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Occurrence of Endophytic fungi on sal tree (*Shorea robusta* G. f.)) in forest of Chhattisgarh

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During the present investigation extensive survey was made in the Chhattisgarh forest areas for the occurrence of endophytic fungi in leaf tissues of Sal *(Shorea robusta* G.f.*)* tree. Study indicated that highest 90% occurrence of endophytic fungi was observed in old leaves compared to other age group whereas, the colonization of endophytes was maximum in wet season and minimum in hot season. Among different culture media tested, better growth of these fungi was observed in potato dextrose agar media. Moreover, the solid media enzymatic assay indicated the production of various extracellular enzymes by some isolates which indicated their potential role in degradation of complex organic substance present in plants.

Key words: Endophytic fungi, Sal *(Shorea robusta* G.f.*)* extracellular enzyme

INTRODUCTION

Chhattisgarh state, lying between 17°46' N to 24°6' S latiutude and 80°151' E to 84°51' W longitude has about 40% of its geographical area (1,35,244 sq. km.) under Sal forest. Sal *(Shorea robusta* G.f*) an* evergreen tree is the lifeline of the state to sustain the environment, biodiversity values, agricultural productivity, soil and water conservation and nutrient cycling. Looking the importance of this dominating tree species in the forests the government of Chhattisgarh has declared it as the "State Tree". Undoubtedly, tropical forests of the state can be expected for the more abundant and more fungal species diversity than in other geographical regions of the country.

The endophytic fungi is a group which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease (Wilson, 1995). The term endophyte has been introduced by de Bary (1866) and was initially applied to any organism found within a plant. Carroll (1988) has defined endophyte as an organism that causes asymptomatic infections within plants and exclude of pathogenic and mycorrhizal fungi. Endophytes isolated from plants are important for their potential to produce unique metabolites of pharmaceutical and agricultural importance (Petrini, 1986). Moreover, endophytic fungi have been observed to produce extra-cellular enzymes, which are indicative of their potential role in litter degradation (Kumaresan and Suryanarayanan, 2002). Most works on endophytic fungi have been carried out in temperate regions (Rodrigues and Petrini, 1997) whereas, few tropical plants are studied for endophytes and include *Stylosanthes, Azadirachta, Musa, Citrus,* and *Zea mays* (Azevedo, 1998); members of the Piperaceae and Crassulaceae (Dreyfuss, 1986); *Opuntia stricta* (Fisher, 1996); Palm from Australia etc.. In India mangrove tree species are studied for the occurrence of endophytes in Southern part of the country (Suryanarayanan *et al.*,1998)

The endophytic mycota of tropical plants differ markedly from that of temperate ones (Rodrigues and Petrini, 1997). In tropics that are rich in vascular plant diversity, the study of endophytes has been placed to the forefront and the chance of encountering new species of fungi is equally high. In view of the above perspectives the present investigation

was performed with the objectives to study the occurrence of endophytic fungi in leaves of Sal tree at different age group and within seasons (hot, wet and cold). In addition, some isolates fungal endophytes were evaluated for nutritional study and solid media enzymatic assay to determine the preferred growth media and production of extracellular enzymes respectively.

MATERIAL AND METHODS

Sampling and Isolation

During the present study extensive and intensive surveys were made in the forest areas of Pali, Chaturgarh and Korba for the collection of Sal leaves of various ages. The area of 500 sqm (100 m x 5 m) was selected with ten sample points (each point after 10 m) in each site. Five leaves from each sample point were collected at a height of 10-15 ft as per the degree of variation of the leaf age i.e. new born (young with yellow pink color of size 3-6 cm); young (yellowish light green, thin, tender veins of size 16-24 cm); mature (dark green, attaining full size, much harder, thicker than young one of size 20-28 cm); and old/ senescent (older leaves, harder rough leaf blades, thicker vein & petiole, caduceus of size 20-25 cm). Samplings were done hot (16th May), wet'(16th August) and cold (17th December) seasons of the year 2007.

The leaf samples were brought to the laboratory in sterile polythene bags and processed within 48 hrs of collection. The leaves were washed thoroughly in running tap water especially in the midrib portion. Surface sterilization was performed using the modified method of Guo *et al.* (2003). Segments of 0.5-2.0 cm were cut from the midrib portion of each leaf. The leaf bits were then surface sterilized to eliminate the obligate epiphyllous organisms by first sterilizing in 70% ethanol for 5 sec followed by 4% NaOCl for 90 sec. Leaf bits were then washed in sterile water for 10 sec. Ten leaf segments were plated in each Petri dish (90 cm in diameter) containing potato dextrose agar (PDA) medium with chloramphenicol (150 mg/L) to control the growth of bacteria. The Petri dishes were then sealed with Parafilm™ and incubated under cool daylight fluroscent lamps (12 hrs of light and dark photoperiod) at 26±1°C. Fungi that grew out from the segments were periodically isolated and subcultured separately. Isolates were identified morphologically using standards keys whereas, other which could not be identified were given code no. for further study. Identified isolates were recorded for all the seasons. All the fungal endophytes were maintained in PDA slants at 4°C until use. Isolation rate of endophytic fungi was calculated using the following formula :-Isolation rate = Total no. of isolates yielded / Total no. of leaf segments plated.

Nutritional study

Eight identified endophytic fungal isolates *i.e. Acremonium* sp., *Aspergillus fumigatus*, *Chaetomium* sp., *Colletotrichum* sp., *Fusarium* sp., *Pestalotia* sp., and *Phoma* sp., *Phyllosticta* sp., were raised on four different culture media viz. Sabouraud's Dextrose Agar (SDA); Potato Dextrose Agar (PDA); Malt Extract Agar (MEA); Emerson's Yeast Peptone Starch Agar (EYPPSA) at 26±1°C for 14 days. Point inoculation at the centre of the 90 mm Petri plate was done for this experiment. Radial mycelial growth was measured twice perpendicularly using mm ruler. The experiment was conducted twice each time with triplicates.

Solid media enzymatic assay

Endophytic fungal isolates selected for the nutritional study were also examined for their potential for extra-cellular enzyme production. All tests were made on pre-poured Petri plates by either a spread plate technique with spores and by point inoculation with mycelium wherever necessary. Plates were incubated at 21-23° ± 1° C for 5 days and enzyme detection tests were performed which was perceived by clear zone around the colony. All media contained sufficient substrate to allow good growth of the test fungi. The methods described by Hankin and Anagnostakis (1975) and Rohrmann and Molitoris (1992) were used to detect the production of extracellular enzymes by fungal endophytes which is as follows. The experiment was conducted twice each time with triplicates.

Amylolytic activity : G.Y.P. (Glucose Yeast Peotone) medium (i.e., 1 g Glucose + 0.1 g yeast extract + 0.5 g peptone, 16 g agar in 1000 ml distilled water) plus 0.2% soluble starch, at pH 6 was used. For detection the plates were flooded with iodine solution. A yellow zone around the fungal colony in an otherwise blue medium indicated amylolytic activity.

Cellulase activity : Y.P. (Yeast Peptone) medium containing Na-carboxy-methyl cellulose (0.5%) was used. For detection the plates were flooded with 0.2% congo red solution and destained with 1M NaCl (15 min). Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity.

Lipolytic activity : Tween 20 was sterilised by autoclaving and 1 ml was added to 100 ml of sterile cooled agar medium (Peptone 10 g, NaCl 5 g, $CaCl_2$, $2H_20$ 0.1 g, agar 20 g in 1000 ml distilled water at pH 6). Clearing or precipitation around the fungal colony indicated lipolytic activity.

Pectinolytic activity : Agar medium containing 1 g Yeast extract, 5 g Pectin, 15 g agar in 1000 ml distilled water was used maintaining pH 5. For detection the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. A clear zone formed around the fungal colony indicated pectinolytic activity.

Proteolytic activity : G.Y.P. (Glucose Yeast Peotone) medium amended with 0.4% gelatin (pH 6) was used. A solution of gelatin in water (8%) was sterilised separately and added to G.Y.P. medium at the rate of 5 ml per 100 ml of media (constituting 0.4% gelatine). After incubation, degradation of the gelatine was seen as clearing in the somewhat opaque agar around the colonies. The plate was then flooded with an aqueous saturated solution of ammonium sulphate, which resulted in the formation of a precipitate. This made the agar opaque and enhances the clear zones around the fungal colony.

RESULTS :

Occurrence of endophytic fungi

The present study showed that new born, young, mature and old leaves of *Shorea robusta* were all harboured by fungal endophytes at various degrees. The isolation rate of endophytic fungi was maximum from the old/ senescent leaf (90.1%) followed by mature leaf (75.7%), young leaf (50.5%) and least was observed in new born leaf (30.2%) (Table 1). A total of 17 groups of endophytic fungal isolates including 14 identified and a group of unidentified genera and mycelia sterilia were isolated from the Sal leaves of various ages irrespective of seasons (Table 2). Hot season yielded 10 fungal isolates

Table 1 : Occurrence of endophytic fungi in leaf tissues of different age group from tree (*Shorea robusta*).

Leaf age	Isolation rate (%)
New born	30
Young	50
Mature	75
Old/ senescent	90

Table 2 : Seasonal occurrence of endophytic fungi of Sal tree (*Shorea robusta*)

	Seasons		
Endophytes	Hot	Wet	Cold
Alternaria sp.	-	1	1
Acremonium sp.	-	1	1
Aspergillus fumigatus	1	3	1
Aspergillus nidulans	1	1	-
Aspergillus niger	-	2	2
Chaetornium sp.	1	2	-
Cladosporium sp.	-	3	1
Colletotrichum sp.	-	2	1
Curvularia sp.	1	2	-
Dreschlera sp.	-	-	1
Emericela nidulans	1	1	-
Pestalotia sp.	2	4	2
Phoma sp.	1	3	1
Phyllosticta sp.	-	3	3
Unidentified genera	2	6	4
Mycelia sterilia	-	1	1
Total	10	35	19

whereas, in wet and cold seasons 34 and 18 isolates were recovered respectively *Pestalotia* sp. was isolated maximum (8 isolates) followed by *Phyllosticta* sp. (6 isolates), *Phoma* sp. (5 isolates) and *Aspergillus fumigatus* (5 isolates) whereas, single isolates of *Dreschlera* sp. was found. *Aspergillus nidulans, Curvularia* sp., *Emericela nidulans* were found in hot and wet season but was absent in cold season while, *Aspergillus fumigatus, Pestalotia* sp., *Phoma* sp. was found ubiquitously in all seasons (Table 2). The survey showed that leaves of Sal tree was harboured by anamorphic fungi and Ascomycetes as endophytes. In the present study anamorphic fungi were isolated greater than Ascomycetes similarly more number of sporulating endophytes was recovered. The Hyphomycetes genera such as *Alternaria* sp., *Cladosporium* sp. which are common phylloplane fungi, were also isolated as endophytes from Sal leaves. (Fig.1).

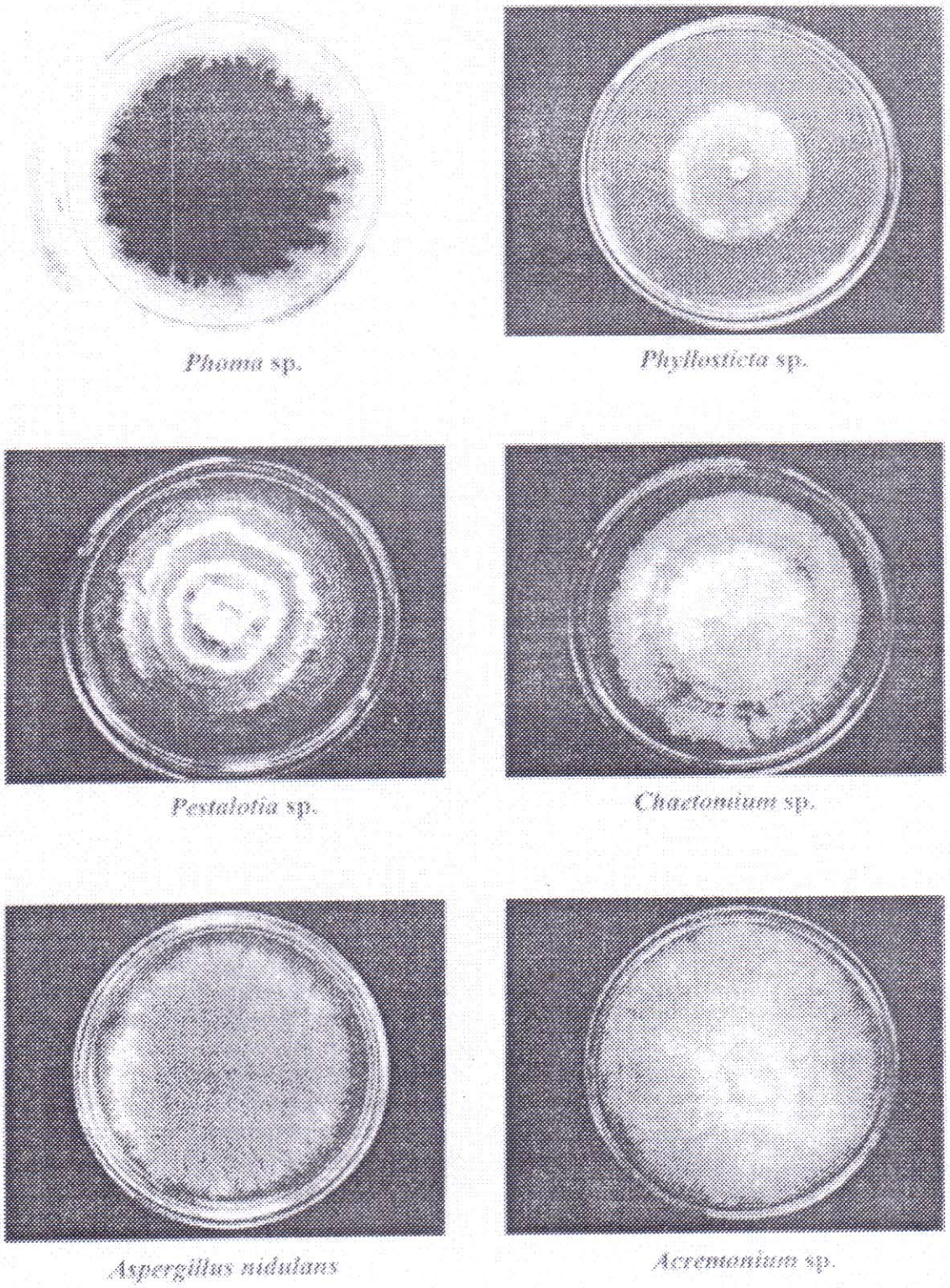

Fig 1. Displaying some identified fungal endophytes isolated from Sal tree.

Nutritional study

All the eight endophytic fungi experienced considerable variability in terms of radial growth in four different culture media (Table 3). Irrespective of fungal genera mean radial growth was maximum in PDA (80.1 mm) followed by MEA (78.6 mm) and minimum in SDA (52.3 mm). The average radial growth irrespective of culture media used was found in descending order as *Phyllosticta* sp. (80.8 mm) > *Acremonium* sp. (80.5 mm) > *Colletotrichum* sp. (80.5 mm) > *Aspergillus fumigatus* (74.0 mm) > *Fusarium* sp. (74.0 mm) > *Chaetomium* sp. (67.5 mm) > *Pestalotia* sp., (63.8 mm) *Phoma* sp. (44.0 mm). Complete mycelial growth (90.0 mm) of *Acremlmium* sp., *Colletotrichum* sp., was observed in PDA medium whereas, in MEA it was witnessed for *Acremonium* sp., *Colletotrichum* sp. and *Phyllosticta* sp.

Solid media enzymatic assay

All the fungal endophytes showed a diverse activity in terms of extra-cellular enzyme production in solid media (Table 4). High amylolytic activity was governed by *Pestalotia* sp. whereas, it was observed in *Aspergillus fumigatus, Chaetomium* sp., *Phoma* sp. for cellulolytic activity. Pectinase activity was witnessed maximum in *Colletotrichum* sp. and whereas, it was shown by *Acremonium* sp. and *Pestalotia* sp. in terms of proteolytic activity. Amylolytic activity was not detected for *Chaetomium* sp. *Phyllosticta* sp. whereas, *Colletotrichum* sp. *Pestalotia* sp. lacked cellulolytic activity. Similarly, Lypolytic activity was not observed for *Acremonium* sp. *Chaetomium* sp. *Phoma* sp. whereas, pectinase production was not detected for *Fusarium* sp. *Phoma* sp. and proteolytic activity was absent in *Aspergillus fumigatus, Colletotrichum* sp. *Phyllosticta* sp.

Table 3 : Radial growth of endophytic fungi in different culture media after 14 days of incubation at 26±1°C temperature. Value represent mean±SEm.

Endophytes	SDA	PDA	MEA	EYPPSA	Mean
Acremonium	61±0.34	90±0.64	90±0.45	81±0.11	80.5
Aspergillus fumigatus	53±0.14	82±0.43	76±0.32	85±0.34	74.0
Chaetomium sp.I	35±0.33	78±0.36	71±0.13	86±0.32	67.5
Colletotrichum sp.II	73±0.87	90±0.84	90±0.54	69±0.12	80.5
Fusarium sp.	55±0.12	86±0.61	80±0.37	75±0.45	74.0
Pestalotia sp. V	38±0.39	75±0.17	70±0.76	72±0.73	63.8
Phoma sp. III	37±0.22	58±0.45	62±0.34	19±0.41	44.0
Phyllosticta sp. I	66±0.28	82±0.26	90±0.44	85±0.31	80.8
Trail mean	52.3	80.1	78.6	71.5	

Table 4 : Detection of extra-cellular enzyme production by tropical endophytic fungi of Sal tree

	Extra-cellular enzymatic activity				
Endophytes	Amylase	Cellulase	Lipolytic	Pectinase	Proteolytic
Acremonium sp.	+	+	+	-	++
Aspergillus fumigatus	+	++	+	+	-
Chaetomium sp.I	-	++	-	+	+
Colletotrichum sp.II	+	-	+	++	-
Fusarium sp.	+	-	+	+	+
Pestalotia sp. V	++	-	+	+	++
Phoma sp. III	+	++	-	-	+
Phyllosticta sp. I	-	+	+	+	-

++ = High activity; + = Moderate activity; - = No activity

DISCUSSION

Occurrence of endophytic fungi

From the present study it was witnessed that the isolation of endophytic fungi depends on the age of the leaf, old or senescent leaves were significantly more densely colonised by fungal endophytes compared with other age group. Similar to our finding earlier reports showed in temperate trees there exist a positive correlation between leaf age and endophytic species richness (Stone, 1987; Espinosa-Garcia and Langenheim, 1999; Taylor *et al.*, 1999). The increased density of colonisation of these fungi in older leaves is due to the repeated re-infection of the leaf probably from air borne inoculum (Carroll *et al*, 1997; Bertoni and Cabral, 1988; Rodrigues *et al.*, 1993). This is also relevant especially in the case of Sal which is an evergreen tree species and its leaves persist on for several months thus enabling the repeated colonization of endophytic fungal flora. In addition, in the present study it was found that the endophytic assemblage of old leaves were more diverse suggesting the high susceptibility of old leaves to fungal endophytes. Due to small size of newborn leaf, there is a little infection of endophytic fungi. Moreover, old leaves contain a higher percentage of cellulose and complex sugar compared with new born and young leaf, which act as a good substrate for a large amount of endophytes.

Marked seasonal variation in the rate of colonisation of fungal endophytes has also been observed in the survey. The extent of endophyte colonization of leaves is known to be influenced by environmental factors (Collado *et al.* 1999). The present investigation showed that the occurrence of endophytes were higher in wet season followed by cold and was least in hot season. Similar to our study Willson and Carroll (1994) and Rodrigues (1994) found respectively in their study that the leaves of temperate and tropical trees harbour more endophytes during the wet season. Also, Rajagopal and Suryanarayanan (2000) studied endophytes of Neem leaves for a period of two years and concluded that the colonisation frequency of the endophytes increased with rainfall. *Pestalotia* sp. *and Phoma* sp. found maximum during rainy season among the identified genera. There were four species of *Pestalotia* sp. and three species of *Phoma* sp. isolated during rainy season showing a seasonal dominance of these endophytic fungal genera in Sal tree. Anamorphic genera such as *Alternaria* sp., *Cladosporium* sp. *Dreschlera* sp. and *Colletotrichum* sp. were exclusively found during winter season which depicted their preference for moderate temperature and low humid condition whereas, *Phyllosticta* sp. was isolated invariably in both wet and cold season. *Chaetomium* sp., *Curvularia* sp. and few species of *Aspergillus* were obtained in higher percentage during hot season as they are highly cellulolytic and happened to be thermophilic compared to other genera. Pigmented strain (green pigmentation) of *Aspergillus nidulans* were found in summer. This suggests the production of heat shock protein and accumulation of melanin in fungal mycelial to bear the surrounding temperature.

Nutritional study

Temperature and precipitation are the prime crucial factors deciding the distribution of endophytic fungi. Endophytes belong to mesophilic group of fungi which are generally sensitive to higher temperature. The normal temperature for the growth of endophytic fungi is reported to be of 2[illegible]°C (Suryanarayanan and Kumaresan, 2000) and thus the nutritional study was set at the same temperature. From the present study it was clear that the fungal endophytes can grow better in potato dextrose agar medium followed by malt extract agar media,, and poorer in Sabraud's dextrose agar medium, However, according to Campbell and Williams (1953) and Langridge, (1963) nutritional supplementation is required to overcome low thermal tolerance in some organisms while in others growth at elevated temperatures is accomplished by an increased nutritional sufficiency. Although each species has a very specific nutritional requirements for its optimum growth and activity, the necessity to identify a unique nutrient medium having a capacity to support a wide range of endophytic fungal flora so as to maintain the cultures and to conduct further studies on enzymatic systems.

Solid media enzymatic assay

Endophytic fungi usually produce the enzymes necessary for the colonization inside plant tissues. The solid media enzymatic assay had demonstrated that most endophytes can able to utilize organic compounds which are major components of plant tissues. The degradative enzymes produced by endophytic fungi are important in host infection, host pro-

tection and breakdown of organic matter (Hankin and Anagnostakis, 1975). In the present study the enzymatic assay make it to understand the functional roles of tropical endophytes in the production of different degradative enzymes. In the present study *Aspergillus fumigatus, Chaetomium* sp. and *Phoma* sp. proved to be highly cellulolytic which can be correlated to their potential role in litter degradation as reported by Kumaresan and Suryanarayanan (2002). *Acremonium* sp. and *Pestalotia* sp. were highly proteolytic whereas, *Colletotrichum* sp. was more pectinolytic. It is reported that if the endophytic fungi are weak parasites or latent pathogens they produce proteinase and pectinase (Brett, 1990).

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