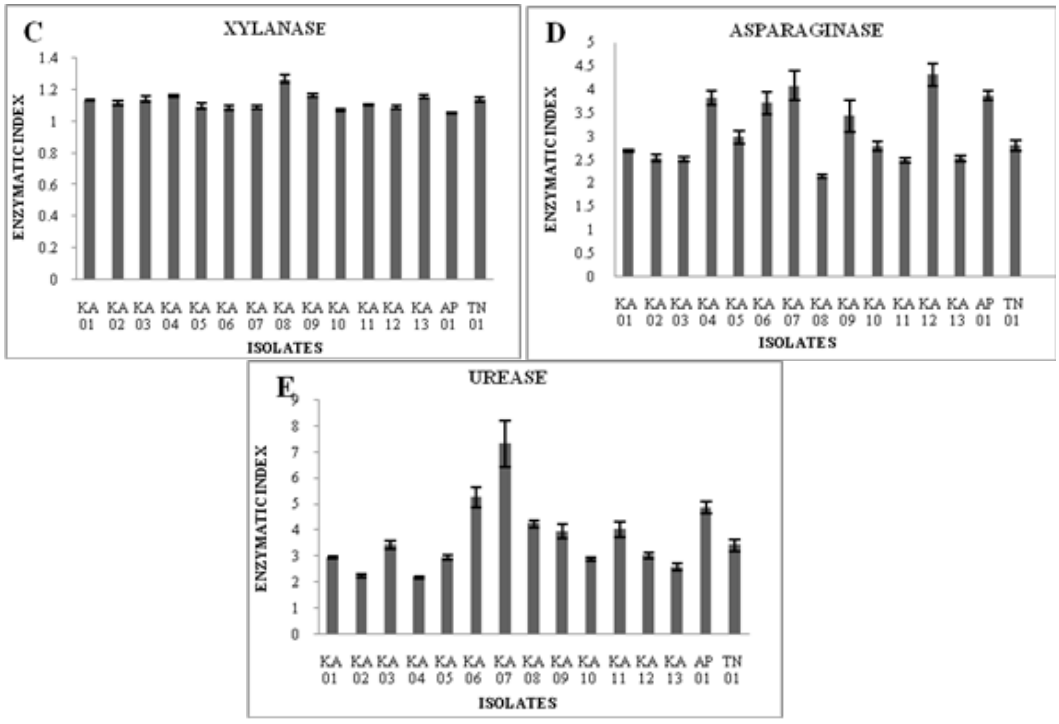


Fig. 2. Graph showing different enzyme activity of *Phomopsis azadirachtae*. A: Polygalacturonase; B: Laccase; C: Xylanase; D: L-asparaginase and E: Urease (expressed as enzymatic index± Standard error)

Assay of enzyme activity

Cell-free culture filtrate was used as crude enzyme to determine the activities of polygalacturonase, xylanase and L-asparaginase. Highest enzyme activity was observed in KA 03 for polygalacturonase (0.651 IU), AP 01 for xylanase (2.342 IU) and KA 08 (1.77 IU) for L-asparaginase. All isolates were assayed for three different enzymes and the enzyme activity obtained is presented in Table 4. Fig. 4 illustrates the enzyme production from all the isolates of *Phomopsis azadirachtae.*

For successful pathogenesis, phytopathogens must degrade the hard cell wall of their host in order to establish pathogenicity either by specific or non-specific events between the pathogen and the host cell wall (Knogee, 1998; Pannecoucque, *et al.*, 2009; King *et al.*, 2011). In order to penetrate the host, phytopathogen must possess either any of the following mechanisms: it should be able to produce an enzyme(s) that facilitates entry through any part of the plant material such as stem, root and leaf; or role of any vector in order to enter the plant tissue such as insect or any other biological factors; penetration of the pathogen will be preceded by an exchange of signals between both organisms

(Schaffer, 1993; Knogee 1998; Dai*et al.*, 2010). Most phytopathogens have the genetic capacity to produce enzymes for the degradation of the major structural polysaccharides found in the cell wall namely: cellulose, xylan, lignin and pectin (Anderson, 1978; King *et al.*, 2011). Plant pathogenic fungi actively kill and degrade plant tissue and utilize liberated carbohydrates and proteins for growth and reproduction (King *et al.*, 2011).The mechanism involved in pathogenicity is by mainly secreting enzymes (Anderson, 1978; Knogee, 1998; Pannecoucque and Hofte, 2009; King *et al.* 2011) and hence the pathogen was screened for different enzymes.

All of the isolates that were screened in the present study, produced an appreciable number of protein and polysaccharide hydrolyzing enzymes i.e., polygalacturonase, xylanase, protease and laccase, which could be useful in the degradation of complex cell wall components of plant. This also indicates genetic and biochemical variability within the isolates. In the *in vivo* condition, the sequence of enzyme secretion corresponds to the sequence of polymers present in the cell wall (Alves, 2002; Mustafa and Kaur, 2010). In qualitative analysis, enzyme production is typically indicated by formation of clear zone around the colony for example polygalacturonase, protease, xylanase etc or formation of colored product as in case of L-asparaginase, laccase, urease, glutaminase etc. Qualitative assays are significant tools used in screening fungi for different enzyme production, since such tests give a positive or negative indication of enzyme production. They are also very useful in screening large numbers of fungal isolates for several classes of enzymes in less period of time compared to quantitative assay where definitive quantitative data are not required and hence after indication of positive or negative, quantitative assay can be done (Hankin*et al.*, 1975; Pointing, 1999; Alves, 2002).

The most significant inference from the current work carried out is the surprising degree of variability in enzymatic index by the isolates of *P. azadirachtae*. This signifies the isolate variability in the level of enzyme production which correlates with the level of pathogenicity of different strains (Asoufi *et al.*, 2007). Level of enzyme production by plant pathogens corresponds to its virulent nature (Anderson, 1978; Asoufi *et al.*, 2007; King *et al.*, 2011). Role of enzymes in entering the host by *Phomopsis* and other endophytes has also been reported in several studies (Cairney *et al.*, 1994; Dai *et al.*, 2010).Variations were also seen in the colony morphology such as colony diameter in the same day old culture. This indicates the genetic versatility within the isolates. Similar type of work i.e., screening for different enzymes has been carried out with different fungi since 1970's (Hankin *et al.*, 1975; Pelaez.*et al.*, 1995) and is still being done till now because of the high significance of pathogenicity related and industrially important enzymes

(Papinutti and Forchiassin, 2007; Mustafa and Kaur, 2010; Promputtha *et al.*, 2010; King *et al.*, 2011). Highly active plant pathogenic fungi are promising targets for identification and characterization of novel cell wall degrading enzymes for industrial applications (King *et al.* 2011).

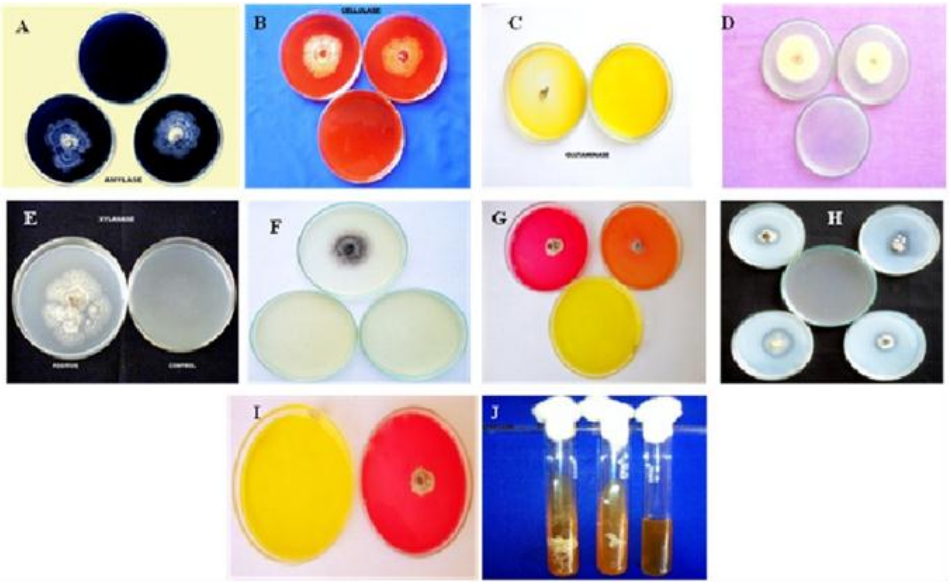
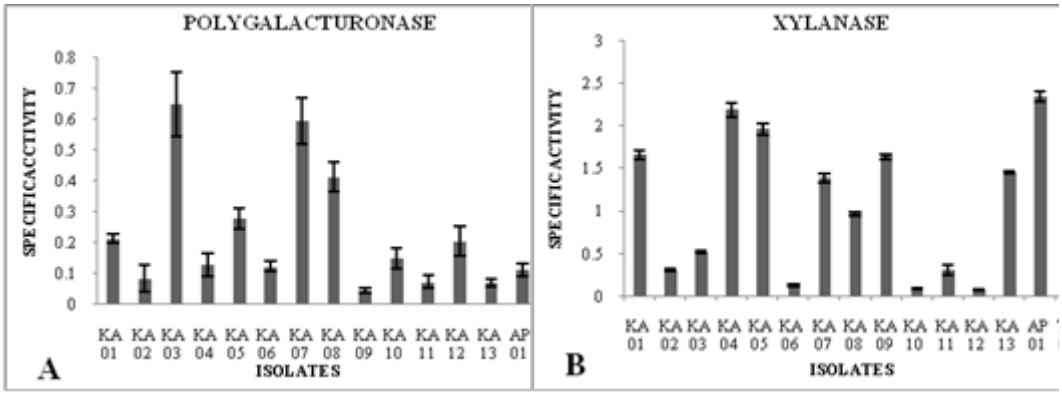


Fig. 3. Different enzyme activity A: Amylase; B: Cellulase; C: Glutaminase; D: Lipase; E: Xylanase; F: Laccase; I: G-asparaginase; H: Polygalacturonase; I: Urease and J: Protease.



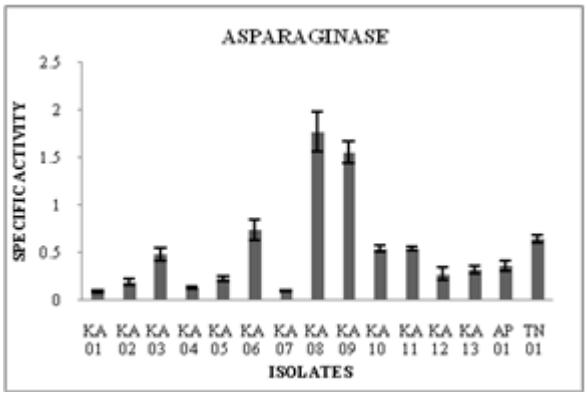


Fig. 4. Assay of different enzymes A. polygalacturonase, B. xylanase and C. L-asparaginase from isolates of *Phomopsis azadirachtae* (Enzyme activity expressed in specific activity ± standard error)

Conclusion

All the isolates were found to produce polygalacturonase, laccase, protease and xylanase. Polygalacturonase and xylanase were further assayed in order to quantify the enzyme produced in all the isolates. In addition to pathogenicity enzymes, isolates were also found to produce some industrially important enzymes such as L-asparaginase and urease. The results presented in this paper are information for the first time regarding different enzymes produced by *P. azadirachtae*. In the coming months, role of each enzyme in establishing pathogenicity will be identified *in vivo* by pathogenicity test and activity of the enzyme inside the host will be assayed. Isolates with high enzyme activity can further be optimized and purified for high enzyme production and can be exploited for various industrial uses since these enzymes are industrially very important.

Acknowledgements

The authors are very grateful to University Grants Commission, New Delhi for sanctioning this major research project, Department of Microbiology and Biotechnology for providing laboratory facility.

References

Abbasi, P.A., Cuppels, D.A. and Lazarovits, G. (2003). Effect of foliar applications of neem oil and fish emulsion on bacterial spot and yield of tomatoes and peppers. Can J Plant Pathol. 25:41–48.

Alves, M.H., Takaki, G.M., Porto, A.L.F. and Milanez, A.I. (2002). Screening of *Mucor spp.* for the production of amylase, lipase, polygalacturonase and protease.Braz. J. Microbiol. 33:325-330.

Anderson, A.J. (1978). Extracellular enzymes produced by *Colletotrichumlindemuthianum* and *Helminthosporiummaydis* during growth on isolated bean and corn cell walls. Phytopathology 68:1585-1589.

Asoufi, H. Hameed, K.M. and Mahasneh, A. (2005). The cellulase and pectinase activities associated with the virulence of indigenous *Sclerotiniasclerotiorum* isolates in Jordan valley. Plant Pathol. J. 23(4):233-238.

Bhat, M.K. (2000). Cellulases and related enzymes in biotechnology. Biotech. Adv. 18:355- 383.

Cairney, J.W.G. and Burke, R.M. (1994). Fungal enzymes degrading plant cell walls: their possible significance in the ectomycorrhizal symbiosis. Mycological Research 98(12):345-1356.

Dai, C.C., Chen, Y., Tian, L.S. and Shi, Y. (2010). Correlation between invasion by endophytic fungus *Phomopsis* sp. and enzyme production.African Journal of Agricultural Research 5(11):1324-1330.

Farr, D.F, Castlebury, L.A. and Rossman, A.Y. (2002). Morphological and molecular characterization of *Phomopsisvaccinii* and additional isolates of *Phomopsis* from blueberry and cranberry in the eastern United States Mycologia 94:494-504.

Goodenough, P.W., Clark, D.C., Durrant, A.J.,Gilbert, H.J., Hazlewood, G.P. and Waksman, G. (1991). Structural-analysis by circular-dichroism of some enzymes involved in plant-cell wall degradation. Febs Lett. 282(2):355 - 358.

Hankin, L. and Anagnostakis, S.L. (1975). The Use of Solid Media for Detection of Enzyme Production by Fungi. Mycologia 67(3):597-607.

Houben, K., Jolie, R.P, Fraeye, I, Loey. A.M.V. and Hendrickx, Me (2011). Comparative study of the cell wall composition of broccoli, carrot, and tomato: Structural characterization of the extractable pectins and hemicelluloses. Carbohydrate Research 346:1105–1111.

Imada, A., Igarasi, S., Nakahama, K. and Isono, M. (1973). Asparaginase and glutaminase activities of micro organisms. Journal of General Microbiology 76:85-99.

Junior, C.J.F.O., Cavalari, A.A., Carpita, N.C., Buckeridge, M.S. and Braga, M.R. (2010). Cell wall polysaccharides from cell suspension cultures of the Atlantic Forest tree Rudgeajasminoides (Rubiaceae). Trees 24:713–722.

King, B.C., Waxman, K.D., Nicholas, V., Nenni, N.V., Walker, L.P., Bergstrom, G.C. and Gibson, D.M. (2011). Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic Fungi. Biotechnology for Biofuels 4: pp.4.

Kitancharoen, N. and Hatai, K. (1998). Some biochemical characteristics of fungi isolated from salmonid eggs. Mycoscience 39:249-255.

Knogee (1998). Fungal pathogenicity.Plant Microbe Interactions 1:324-328.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement by Folin phenol reagent. J Biol Chem.193:265-275.

Marquez, A.L., Paramo, M.G.Z., Romero, E.L. and Camacho, H.C. (2011). Biotechnological potential of pectinolytic complexes of fungi. Biotechnol Lett. 33:859-868.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31:426-428.

Mustafa, U. and Kaur, G. (2010). Studies on extracellular enzyme production in *Beauveriabassiana*isolates.International Journal of Biotechnology and Biochemistry 6(5):701-713.

National Research Council (1992). NEEM :A Tree For Solving Global Problems Report of an Ad Hoc Panel of the Board on Science and Technology for International Development National Research Council NATIONAL ACADEMY PRESS Washington, DC.

Ogbuewu, I.P., Odoemenam, V.U., Obikaonu, H.O., Opara, M.N., Emenalom, O.O., Uchegbu, M.C., Okoli, I.C, Esonu, B.O and Iloeje, M.U. (2011). Research Journal of Medicinal Plant 53:230-245.

Pannecoucque, J. and Hofte, M. (2009). Interactions between cauliflower and *Rhizoctonia*anastomosis groups with different levels of aggressiveness. BMC Plant Biology 9: pp. 95.

Papinutti, V.L. and Forchiassin, F. (2007). Lignocellulolytic enzymes from *Fomessclerodermeus* growing in solid-state fermentation. Journal of Food Engineering 81:54–59.

Pelaez, M.J., Martinez, M.J. and Martinez, A.T. (1995). Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation.Mycol Res. 99(1):37-42.

Pointing, S.B. (1999). Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2:17-33.

Promputtha, I., Kevin, D., Hyde, Eric, H.C., Mc Kenzie., John, F., Peberdy and Lumyong, S. (2010). Can leaf degrading enzymes provide evidence that endophytic fungi becoming saprobes? Fungal Diversity 41:89–99.

Rehner, S.A. and Uecker, F.A. (1994). Nuclear ribosomal internal transcribed spacer phylogeny and host diversity in the coelomycete *Phomopsis.*Canadian Journal of Botany 72:1666-1674.

Rembold, H. (1996). Neem and its general development for pest control. In: Neem and Environment Vol I. (Eds RP Singh, MS Chari, AK Raheja, W Kraus). Oxford & IBH Publishing Co Pvt Ltd, New Delhi.

Saparrat, M.C.N., Martinez, M.J., Cabello, M.N. and Arambarri, A.M. (2002). Screening for ligninolytic enzymes in autochthonous fungal strains from Argentina isolated from different substrata. Rev Iberoam Micol. 19:181-185.

Sateesh, M.K. and Shankara Bhat, S. (1999). Detection of see-borne *Phomopsis azadirachtae* and its transmission in *Azadirachta indica* (Neem). Seed Science and Technology. 27:753-759.

Sateesh, M.K. and Shankara Bhat, S. and Devaki, N.S. (1997). *Phomopsis azadirachtae*sp. nov from India. Mycotaxon 65:517-520.

Sateesh, M.K. and Shankara Bhat, S. (1998). A new fungal threat to neem trees in India. Global Neem Update, pp. 9.

Sateesh. M.K. (1998). Microbiological investigations on die-back disease of neem (*Azadirachta indica*A. Juss.).PhD dissertation, University of Mysore, Mysore, India.

Schaffer, W. (1993). The role of cutinase in fungal pathogenicity. Trends Microbiol. 1:69-71.

Shankara Bhat, S., Sateesh, M.K. and Devaki, N.S. (1998).A new destructive disease of neem trees incited by *Phomopsis azadirachtae*.Current Science 74:17–19.

Sohail, M., Naseeb, S., Sherwani, S.K., Sultana, S., Aftab, S., Shahzad, S., Ahmad, A. and Khan, S.A. (2009). Distribution of hydrolytic enzymes among native fungi: *Aspergillus the pre-dominant genus* of hydrolase producer. Pak. J. Bot. 41(5):2567-2582.

Subapriya, R. and Nagini, S. (2005). Medicinal Properties of Neem Leaves: A Review. Current Medicinal Chemistry - Anti-Cancer Agents 5:149-156.

Uecker, F.A. (1988). A world list of *Phomopsis* names with notes on nomenclature, morphology and biology Mycologia Memoir 13:1-323.

Wriston, J.C. and Yellin, T.O. (1973). L-asparaginase: A review. Adv. Enz. 39:185-248.

(Received 11 April 2013; accepted 30 June 2013)