
Enzyme production by endophytes of *Brucea javanica*

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Twenty-one endophytic isolates from *Brucea javanica* were tested for their ability to produce extracellular cellulase and extracellular and intracellular amylase, ligninase, pectinase and xylanase. The same fungi were tested for their ability to cause weight loss in wood blocks. All fungi produced amylase and cellulase, while only one sterile mycelium produced ligninase and no isolates produced pectinase. The enzyme tests indicate that most endophytes are degraders of the simpler sugars and cellulose available in recently dead leaves and possibly wood. Only one slow growing species of sterile mycelium however, appeared to be capable of degrading lignin that would be available in dead wood. No fungi appeared to be latent pathogens. A discussion of enzyme production in relation to possible roles of endophytes is provided.

Key words: endophytes, enzyme production, latent pathogens, ligninases.

Introduction

Brucea javanica (*Simarubeaceae*) is a tropical, 3 m high, woody shrub, clothed in yellow fluff. It is found from southern China to northern Australia. The seeds are used in Traditional Chinese Medicine for the treatment of dysentery, malaria and cancer (Lin *et al.*, 1990), while the leaves are used in folk medicine for poultices on boils, ringworm, scurf, centipede bites and enlarged spleens (Perry, 1980). We are interested in establishing the composition of endophytic communities within *B. javanica* plants and their functional roles, either before or after plant death, and whether the endophytes possess any of the medicinal properties of the plant.

This report is part of a study where we isolated endophytes from *B. javanica*. We selected 21 unique taxa of fungal endophytes and examined their ability to produce enzymes *in vitro*. Qualitative assays were used to screen the endophytic fungi for their ability to produce lignocellulose degrading and other enzymes, including amylase, cellulase, ligninase, pectinase and xylanase. The enzyme tests may help us to understand the functional roles of endophytes and

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test whether fungi can change their mode of life from an endophyte, to a saprobe or pathogen. Weight loss tests using wood blocks were also used to compare the results of the enzyme tests and investigate the effectiveness of the wood degrading enzymes produced by the endophytes (Pointing *et al.*, 2003; Bucher *et al.*, 2004).

Methods and materials

Twenty-one taxa of endophytic fungi, comprising 7 species identified to genus, 8 identified to a major taxonomic group, and 7 unidentified sterile mycelia were selected to perform the enzyme assays and are listed with their collection details in Table 1. The endophytic fungi were isolated from stems, branches or leaves of *B. javanica* from different sites and during different seasons. The endophytes selected included potential pathogens, such as *Colletotrichum* spp., *Fusarium* spp., *Phomopsis* spp. and *Phoma* sp., as well as unidentified species of coelomycetes, hyphomycetes, xylariaceous taxa and sterile mycelia.

Table 1. *Brucea* endophytes used in this study.

Name	Code	HKUCC No.	Location	Collection Date
<i>Colletotrichum</i> sp.	B10810	6055	North Queensland, Australia	June, 1999
<i>Colletotrichum</i> sp.	L27103	6061	Hong Kong	September, 1999
<i>Fusarium</i> sp.	S29905	6063	Hong Kong	September, 1999
<i>Phomopsis</i> sp.	S12803	6056	North Queensland, Australia	June, 1999
<i>Phomopsis</i> sp.	S29904	6062	Hong Kong	September, 1999
<i>Phoma</i> sp.	B25603	6058	Hong Kong	September, 1999
Coelomycete sp. 4	B0605	5881	Hong Kong	March, 1999
Coelomycete sp. 6	S2502	5883	Hong Kong	March, 1999
Coelomycete sp. 8	S3601	5885	Hong Kong	March, 1999
Hyphomycete sp. 4	S0903a	7118	Hong Kong	March, 1999
Hyphomycete sp. 3	L0302	5866	Hong Kong	March, 1999
Hyphomycete sp. 2	L0901	5658	Hong Kong	March, 1999
Xylariaceae sp.	B25605	6059	Hong Kong	September, 1999
Xylariaceae sp.	S26807	6060	Hong Kong	September, 1999
Sterile Mycelia	B23904	6942	Hong Kong	September, 1999
Sterile Mycelia	S10702	6054	North Queensland, Australia	June, 1999
Sterile Mycelia	S11303	7119	North Queensland, Australia	June, 1999
Sterile Mycelia	S11309	7120	North Queensland, Australia	June, 1999
Sterile Mycelia	S13602	6057	North Queensland, Australia	June, 1999
Sterile Mycelia	S31105	6064	Hong Kong	September, 1999
Sterile Mycelia	S31106	6965	Hong Kong	September, 1999

Growth rates

The growth rates of the fungi were measured in triplicate on PDA.

Enzyme assays

Five different enzyme assays, plus weight loss tests were conducted to investigate the abilities of the endophytes to produce wood degrading and other enzymes. Enzyme production can be separated into intracellular and extracellular production. The cellulase test only measured extracellular cellulase production, while the other tests measured both intracellular and extracellular enzyme production.

Extracellular enzyme production ratio = the ratio of clear zone diameter to that of colony diameter.

The extracellular enzymatic reactions of the following tests were classified into 4 types:

Strong reaction: the extracellular enzyme ratio was higher than or equal to 2.

Medium reaction: the extracellular enzyme ratio was less than 2 but more than 1.

Weak reaction: the extracellular enzyme ratio was equal to or less than 1.

No reaction: there is no reaction at all.

For intracellular enzymes (those endophytes with extracellular enzyme ratio equal to or less than 1), the diameter of the clear zone was used as a measurement of the amount of enzyme production.

Amylase

Starch agar was prepared and autoclaved. The test fungi were inoculated onto the agar plates and incubated for ten days. The plates were flooded with a dilute iodine solution (Lugol's iodine). After flooding with iodine, the starch stains blue-black and the zone of degradation around the colonies is either stained brown or remains colourless (Peterson and Bridge, 1994).

Starch solution was prepared by dissolving 10 g soluble starch in 50 ml distilled water. This was stirred to give an even paste and then heated at 70-80°C for 1-2 minutes before adding to medium.

Cellulase

Cellulose azure agar was used to test for the presence of cellulase. Water agar (1.6% agar in d.H₂O) was prepared and 10 ml aliquots were transferred to

glass tubes, autoclaved, and then allowed to solidify. Cellulose azure (1%), yeast extract (0.1%) and agar (1.6%) were prepared and autoclaved. This was allowed to cool until viscous, mixed gently, and 1 ml aliquots were transferred aseptically onto the surface of the solidified water agar as an overlay.

The test fungi were inoculated and an uninoculated bottle was retained as a control. The bottle caps were loosely fitted to allow adequate gaseous exchange. They were incubated at 25°C in darkness and examined daily for 10 days. When cellulase degrades cellulose azure agar, the blue colour at the top layer migrates to the lower layer. This cellulolytic reaction can be divided into 4 classes (Pointing, 1999):

Strong reaction (3+): the blue colour migrates to the bottom of the universal bottle in 10 days and the blue colour is decolourised afterwards.

Medium reaction (2+): the blue colour migrates to the bottom of the universal bottle in 10 days and the blue colour is not decolourised afterwards.

Weak reaction (+): the blue colour migrates to half the depth of the universal bottle in 10 days.

No reaction (~): there is no blue colour migration at all.

Ligninase

Poly R agar was made with 0.2% Poly R, 0.2% glucose, 0.1% yeast extract and 1.6% agar. The test fungi were inoculated onto the agar plates. Clearance can be considered as an indication of ligninase production (Pointing, 1999).

Pectinase

Pectin agar was prepared and autoclaved. The test fungi were inoculated onto the agar plates and incubated for 7 to 10 days. The colony diameters were recorded. The agar plates were flooded with 0.1 M aqueous malic acid and left to stand at room temperature for 1 hour. The malic acid was drained and the plates were flooded with 0.1% aqueous ruthenium red and left for 2 days at 4°C. The ruthenium red was drained off and the plates were washed for 1 hour in distilled water. They were washed with 0.1% aqueous ammonium persulphate to increase contrast. The agar plates are pink after staining and dark pink zones around colonies indicate pectinase activity (Peterson and Bridge, 1994).

Xylanase

Xylan agar was prepared with 1% xylan, 0.1% yeast extract and 1.6% agar, and autoclaved. The test fungi were inoculated onto the agar plates.

Clearance can be considered as an indication of xylan utilization (Pointing, 1999). Dilute iodine solution was used to stain the agar plates, and a yellow-opaque area around colonies indicated xylan degradation as compared to a reddish purple colour for undegraded xylan.

Weight loss test

Debarked branches of *Brucea javanica* were cut into 3 cm test blocks. They were soaked in water for 24 hours and dried at 60°C for 48 hours. Test blocks were picked randomly, weighed and marked with a number (from 1 to 142) to obtain the initial dry weight. They were then wrapped with aluminium foil and autoclaved twice (121°C, 15 minutes).

Corn meal agar (CMA) plates were prepared and the test fungi were inoculated separately onto the agar plates. Each test fungus had 5 replicates and they were incubated at 25°C for 10 days. The test blocks were placed onto agar plates with growing mycelia. The plates were incubated at 25°C for 6 weeks. The test blocks were removed and dried at 60°C for 48 hours. The final dry weight of the test blocks were obtained and the percentage weight loss was then calculated.

Percentage weight loss = (Initial dry weight – final dry weight) / Initial dry weight × 100%

Results

Enzyme assay

Amylase

All strains produced amylase, one sterile mycelium (S11309) had a strong reaction (an extracellular enzyme ratio of 3.9), five strains had medium reactions and 15 had weak reactions (Table 2). Thirteen strains produced intracellular amylase. Hyphomycete sp. 2 and 3 produced the highest amounts of intracellular amylase.

No purple reaction with Lugol's solution occurred under the growing mycelium, while in some species clear halos extended 3-4 mm beyond the growing hyphae. The amylase production was therefore internal and external. A clear zone beyond the mycelia (extracellular enzyme production ratio greater than 1) indicated that external amylase was produced to degrade starch, while those that only produced a clear zone below the colonies (extracellular enzyme production ratio less than 1) were indicative of internal amylase production.

Table 2. Summary of 5 enzymatic tests, weight loss assay and average growth rates.

Taxa	Amylase	Cellulase	Ligninase	Pectinase	Xylanase	Weight loss	Growth rate
	1	2	3	4	5	6	7
Coelomycete sp. 4	0.9	-*	-	-	0.9 (4)	15.3	5.6
Coelomycete sp. 6	0.8	2+ (6)	-	-	1.1 (4)	23.9	8.6
Coelomycete sp. 8	0.9	2+ (6)	-	-	1 (6)	20.3	8.8
<i>Colletotrichum</i> sp.	0.9	2+ (9)	-	-	1.1 (4)	23.3	9.1
<i>Colletotrichum</i> sp.	0.9	2+ (6)	-	-	1.1 (4)	22.4	10.2
<i>Fusarium</i> sp.	1.1	-	-	-	-	13.4	6.4
Hyphomycete sp. 2	1	+ (13)	-	-	1 (6)	19.9	10
Hyphomycete sp. 3	1	2+ (8)	-	-	1 (4)	12.6	9.8
Hyphomycete sp. 4	0.9	+ (9)	-	-	-	17.6	9.2
<i>Phoma</i> sp.	0.6	2+ (6)	-	-	0.9 (4)	13.9	12.1
<i>Phomopsis</i> sp.	0.8	+ (13)	-	-	-	17.5	10
<i>Phomopsis</i> sp.	1.3	-	-	-	1.2 (8)	14.8	6.4
Sterile mycelium	1.3	2+ (6)	-	-	1.4 (8)	23.2	3.2
Sterile mycelium	0.8	-	-	-	-	19.5	6.4
Sterile mycelium	1	+ (8)	-	-	-	18.8	6.4
Sterile mycelium	1.5	+ (10)	-	-	1.8 (10)	18	3.2
Sterile mycelium	0.8	+ (10)	-	-	1 (4)	14.4	10.8
Sterile mycelium	0.5	-	-	-	-	13.7	10
Sterile mycelium	3.9	-	1.2 (8)	-	6.4 (5)	4.9	1.1
Xylariaceae sp.	1.1	+ (13)	-	-	1 (8)	23.4	8.1
Xylariaceae sp.	0.6	-	-	-	1 (4)	17.6	12.1
Negative control	-	-	-	-	-	0	0
<i>Trametes versicolor</i>	0.9	+ (6)	0.68 (4)	1 (7)	1.2(6)	59.7	11.6

1. Extracellular enzyme production rate (clear zone in cm, colony diameter in cm) over 10 days.
 2. Strength of reaction: 2+ = medium; + = weak; - no reaction in (x) days.
 3. As 1. but in (x) days.
 4. As 3.
 5. As 3.
 6. Actual weight loss %.
 7. Average growth rate mm/day.
- *10 days if not stated

Cellulase

Fourteen endophytes produced cellulase (Table 2). None had a strong reaction, seven had medium reactions (2+) and seven had weak reaction (+).

Ligninase

Only one endophyte, sterile mycelium strain (S11309), produced extracellular ligninase (Table 3). Over 8 days it extracellular enzyme production was 1.2 and it produced a clear zone at a rate of 0.3 cm per day. *Trametes versicolor* produced intracellular ligninase with a clear zone production of 1.1 cm per day and over 4 days its ratio was 0.68. When comparing the amount of ligninase produced, *Trametes versicolor*, the positive control, was a better producer of ligninase than strain S11309.

Pectinase

None of the endophytes were found to produce pectinase. The positive control *Trametes versicolor* produced extracellular pectinase, with a clear zone of 1.2 cm diameter per day, and an extracellular ratio of 1.1.

Xylanase

Fifteen of the endophytes tested produced extracellular xylanase, but only one had a strong reaction (6.4-Strain S11309), six had medium reactions (1.1-1.8) and eight had weak reactions (0.9-1) (Table 2). Eight strains were intracellular producers of xylanase. *Colletotrichum* species B10810 and L27103, *Phoma* species (B25603) and the xylariaceous taxa (B25605) produced higher amounts of extracellular or intracellular xylanase than the other strains.

Wood block weight loss test

The results of the wood block weight loss tests are shown in Table 3. The “actual weight loss %” was calculated by deducing the average weight loss due to leaching in the negative control (11.8%) from the average weight loss due to each isolate. Six of the endophytes caused more than 20% weight loss, and the other 15 caused less than 20% weight loss. One strain, the sterile mycelium strain S11309 caused only 4.9% weight loss. The average growth rates of the endophytes on corn meal agar plates are also shown for comparison in Table 3.

Comparison of results of enzymatic tests and the wood block weight loss test

From the combined results of the five enzymatic tests and wood block weight loss tests shown in Table 4 it can be seen that those endophytes that caused more than 20% weight loss produced all three types of enzyme, i.e. amylase, cellulase and xylanase. There were no clear patterns of enzyme production in those taxa that produced less than 20% weight loss. Two of the three hyphomycetes tested (L0901 and L0302), which caused less than 20% weight loss, produced cellulase, xylanase and amylase. *Trametes versicolor*, which is a white rot fungus, produced all types of enzymes tested in this study, and caused a 59.7% weight loss.

Discussion

Methodology

Priest (1984) showed that there were several possible regulatory mechanisms in enzyme production, including enzyme induction. In the enzyme production tests used in this study, cellulose, pectin, Poly-R, starch and xylan were used as substrates to induce enzyme production. They were mixed with low nutrient content agar that had just enough nutrient for mycelial growth to spread across the agar plates.

Decay caused by *Gendarme colossus* has previously been found to be dependent on time of incubation and temperature (Adaskaveg *et al.*, 1995). Weight loss increased as the incubation time increased and the optimal temperature for the highest weight loss was 30 to 40°C. In this study, the containers were 90 mm Petri dishes, which are not air-tight and the incubation time was 6 weeks. As a result, the incubation temperature was never greater than 25°C. As the wood branches used in this study were low in weight (~ 0.1 g), and since most of the endophytes tested were fast-growing fungi, a shorter incubation time, 6 weeks, was chosen.

Enzyme production

In order to establish the functional role of endophytes it would be useful to establish their patterns of substrate utilization and which enzymes they produce (Carroll and Petrini, 1983). If they are weak parasites or latent pathogens they may produce proteinase and pectinase (Brett, 1990b; Reddy *et al.*, 1997), while if they are mutualistic, eventually being saprobes they are

likely to produce cellulase, mannanase and xylanase (Pointing, 1999). We have therefore tested the ability of endophytes from within seedlings to produce amylase, cellulase, mannanase, proteinase and xylanase in order to allude possible roles.

Amylase

All endophytes tested in this study were found to degrade soluble starch. The results are similar to the findings of Adaskaveg *et al.* (1991), who found that the white and brown rot fungi utilized starch in palm wood. These fungi also utilized starch when grown on starch agar media. Endophytes are likely to be the first colonizers of dead plant tissue as they are already living within the plant when it is still alive (Guo *et al.*, 1998; Fröhlich *et al.*, 2000). Plant tissues store starch as a food source and this starch is one of the most easily digestive food sources within plant tissues. When the plant dies, the starch becomes available and the endophytes can consume the starch before other new colonizers appear.

Cellulase and xylanase

Endophytes that degraded xylan, are also likely to have the ability to degrade cellulose and are discussed together here. Xylan (hemicellulose) and cellulose occupy between 25 to 40% and 40 to 50% of the wood mass respectively. After all simple food sources, such as glucose and starch, are used up, wood degrading fungi will start to degrade the cell wall components. As a result, endophytes that produce both cellulases and hemicellulases, such as xylanases should have the ability to compete with other types of fungi surviving on dead wood and leaves (Carroll and Petrini, 1983).

Pectinase

No endophytes tested had the ability to degrade pectin. Only the positive control, *Trametes versicolor*, degraded pectin. Pectic substances are predominantly located in the middle lamella and primary wall and they are highly susceptible to degradation under relatively mild conditions (Brett, 1990b), but in small amounts (Tsoumis, 1991).

Ligninase

Only one of the endophytes tested (S11309) and the positive control, *Trametes versicolor* produced ligninase. This indicates that most of the endophytes in the plant tissues may not have the ability to degrade lignin. It is thought that most endophytes start to grow when the plant is weakened or dies. This and other studies (e.g. Carroll and Petrini, 1983) indicate that endophytes appear to be able to degrade more simple substrates, such as starch, cellulose and hemicelluloses, rather than very complex substrates like lignin.

Strain S11309 is a slow growing endophyte (1.1 mm/day) that had very high extracellular enzyme production ratios in xylanase (6.4) and amylase (3.9) tests and a higher ratio (1.2) in the ligninase test than the positive control *Trametes versicolor* (0.68). All of the enzymes tested were produced externally. The fungus digested most of the food sources offered to it. This implies that this slow growing fungus is able to produce a wide range of enzymes externally.

Pathogenesis

The production of fungal enzymes by fungi in nature has a role in their pathogenicity or degradative capacity (Archer and Wood, 1995). Sieber *et al.* (1991), Carroll and Petrini (1983) and Savorie and Gourbiere (1989) found that the endophyte *Leptostroma* strongly produced extracellular cellulases, indicative of its ability to digest cell wall components. Sieber *et al.* (1991) also stated that the fervent production of extracellular cellulases together with that of pectinases in the *Leptostroma* endophyte could imply that the fungus is well equipped for both penetration of living cells and decomposition of dead tissues. Brett (1990a) stated that the major enzymes involved in microorganisms attacking living higher plant tissues are pectic enzymes. Pectic enzymes are induced in the presence of pectic substances by both pathogenic fungi and pathogenic bacteria.

No endophyte tested in this study had the ability to degrade pectin. Pectic substances are predominantly located in the primary wall, the middle lamella in small proportions (or are absent in older wood), and cambial tissue, where they form the membrane that separates the young daughter cells produced by the cambium (Tsoumis, 1991). If an endophyte can degrade pectic substances, this implies that the fungus is likely to be a latent pathogen. As the result of this study, where only *Trametes versicolor*, a wood degrading fungus, degraded pectin, the endophytes tested are unlikely to be latent pathogens, even though some belong to pathogenic genera (e.g. *Colletotrichum* spp.).

Wood block weight loss test

There was considerable weight loss (11.8%) in the control wood blocks. This was probably due to the wood component breakdown during the double autoclaving (121°C, 15 minutes) so that it became water-soluble and heavily leached during the incubation period. Brown liquid droplets were found on the surface of the wood block in the negative control chamber. Adaskaveg *et al.* (1990) suggested that cellulose degradation may be due to the long period of time the blocks remained in the decay chambers and this may be the other source of weight loss in the negative control.

Most strains caused some weight loss and this probably reflects their ability to degrade cellulose. Similar low weight losses have been recorded in other fungi. Strain S11309 caused very little weight loss (4.9%) in the wood block weight loss test. The fungus appeared to colonize 10% of the wood block surface within 6 weeks, which indicates that, against the control, it is not a good wood degrader. Strain S11309 however, had the ability to produce extracellular wood degrading enzymes, including amylase, ligninase and xylanase. It had very high extracellular enzyme production ratios in the xylanase (6.4) and amylase (3.9) tests and a medium reaction in the ligninase test. The fungus, therefore, produces a wide range of wood degrading enzymes, but can only degrade wood blocks with a negligible weight loss within 6 weeks. This is almost certainly due to its slow growth rate (Table 2). It may have degraded wood blocks to a much greater extent if it had a longer time to colonize the whole wood block surface.

The slow growing strain S11309 would find it difficult to compete in nature against the faster growing strains and this may reflect its role in nature. Unlike most other *Brucea* endophytes studied, this taxon has the ability (*in vitro*) to degrade ligninases. This species may therefore be a late coloniser of dead *Brucea* or other wood, degrading lignin to release cellulases for its growth. This may also be an interesting fungus to test for its competitive ability against other fungi, since it is so slow growing and may need to produce anti-fungals to compete for territory (Hyde, 2001).

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