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Characterization of embryogenic mango cultures selected for resistance to *Colletotrichum gloeosporioides* culture filtrate and phytotoxin

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Abstract Embryogenic nucellar cultures of two polyembryonic mango cultivars, 'Hindi' and 'Carabao', were selected for resistance to the culture filtrate and phytotoxin of a virulent strain of *Colletotrichum gloeosporioides* Penz. that was isolated from mango leaves. The cultures were recurrently selected either with progressively increasing concentrations of culture filtrate or by continuous challenge with the same concentration of either culture filtrate phytotoxin. Mycelium growth was inhibited when the pathogen was cocultured with the selected, resistant embryogenic cultures. Conditioned plant growth medium containing macerated resistant embryogenic cultures did not inhibit mycelium growth, confirming that extracellular antifungal compounds were involved in the defense response. Enhanced secretion of chitinase and glucanase was observed in the plant growth medium in which resistant embryogenic cultures and regenerated somatic embryos were grown in comparison with the controls.

Key words *Mangifera indica* L. · Anthracnose · Somatic embryogenesis · Dual culture · RAPD

Introduction

Anthracnose is the major production and post-harvest problem of mango (*Mangifera indica* L.) and is caused by the fungus *Colletotrichum gloeosporioides* Penz. Most commercial mango cultivars are highly susceptible to this disease. Although southeast Asian cultivars appear to be more tolerant of this disease in their main centers of cultivation (Mathews and Litz 1992), they appear to be susceptible when they are grown elsewhere (Knight 1993), indicating that the true genetic resistance to this disease is absent in the species. The use of conventional breeding to introduce greater resistance to anthracnose into improved mango cultivars has been futile due to extremely poor fruit set, long pre-bearing age of seedlings, polyembryony and unpredictable performance of the F_1 progeny due to heterozygosity. Alternative approaches could involve the use of in vitro selection and genetic transformation to enhance resistance to anthracnose in existing cultivars.

Disease resistance has been identified in somaclonal variants of several species, either by selection for resistance in vitro or by evaluating large numbers of regenerants in the field for disease resistance (Hammerschlag 1992); however, recovery of disease resistant plants by in vitro selection using appropriate selective agents has rarely been applied to woody species (Hammerschlag 1992). The most comprehensively documented studies have involved peach (*Prunus persica* L.) and lemon (*Citrus limon* L.). Hammerschlag (1988) screened embryogenic callus derived from immature zygotic embryos (i.e., nonclonal) of peach against the culture filtrate produced by the bacterial pathogen *Xanthomonas compestris* pv. *pruni* and reported that regenerated plants were more resistant to the pathogen. Gentile et al. (1992, 1993) regenerated mal secco resistant lemon by selecting nucellar (clonal) embryogenic cultures for resistance to a partially purified phytotoxin produced by the causal fungus *Phoma tracheifila*.

Resistance expressed in vitro has been demonstrated to be associated with the elicitation of defense genes, which also occurs during the infection of plants by a pathogen (Roby et al. 1990). Among the polypeptides that are expressed in greater quantities are antifungal

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hydrolases or pathogenesis-related proteins (PR proteins) (Legrand et al. 1987; Mauch et al. 1988), e.g., chitinase and β-1,3-glucanase. Plant chitinase (EC 3.2.1.14) and β-1,3-glucanase (EC 3.2.1.6) occur in several isoforms that differ in physical and molecular properties, enzyme activity (Melchers et al. 1993), cellular compartmentalization, localization and antifungal properties (Cabello et al. 1994). Both of these enzymes are encoded by a small multigene family (Linthorst 1991). This isozyme multiplicity enables plant cells to deploy one form or another in a tissue-specific or stimulus-specific manner (Mardis-Pinheiro et al. 1991). Extracellular chitinases and β-1,3-glucanases probably function as the first line of defense that is deployed by plants against pathogens (Collinge et al. 1993) because chitins and β-1,3-glucans are key components of the cell walls of many plant pathogenic fungi (Wessels and Sietsma 1981; Cornellissen and Melchers 1993).

In this report, we describe the selection and characterization of embryogenic nucellar cultures of two important mango cultivars for resistance to the culture filtrate and phytotoxin of *C. gloeosporioides*.

Materials and methods

Induction and maintenance of embryogenic cultures

Young fruits, approximately 45 days post-anthesis, were harvested from the germplasm collections of the USDA ARS Subtropical Horticultural Research Station, Miami, Florida ('Carabao') and the University of Florida Tropical Research and Education Center, Homestead, Florida, ('Hindi'). Following surface-disinfestation for 30 min in 20% (v/v) commercial bleach containing a few drops of Tween-20, the fruits were thoroughly washed with sterile deionized water and then bisected longitudinally under sterile conditions. The immature seeds were removed and also bisected longitudinally under sterile conditions. The immature seeds were removed and also bisected longitudinally. The polyembryonic masses were removed and discarded. The seed halves were cultured with the nucellus in direct contact with the induction medium (Mango Medium I; MM I), which consisted of B5 major salts (Gamborg et al. 1968) and MS minor salts and organics (Murashige and Skoog 1962) supplemented with 400 mg l^{-1} glutamine, 60 g l^{-1} sucrose, 1.0 mg l^{-1} 2,4-D (DeWald et al. 1989a) and 2.0 g l^{-1} gellan gum. Sterile MM I was dispensed into 90 × 15-mm disposable plastic petri dishes. Inoculated petri dishes were sealed with Parafilm™. The cultures were incubated in darkness at 25° ± 2°C, subcultured 3 days after explanting, and at 3-week intervals thereafter. Embryogenic cultures were established in approximately 8 weeks with 'Hindi' and 10 weeks with 'Carabao'. After proliferation for 3 months on semi-solid MM I, suspension cultures were initiated by transferring approximately 2.0 g embryogenic cultures from a single nucellus into 40 ml of sterile liquid MM I in 125-ml Erlenmeyer flasks that were sealed with aluminum foil and Parafilm™. Suspension cultures were maintained on a rotary shaker in semi-darkness at 100 rpm at 25° ± 2°C. Embryogenic suspension cultures were transferred weekly to fresh liquid MM I. Unless otherwise stated, all plant growth media were sterilized by autoclaving at 1.1 kg cm^{-2} and 120°C for 15 min.

Somatic embryo maturation

Maturation of somatic embryos from embryogenic cultures was initiated by the subculture of proembryonic masses to plant growth medium without 2,4-dichlorophenoxyacetic acid (2,4-D) (MM II) (DeWald et al. 1989b). Early maturation of 'Hindi' occurred in liquid MM II, whereas 'Carabao' development occurred entirely on semi-solid MM II. Post-heart stage somatic embryos of both mango cultivars were grown on semi-solid MM II.

Preparation of culture filtrate and phytotoxin

Spores from a virulent strain of *C. gloeosporioides* isolated from mango leaves were suspended in sterile, deionized water, and the density was adjusted to 10,000 spores per milliliter. Sterile Czapek-Dox medium in 20-ml aliquots in 50-ml Erlenmeyer flasks was inoculated with 100 µl of spore suspension and incubated on a rotary shaker at 100 rpm under low light conditions. After 5 days, the contents of each flask were transferred to a 250-ml Erlenmeyer flask; the final volume of the medium was adjusted to 100 ml with sterile Czapek-Dox liquid broth. After 7 days, the contents from the two flasks were transferred to a 1.0-l flask containing 300 ml fresh broth. The fungus was allowed to grow for 9 more days, after which time the cultures were filtered through a double layer of cheese cloth and refiltered through Whatman #1 filter paper. This culture filtrate was used for in vitro selection and for purification of the phytotoxin.

The procedure for extraction of the phytotoxin was adapted from Gohbara et al. (1978). Four liters of culture filtrate were acidified (pH 3.0) with HCl and extracted repeatedly with ethyl acetate (v/v) in a separatory funnel. The aqueous layer was discarded, and the organic layer was concentrated to 500 ml in vacuo. The concentrate was washed once with an equal volume of saturated sodium bicarbonate and twice with sterile deionized water in order to obtain the neutral extract. The neutral extract was dissolved in an equal volume of 80% (v/v) methanol and shaken with *n*-hexane. The *n*-hexane layer was discarded, and the aqueous methanol fraction was concentrated in vacuo to 20% of its original volume and then filter-sterilized. Four liters of full-strength Czapek Dox broth was extracted in the same manner and used as a medium control.

Recurrent selection with increasing concentrations of culture filtrate

Approximately 500 mg of embryogenic culture (<960-µm diameter proembryonic masses) was challenged for 1 week in 40 ml total volume of MM I with 0% (control), 20%, 40%, 60% and 75% (v/v) of culture filtrate. There were seven 125-ml Erlenmeyer flasks for each treatment and the control. Cultures were transferred to liquid MM I without culture filtrate, grown for 3 weeks and then rechallenged with the next concentration for four successive recurrent selections. After exposure to the highest concentration of culture filtrate, selected embryogenic cultures were grown in liquid MM I. The selected embryogenic culture that was initially challenged with 60% culture filtrate followed by 75% culture filtrate was designated putatively resistant line I (PRL I) (Table 1). Cultures that had been recurrently selected with 40%, 60% and 75% culture filtrate were designated PRL II (Table 1). PRL III was recurrently selected with 20%, 40%, 60% and 75% culture filtrate (Table 1). Surviving (putatively resistant) and dead (susceptible) proembryonic masses were counted after the first and second challenges only, as there was excessive browning after the second challenge.

Continuous challenge with culture filtrate

Embryogenic cultures were incubated with 40% (v/v) culture filtrate in liquid MM I for 4 weeks with weekly transfers to fresh medium of

Table 1 Time frame for experimental protocols, including recurrent and continuous selection, recovery and maintenance after selection, dual culture, conditioned medium study, somatic embryo (SE), germination and extraction of extracellular proteins from embryogenic cultures and germinating somatic embryos

Selection no.	Recurrent selection					Continuous selection			
	Culture filtrate (%)	Culture filtrate (%)	Culture filtrate (%)	Culture filtrate (%)	Time (days)	Culture filtrate (%)	Time (days)	Phytotoxin (%)	Time (days)
1	60	40	20	10	7 21 recovery	40 PRL V	28	7.5 PRL	21
2	75 PRL I	60	40	20	7 21 recovery				
3		75 PRL II	60	40	7 21 recovery				
4			75 PRL III	60	7 21 recovery				
5				75 PRL IV	7 21 recovery				
Dual culture (days after selection)	314	286	258	Dead		241		179	
Extracellular (EC) proteins (days after selection)	314	286	258	Dead		229		167	
Conditioned medium (days after selection)	326	298	270	Dead		241		179	
SE germination (days after selection)	–	–	434	Dead		433		NA	
Extracellular SE proteins (days after selection)	–	–	533	Dead		532		NA	

the same composition that contained culture filtrate. In addition to an unchallenged control, a medium control consisting of 40% (v/v) Czapek-Dox broth in MM I was also used. The broth was freshly prepared, filter-sterilized, the pH adjusted to 5.8 and then it was added to the medium after autoclaving and cooling. There were seven replicates of 40 ml medium in 125-ml Erlenmeyer flasks for each treatment. After 4 weeks of recurrent selection, embryogenic cultures were transferred to liquid MM I. Cultures that were recovered after continuous challenge with culture filtrate were designated PRL V (Table 1).

Continuous challenge with phytotoxin

Embryogenic cultures (<960 μm) were challenged with 7.5% (v/v) phytotoxin in liquid MM I for 3 weeks with a weekly transfer to fresh medium of the same composition. There were two controls: an unchallenged control and a medium control with 7.5% (v/v) Czapek-Dox extract extracted as the phytotoxin. There were seven 50-ml Erlenmeyer flasks for each treatment, and the total medium volume for each flask was 15 ml. Each flask was inoculated with 500 mg inoculum. Cultures were incubated for 7 days. After 3 weeks, the cultures were transferred to liquid MM I. Cultures that were recovered after continuous selection with phytotoxin were designated phytotoxin-resistant line PRL (Table 1).

Dual culture

Putatively resistant, selected embryogenic cultures and controls of both cultivars were maintained in liquid MM I for 16 weeks after recurrent selection. The procedure for dual culture was adapted from Gentile et al. (1993). Embryogenic cultures were collected on sterile filter paper, and 1.0 g embryogenic culture was inoculated onto semi-solid MM I at opposite sides of a 90×15-mm petri dish, sealed with Parafilm™ and incubated in darkness at $25^\circ \pm 2^\circ$C. After 4 weeks, the center of each petri dish was inoculated with a mycelial plug (5 mm in diameter) from the virulent *C. gloeosporioides* strain that produced the phytotoxin. The petri dishes were resealed with parafilm and incubated at $25^\circ \pm 2^\circ$C. There were five petri dishes for each treatment and the control. After 7 days, the area of the mycelium in each petri dish was determined from the colony diameter. The experiment was repeated twice.

Extraction of extracellular proteins from embryogenic cultures and mature somatic embryos

Extracellular proteins were extracted from the PRLs and unchallenged controls were maintained in liquid MM I for at least 8 months after either recurrent or continuous selection. On day 6 and day 12 after subculture, the embryogenic cultures were transferred to fresh medium, and the spent MM I medium was collected. The cellular debris was removed by low-speed centrifugation (2,000 rpm) for 5 min.

Mature and germinating somatic embryos normally developed from resistant and control cultures 5–7-months after transfer from MM I to MM II. Semi-solid MM II (from three Petri dishes) on which mature somatic embryos had been grown for 1 month before germination was decanted into a sterile beaker. The spent MM II was gently homogenized. An equal volume of sterile, deionized water was added to the growth medium extract, and the mixture was incubated overnight at room temperature with continuous stirring. The slurry thus obtained was filtered through a sterile Whatman #1 filter paper.

Proteins were precipitated overnight from the filtrates with two to three volumes of cold 95% ethanol at -4°C. The proteins were collected by centrifugation at 10,000 rpm for 10 min at 4°C, resuspended in 1 ml of 20 m*M* TRIS-HCl and quantified with bovine albumin as a standard. Protein profiles were less clearly resolved in extracellular proteins isolated 6 days after subculture than after 12 days. Therefore, the latter were used for all analyses.

SDS-PAGE of extracellular protein

SDS PAGE was carried out using 12% (v/v) polyacrylamide gels ($160 \times 180 \times 1.5$ mm) containing 0.1% (w/v) SDS (Laemmli 1970). The stacking gel consisted of 5% (w/v) polyacrylamide and 0.1% (w/v) SDS. Samples consisting of 30 µg of protein from each selected embryogenic culture and the controls were analyzed in a LKB 2001 vertical electrophoresis unit at 100 mA constant current. Samples were diluted with 20 µl of loading buffer containing 400 mg SDS, 2 ml glycerol, 1 ml mercaptoethanol, 4 mg bromophenol blue and 0.125 *M* TRIS-HCl in a total volume of 10 ml and boiled for 10 min before loading. Electrophoresis was carried out in TRIS-Glycine-SDS buffer at pH 8.9. Protein standards (Sigma, St. Louis Mo) were used as molecular weight markers. After electrophoresis, the gels were stained with either Coomassie Brilliant Blue R 250 or a silver-staining kit (Sigma).

Native PAGE and detection of chitinase activity

Polyacrylamide resolving gels (15%, $160 \times 180 \times 1.5$ mm) were prepared by casting a mixture of 15.0 ml of 30% polyacrylamide mixture (acrlamide/Bis 30:0.8), 3.75 ml 3.0 *M* TRIS-HCl (pH 8.8), 600 µl of 10% ammonium presulfate, 9.75 ml sterile sterile deionized water and 20 µl of TEMED. The stacking gel included 2.5 ml of 30% acrylamide mixture, 5.0 ml 500 m*M* TRIS-HCl (pH 6.8), 400 µl of 10% ammonium persulfate, 11.5 ml sterile deionized water and 20 µl of TEMED. Protein samples were diluted with equal volumes of loading buffer (Laemmli 1970) lacking SDS and mercaptoethanol. Electrophoresis was carried out at 10°C at 100 mA constant current in an LKB 2001 vertical electrophoresis unit. After electrophoresis, chitinase activity was detected using an overlay gel containing glycol chitin (Pan et al. 1991). The gels were incubated in 150 m*M* sodium acetate buffer (200 ml/gel) at pH 5.0 for 10 min with gentle shaking. They were then placed on a clean glass plate and covered with a 7.5% polyacrylamide overlay gel ($160 \times 180 \times 0.75$ mm), containing 0.01% (w/v) glycol chitin in 100 m*M* sodium acetate buffer (pH 5.0). After all the air bubbles and any liquid between the gels were eliminated, the gels were incubated at 37°C for 2 h under moist conditions. The overlay gel was separated and incubated in freshly prepared 0.01% (w/v) fluorescent brightener 28 (=Calcofluor White M2R) in 500 m*M* TRIS-HCl (pH 8.9) at room temperature with gentle shaking for 10 min. The brightener solution was discarded, and the overlay gel was stored in sterile deionized water for 2 h at room temperature. Chitinase isozymes were visualized as cleared zones or lytic bands when the gel was placed over a UV transilluminator. The gel was photographed with Polaroid type 55 film with UV haze and 02 orange filters.

Detection of glucanase after native PAGE

Native PAGE was carried out as described above, and the gels were washed with sterile deionized water and incubated in 50 m*M* sodium acetate (pH 5.0) for 5 min. Glucanase detection followed the procedure of Pan et al. (1991). Laminarin (500 mg) was dissolved in 40 ml sterile deionized water by warming in a water bath, cooled to room temperature and mixed with 40 ml 50 m*M* sodium acetate (pH 5.0). The gels were removed from the sodium acetate buffer and incubated in laminarin solution at 40°C for 30 min. They were then fixed in a methanol:water:acetic acid (5:5:2) buffer for 5 min. After thorough washing with sterile deionized water, the gels were stained with 300 mg of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1 *M* NaOH in a boiling water bath. Glucanase isozymes appeared as red bands after approximately 10–12 min. Laminarinases from *Penicillium* spp. and mollusk (Sigma) were used as standards. The gels were photographed immediately.

Conditioned medium

Selected PRLs and control embryogenic cultures were rapidly cooled in liquid nitrogen and macerated under aseptic conditions. Macerated cultures were added to warm MM I (20% w/v) before it solidified, and the modified MM I was decanted into 90×15-mm plastic, disposable petri dishes. After 24 h, a mycelial inoculum was placed at the center of each plate, and the plates were sealed with Parafilm™. The plates were incubated under a 16-h photoperiod in an incubator at $25^{\circ} \pm 2^{\circ}$C. The area of the mycelium was determined from the colony diameter 10 days after inoculation. There were five petri dishes for each treatment, and the experiment was repeated twice.

Statistical analysis

Data were analyzed using the SAS program (SAS Institute 1982). The general linear model was used to compute the regression coefficients, and the means were separated by Duncan's multiple range test.

Results

Recovery of culture filtrate- and phytotoxin-resistant embryogenic cultures

There was an increased mortality of embryogenic cultures that were recurrently selected with increasing concentrations of culture filtrate. Significant differences in the mortality of embryogenic cultures were observed between each concentration of culture filtrate during the first challenge with both cultivars (Tables 2 and 3). Increased resistance to culture filtrate and phytotoxin was observed after each challenge with both cultivars (Tables 2 and 3). Few embryogenic cultures survived exposure to 75% culture filtrate without a prior challenge with a lower concentration of culture filtrate. None of the cultures survived more than four recurrent selections, and visual readings of percentage survival could not be made after two recurrent selections due to extensive necrosis of the tissue.

Dual culture

PRL I did not inhibit growth of *C. gloeosporioides* in dual culture; however, PRL III, PRL V and PRL of both cultivars significantly inhibited growth of the fungus (Table 4). Inhibition of mycelium growth increased in relation to length of exposure to selection pressure.

Table 2 Frequency of recovery of resistant embryogenic 'Hindi' cultures after challenge and selection with *C. gloeosporioides* culture filtrate[a,b]

Challenge 1 (% culture filtrate)	Resistance (%)	Challenge 2 (% culture filtrate)	Resistance (%)
0	81.7 ± 2.3a	0	76.2 ± 1.1a
10	55.1 ± 1.8b	20	64.7 ± 1.5b
20	43.9 ± 2.1c	40	62.0 ± 0.9b
40	30.2 ± 1.2d	60	52.7 ± 1.2c
60	15.6 ± 1.1e	75	1.5 ± 1.5d
75	0.0	–	–
Regression coefficient	0.938		0.991

[a] Means are transformed values ± standard error
[b] Means with same letter in a column do not differ by Duncan's multiple range test at $P = 0.05$

Table 3 Frequency of recovery of resistant embryogenic 'Carabao' cultures after challenge and selection with *C. gloeosporioides* culture filtrate[a,b]

Challenge 1 (% culture filtrate)	Resistance (%)	Challenge 2 (% culture filtrate)	Resistance (%)
0	77.2 ± 1.5a	0	81.6 ± 0.9a
10	53.3 ± 1.5b	20	46.2 ± 1.6b
20	42.9 ± 1.2c	40	36.9 ± 1.1c
40	33.1 ± 0.8	60	26.5 ± 1.2d
60	11.6 ± 1.7	75	8.4 ± 2.3e
75	0.0	–	–
Regression coefficient	0.979		0.985

[a] Means are transformed values ± standard error
[b] Means with same letter in a column do not differ by Duncan's multiple range test at $P = 0.05$

Table 4 *Colletotrichum gloeosporioides* mycelium area in dual culture with resistant lines of 'Hindi' and 'Carabao'. Embryogenic cultures were maintained in liquid MMI for 20 weeks after in vitro selection

Treatment	Hindi[a,b]	Carabao[a,b]
Control	39.3 ± 3.4a	41.5 ± 2.1a
PRL I	41.4 ± 3.3a	39.4 ± 1.2a
PRL II	33.5 ± 1.9b	36.2 ± 1.5b
PRL III	8.6 ± 1.4c	10.2 ± 1.2c
PRL V	8.6 ± 1.0c	10.9 ± 0.3c
PRL	8.5 ± 2.3c	8.9 ± 0.8c
Regression coefficient	0.940	0.971

[a] Mean area of fungal colony in square centimeters ± standard error 7 days after inoculation of medium
[b] Means area with same letter do not differ by Duncan's multiple range test at $P = 0.05$

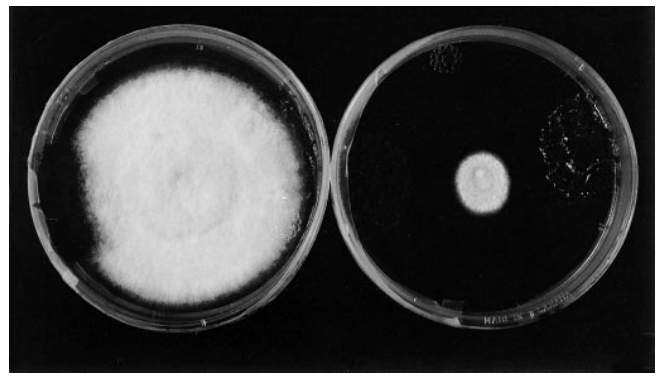


Fig. 1 Dual culture of *C. gloeosporioides* with control culture (*left*) and 'Hindi' culture PRL III (*right*), showing mycelium growth inhibition in the selected culture

With the controls and less resistant embryogenic cultures, mycelium growth was not inhibited (Fig. 1). There was no significant difference between the responses of the two cultivars. The high regression coefficients of both cultivars indicated that increased mycelium growth inhibition was associated with the number of recurrent selections.

Conditioned medium

Fungal growth was not inhibited on MM I containing macerated resistant embryogenic cultures (Table 5). No significant differences were observed between treatments containing macerated selected embryogenic cultures and the unchallenged controls. There were also no significant differences between the cultivars. The very low regression coefficients with 'Hindi' ($r^2 = 0.084$) and 'Carabao' ($r^2 = 0.137$) confirm the absence of any effect of the macerated cultures on fungal growth.

Extracellular proteins

There was no significant difference between unchallenged controls and resistant lines with respect to extracellular protein content 6 days after subculture; however, significant differences were observed between resistant embryogenic cultures and unchallenged controls 12 days after subculture (Table 6). The unchallanged controls of 'Carabao' yielded the lowest amount of protein 6 and 12 days after subculture (18.0 and 20.2 μg/ml, respectively), while PRL III of 'Hindi' and PRL V of 'Carabao' yielded the greatest amount of protein 12 days after subculture (38.3 and 37.8 μg/ml, respectively).

There were differences in the protein profiles following SDS and PAGE between recurrently selected embryogenic cultures and unchallenged controls and

Table 5 *Colletotrichum gloeosporioides* mycelium area in conditioned MMI containing 20% (w/v) macerated resistant embryogenic cultures. Embryogenic cultures were maintained in liquid MMI for 20 weeks after in vitro selection

Treatment	Hindi[a,b]	Carabao[a,b]
Control	41.6 ± 1.1a	40.6 ± 0.6a
PRL I	39.9 ± 1.3a	39.8 ± 1.0a
PRL II	40.4 ± 1.0a	39.5 ± 0.6
PRL III	40.7 ± 1.1a	40.0 ± 0.5a
PRL V	40.2 ± 0.6a	41.5 ± 1.2a
Regression coefficient	0.084	0.137

[a] Mean area in square centimeters of fungal mycelium ± standard error 10 days after inoculation of conditioned medium
[b] Means with same area do not differ by Duncan's multiple range test at $P = 0.05$

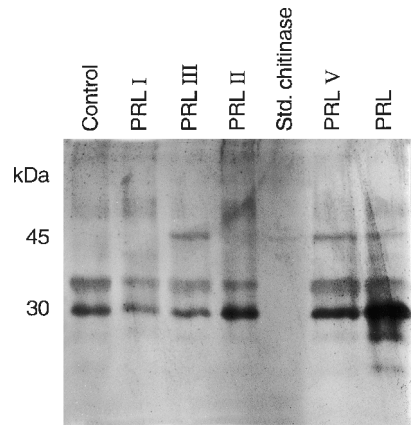


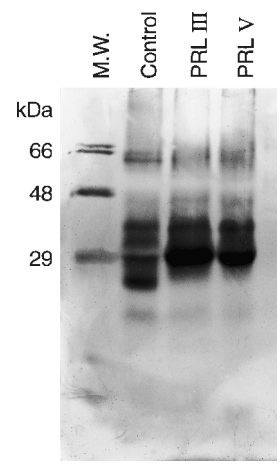
Fig. 2 SDS PAGE separation of extracellular proteins from the spent medium of various selected 'Hindi' cultures. *Lanes: 1* Control, *2* PRL I, *3* PRL III, *4* PRL II, *5* standard chitinase, *6* PRL V, *7* PRL

Table 6 Extracellular protein content (μg/ml) in spent MMI of resistant embryogenic cultures of 'Hindi' and 'Carabao' 6 and 12 days after culture (DAC). Embryogenic cultures were maintained for 8 months in MMI after selection

Treatment	Hindi[a,b]		Carabao[a,b]	
	6 DAC	12 DAC	6 DAC	12 DAC
Control	18.3 ± 0.2a	20.5 ± 1.1a	18.0 ± 0.0a	20.2 ± 0.1a
PRL I	18.4 ± 0.1a	20.4 ± 0.8a	19.0 ± 0.0a	21.7 ± 0.2a
PRL II	18.3 ± 1.2a	27.3 ± 0.3b	18.9 ± 0.1a	26.3 ± 0.2b
PRL III	18.3 ± 0.2a	38.3 ± 0.2c	19.1 ± 0.1a	29.0 ± 0.4b
PRL V	18.7 ± 0.2a	30.6 ± 0.4b	19.0 ± 0.0a	37.8 ± 0.5c
PRL	18.7 ± 0.2a	30.5 ± 0.3b	18.9 ± 0.1a	29.5 ± 0.9b
Regression coefficient	0.331	0.982	0.268	0.996

[a] Mean protein content ± standard error
[b] Means with same letter do not differ by Duncan's multiple range test at $P = 0.05$

Fig. 3 SDS PAGE separation of extracellular proteins from the spent medium of 'Carabao'. *Lanes: 1* Molecular weight marker, *2* control, *3* PRL III, *4* PRL V



between cultivars. With 'Hindi', there were two prominent bands of 31 and 33 kDa in all challenged cultures and their controls (Fig. 2); however, expression of the 31-kDa band increased with respect to the number of challenges. The phytotoxin-resistant lines also expressed this band strongly. In addition, a band of approximately 45 kDa was expressed by the most resistant selected embryogenic cultures and co-migrated with one of the bands produced by chitinase from *Serratia marescens*.

The extracellular protein profile of 'Carabao' was different from that of 'Hindi', with unchallenged controls exhibiting a dense band of approximately 27 kDa, which was only weakly expressed by the resistant selected cultures (Fig. 3). A band of approximately 32 kDa was strongly expressed by the PRLs in comparison with the unchallenged control. A 45-kDa band was also weakly expressed by PRL V and PRL.

Chitinase and β-1,3-glucanase activities associated with embryogenic cultures

Selection of embryogenic cultures with culture filtrate or phytotoxin resulted in enhanced activity of chitinase isozymes and/or induction of new chitinase isozymes in the extracellular proteins. With 'Hindi', there were two chitinase isozymes of approximately 30 and 32 kDa that were associated with the unchallenged controls, PRL, PRL III and PRL V (Fig. 4). The intensity of hydrolyzation was greater in PRLs than in the controls. There was an additional isozyme of approximately 45 kDa associated with PRL, PRL III and PRL V which had greater hydrolytic activity than the isozymes at 30 and 32 kDa.

With 'Carabao', the selected embryogenic lines exhibited a chitinase isozyme at 27 kDa that showed stronger activity with PRL V and PRL and an isozyme of approximately 33 kDa that was weakly expressed (Fig. 5). PRL V and PRL had a weakly lytic zone at 45 kDa. PRL III exhibited a weakly expressed band of

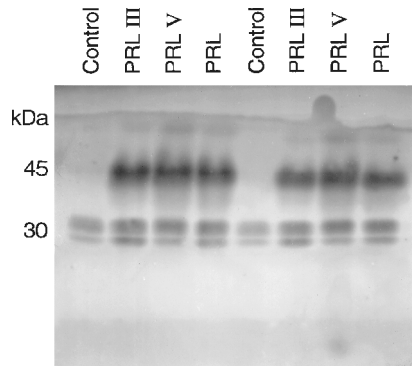


Fig. 4 Chitinase activity associated with extracellular protein of selected embryogenic 'Hindi' cultures in a glycol chitin overlay gel following native PAGE separation. *Lanes: 1* Control, *2* PRL III, *3* PRL V, *4* PRL

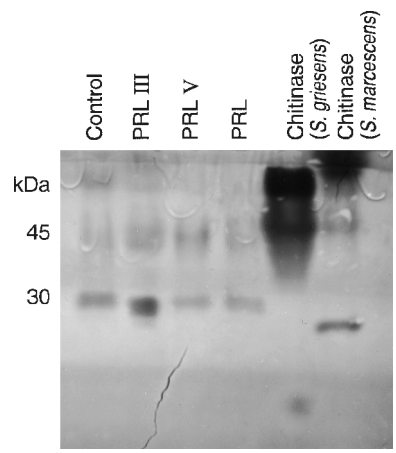


Fig. 5 Chitinase activity associated with extracellular protein of selected 'Carabao' cultures in a chitin overlay gel following native PAGE separation. *Lanes: 1* Control, *2* PRL III, *3* PRL V, *4* PRL, *5*, *6* chitinase standards

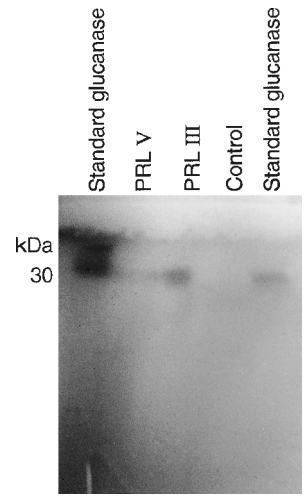
approximately 25 kDa that was not present in any other selected embryogenic culture.

Enhanced glucanase activity was also observed in the extracellular proteins of resistant 'Hindi' embryogenic cultures relative to the unchallenged control (Fig. 6). Only a single isozyme of glucanase was present (ca. 30 kDa), and this band in PRL III showed the greatest activity. In contrast, glucanase activity in the extracellular fractions of 'Carabao' was very weak. All of the 'Carabao' resistant embryogenic cultures demonstrated a glucanase isozyme of approximately 30 kDa; however, PRL V of 'Carabao' also showed a band of approximately 25 kDa.

Chitinase activity associated with mature somatic embryos

Somatic embryo maturation and germination occurred normally from embryogenic cultures of PRL III and PRL V as well as from the control cultures of both cultivars. Somatic embryos germinated approximately 18 months after in vitro selection. Somatic embryo germination and recovery of plantlets are described in Table 7. The assay of chitinase in the spent MM II indicated that there was enhanced expression of all isozymes of the resistant embryogenic cultures in comparison with the controls of both cultivars (Fig. 7), indicating that the resistance responses are stable and not epigenetic.

Fig. 6 Glucanase activity associated with extracellular protein of selected 'Hindi' embryogenic cultures in a laminarin overlay gel following native PAGE separation. Lanes*: 1* Standard glucanase, *2* control, *3* PRL III, *4* PRL V, *5* standard



Discussion

Extracellular PR proteins, i.e., chitinase and β-1,3-glucanase, are associated with mango embryogenic cultures and regenerants that have resulted from in vitro selection with the culture filtrate and phytotoxin of *C. gloeosporioides*. Inhibition of mycelial growth in vitro was correlated with the number of recurrent selections, although at least three recurrent selections were necessary to induce significant levels of resistance to the fungal pathogen in the embryogenic cultures.

Secretion of extracellular proteins by embryogenic cultures of several plant species has been reported (Ohashi and Matsuoka 1987; Esaka et al. 1990; Kragh et al. 1991; Gavish et al. 1992; Gentile et al. 1993; von Arnold et al. 1995). Extracellular secretion of PR proteins, i.e., chitinase and glucanase, has been associated with salt stress (Esaka et al. 1994), culture stress (Flach et al. 1993) and pathogen attack (Lamb et al. 1992; Cabello et al. 1994; Dixon et al. 1994). Wink (1994) suggested that secretion of hydrolases resulting from stress in liquid culture medium could be analogous to extracellular secretion of these enzymes following pathogen attack. The current study provides evidence that PR proteins are associated with the defense response of mango that confers resistance to infection by *C. gloeosporioides*. Gentile et al. (1993) also observed

Table 7 Somatic embryo (SE) recovery and plantlet formation from selected embryogenic cultures of 'Hindi' and 'Carabao'

	Hindi			Carabao		
	Number of SE recovered	Number of germinated SE	Number of plantlets	Number of SE recovered	Number of germinated SE	Number of plantlets
Control	345	320	9	176	154	7
PRL III	328	306	11	203	181	9
PRL V	276	243	7	155	132	4
TOTAL	949	869	27	534	467	20

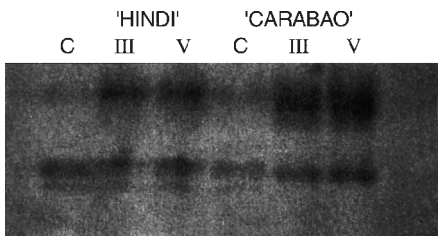


Fig. 7 Chitinase activity associated with extracellular protein of mature and germinating 'Hindi' and 'Carabao' somatic embryos in a chitin overlay gel following native PAGE separation. *Lanes: 1* 'Hindi' Control, *2* 'Hindi' PRL III, *3* 'Hindi' PRL: V: *4* 'Carabao' control, *5* 'Carabao' PRL III, *6* 'Carabao' PRL V

a correlation between enhanced release of PR proteins and host resistance of lemon to infection by *Phoma tracheiphila*.

The extracellular chitinase and glucanase in the current study and in the reports cited above were acidic. In contrast, Kragh et al. (1991) identified three basic chitinases and one basic glucanase in the spent medium of suspension cultures of barely. Basic chitinases and glucanases are localized intracellularly (Melchers et al. 1993). Joosten et al. (1995) reported that extracellular isoforms in tomato are not antifungal, either alone or in combination; however, there is also strong agreement among several published reports that chitinase and glucanase act synergistically in the plant defense response. Extracellular isozymes are implicated in the early line of defense in plants (Collinge et al. 1993), where they can function directly by blocking the growth of fungal hyphae or indirectly by signaling the release of other defensive mechanisms in the host, i.e., elicitors (Barber et al. 1989).

The secretion of PR proteins into the culture medium by mature and germinating mango somatic embryos of the selected resistant embryogenic cultures more than 18 months after the cessation of selection pressure indicates that the expression of chitinase and glucanase are stable. Somaclonal variants are known to be associated with in vitro culture (Lee and Phillips, 1989), and one or more mutations might have affected the promoter regions of the genes for these PR proteins, as observed by Roby et al. (1990), resulting in the activation of these genes. In vitro selection for recovery of mango embryogenic cultures and somatic embryos that are resistant to *C. gloeosporioides* culture filtrate and phytotoxin could be an alternative to genetic transformation for enhancing resistance to anthracnose in important clonal selections of this important perennial fruit crop.

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