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AVOCADO ROOTSTOCK RESISTANT TO *PHYTOPHTHORA* ROOT ROT DISEASE IN CELL CULTURES

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Executive Summary

Root rot disease is still the most devastating disease for most of the avocado growing regions The main objective of the present project was to expand the resistance genetic sources by development of *in vitro* methods for identification and selection of clones resistant to *Phytophthora cinnamomi* and by collecting and screening new avocado strains throughout Philippine wild and avocado growing areas

To meet the mentioned objectives, the project has accomplished the following highlights

1 Tissue cultures were developed from 8 species and varieties of *Persea,* ranging in their resistance to *P cinnamomi* and plantlets were regenerated through somatic embryogenesis †Embryogenesis ranging from 33 to 80% was observed in calli initially cultured on media with BSM + inoculated roots Whole calli obtained from treatments with crude filtrate showed embryogenesis of 33 to 76% Some embryogenic calli obtained from treatments with *P cinnamomi* showed continuous growth as indicated by the increase in the number of callus culture Most embryogenic calli showed differentiation of shoot-like structure This part of the project was summarized as a joint article of both research groups and was published in Plant Tissue Culture and Biotechnology, 4 196-206

2 The question of how much the tissue cultures can truly represent the intact plants, in respect to resistance to *P cinnamomi*, has been addressed A significant correlation was found between the resistance of the plant sources and the response of calli following inoculation with the pathogen

3 Two types of reactions were characterized Necrosis development and peroxidase activity The visual assessment of necrosis was significant correlated with surface colour detection of callus as defined by the 'L' parameter with a Minolta chromameter and with spectroscopic determination of the colour of the extract The resistant originated calli expressed higher peroxidase specific activity than the susceptible ones, which was in significant correlation with the necrosis development This part of the work has been submitted for publication to Plant Cell Reports

4 The consequence of the positive *in vivo / in vitro* correlation is that tissue cultures can be reliably exploited for screening and selection of new resistant avocado clones Actually, a new clone of avocado was identified as a moderate resistant by using the developed *in vitro* system

5 Two major components of the necrosis-inducing factor in the crude filtrates of *P cinnamomi* are the cell wall degrading enzyme polygalacturonase and low MW compounds High activity of polygalacturonase was evident as early as two days after start of culture and increased afterwards This suggests an important role for polygalaturonase in the pathogenesis of avocado by *P cinnamomi* We are still in the process of isolating the components of the low MW fraction that causes tissue necrosis This component is also heat stable as heat-treated

fractions still cause necrosis of callus tissues The isolated components might be used as a selecting pressure for *in vitro* screening and selection of new resistant germplasm

6 Thirty four avocado strains were collected from in 6 locations throughout Philippine various sites, characterized for fruit qualities and screened for resistance to *Phytophthora* root rot Six strains were found to be apparently resistant to *P cinnamomi* under field conditions These strains will be continuously examined for their commercial potential for being introduced in avocado orchards An efficient and reliable screening method of identifying root rot resistant material was developed This method used detached seedlings roots that were inoculated with *P cinnamomi* and followed for their reaction

In conclusion tissue culture system was proved to be reliable for being genetic source of resistance to *P cinnamomi* Methods for identification of resistant calli were developed Inducing necrosis components were identified in the pathogen s culture These might be used for selecting agent of resistant clones First promising steps for plantlet regeneration from undifferentiated tissue cultures were achieved Six avocado strains were selected from root rot infected area of Philippines, and one from the *in vitro* screening The progress achieved in the present project enable using the tissue culture for further intensive selection of resistance individuals through somaclonal variation or mutagenesis, or for rapid detection of new collections from infested fields

Just from looking on the long list of the Philippine research team it is evident that researchers and other scientific staff from various scientific disciplines (such as, tissue culture, plant breeding, pathology, plant physiology, biotechnology and biochemistry) took part in this project There is no doubt that this project improves significantly the professional skills of all the project s participants

Research Objectives

Root rot, caused by *Phytophthora cinnamomi* Rands is the most destructive disease of avocado (*Persea americana* Mill) world-wide including many developing countries in South and Central America, Africa, the Caribbean Islands, and in Asia Chemical control and use of resistant rootstocks are the logical solutions to the problem but these approaches are beset with limitations Owing to the outcrossing habit and perennial nature of avocado, conventional methods of identifying resistant rootstocks are a problem Use of chemicals is a secondary choice because of detrimental effects to human health and the environment

This project aimed to exploit the use of *in vitro* culture to induce resistance in avocado to *P cinnamomi* for production of resistant rootstocks suitable to the crop and particular field conditions

To achieve the overall goal the following studies were undertaken

1 Collection, evaluation and characterization of avocado strains from various parts of the Philippines and from Israel to identify possible sources of root rot resistant rootstocks (Philippines),

2 Establishment of the *in vitro* culture and regeneration system for different avocado species (Israel and Philippines),

3 Development of appropriate screening methods that will correlate *in vitro* resistance with the intact plant response (Israel and Philippines),

4 Partial purification and characterization of necrosis-inducing factor from culture filtrate of *P cinnamomi* for use in the induction of resistance (Philippines),

5 Induction of resistance by somaclonal variation and *in vitro* directed selection and analysis of putative variants (Israel and Philippines)

The project has reached applied and scientific achievements Approximately 7 avocado strains have been identified to be potentially resistant to *P cinnamomi* These strains would have been tested under field conditions The *in vitro* selection system has been developed This system could be almost readily used for carrying out the screening and the selection experiments Avocado is very difficult species for regeneration Successful protocols have been developed resulting plantlets regeneration, however the efficiency of this procedure should be improved The developed cell and callus cultures could be also exploit for genetic engineering and transforming resistance genes to distinguished avocado rootstocks

Methods and Results

The Philippine team

1 0 Collection, Evaluation and Characterization of Avocado Strains

1 1 Objectives

1 To select avocado strains with good horticultural characteristics and resistance to root rot
2 To evaluate fruit qualities of various avocado strains
3 To provide the project with materials needed for screening work

1 2 Introduction

Avocado was first introduced in the Philippines in the early 1890's However, despite its very early introduction it has not gained the popularity enjoyed by other fruits It is grown in almost all parts of the country as a backyard fruit crop but the leading regions are Southern Tagalog, Bicol, Western Visayas, Western Mindanao and Ilocos (Coronel, 1983) It is considered as one of the most promising fruit crops in the Philippines because it is very nutritious It contains high moisture, fat, Vitamin A and calories The avocado oil is highly digestible and is one of the most valuable of the unsaturated fatty acids Moreover, the avocado oils is reported to be the fifth of the most desirable oil known as anti-cholesterol agents which counteract the effect of hard, saturated animal fat (Pierce, 1959) The fruit is well liked for its nutlike flavor and buttery consistency The fruit is usually not eaten alone but always in combination with sugar and milk as dessert It is also used commercially as flavoring for ice cream

In view of the lack of a formal breeding program for avocado in the Philippines, this study aimed to collect, evaluate and characterize avocado from different places In addition to horticultural tracts, the collection was also screened for resistance against *P cinnamomi*

1 3 Highlights Of Accomplishment

On-site selection and evaluation of different avocado strains possessing good horticultural characteristics (e g prolific bearer) and apparent resistance to *Phytophthora* root rot were conducted in various avocado - growing areas where there is high diversity of the crops The different avocado strains were collected in the following places 1) Balete, Batangas, 2) Talisay Batangas, 3) Tagaytay City, 4) Los Baños, Laguna, 5) Cebu City and 6) Davao City Owners were interviewed regarding relevant information about the tree These include the age of the tree, infestation of pests and diseases, bearing habit and other organoleptic characteristics Five to ten fruit samples were taken randomly from each selected trees and samples were brought to the laboratory Fruit quality evaluation was done on ripened fruits while immature fruits were also collected and supplied to the plant tissue culturists

A total of thirty-four (34) avocado strains selected on-site for specific characteristics were evaluated for fruit qualities (Table 1 1) Out of these strains, four (4) were found to be promising selections in terms of fruit quality These selections are medium- to big-fruited, had thick flesh and high edible portion In addition, they had superior organoleptic characters such as nutty and buttery taste, non-fibrous and absence of unpleasant flavors

Table 1 1 Thirty four avocado strains collected on-site and their important fruit characteristics These strains were used in fruit evaluation and screening for resistance to root rot The promising selections in terms of fruit qualities were indicated by asterisk (*)

Strain	Fruit Weight (g)	Flesh Thickness (cm)	% Edible Portion
Balete 1	319 59	1 52	70 60
Balete 2	294 08	1 37	67 21
Balete 3	192 26	0 73	51 07
Balete 4	250 55	1 38	64 91
Balete 5	409 79	1 77	72 66
Balete 6	208 54	1 22	58 29
Balete 7	305 69	1 29	70 22
Balete 8	302 92	1 40	63 28
Balete 9*	290 18	1 35	72 13
Balete 10	336 91	1 37	69 80
Balete 12	355 20	1 30	54 12
Balete 13	204 95	1 15	69 37
Balete 14	252 67	1 19	73 29
Balete 15	187 69	1 05	56 73
Matina 1*	294 54	1 21	70 70
Matina 2	262 62	1 19	63 84
Toril 1	584 33	2 50	78 63
Toril 2	413 55	1 54	70 50
Toril 3	354 32	2 00	72 76
Toril 4	306 28	1 57	75 91
MIT 1	322 65	1 73	63 72
MIT 2	320 35	1 54	72 32
Bayog 1	383 37	2 11	83 34
Bayog 2	541 30	2 31	81 85
Cebu 1	161 06	1 37	70 46
De Leon No 1*	342 98	1 63	68 94
Calma	419 19	1 58	70 76
Cardinal	326 75	1 46	75 23
227*	449 28	1 70	79 82
240	451 69	1 93	78 75
Magnetic Hill	268 45	1 53	68 41
Villegas	456 20	2 09	76 46
Coronel	343 60	1 92	77 27
R3H5	310 71	1 71	73 80

Six (6) other avocado strains were found to be resistant to Phytophthora root rot under field conditions They were Tranca, Jolo, De Leon, Cardinal, Quarry 5 and Semile (Table 1 2) The strain Tranca was derived from scion collected from a tree that survived from root rot infestation from a field of more than 50 trees Additional accessions were obtained from Taiwan and Israel These strains and accessions were asexually propagated by grafting and are being maintained in the screenhouse

Table 1 2 Potential root rot resistant avocado strains

Strain	Number of Grafted Materials
Tranca	99
Jolo	8
De Leon	4
Cardinal	1
Quarry 5	4
Semile	5

(These strains were propagated and the materials are being maintained in the screenhouse These strains were observed to be apparently resistant to root rot under field conditions)

Collection trips were again conducted in nearby avocado-growing areas of Batangas and Cavite Seeds were collected, allowed to germinate and the seedlings are now being maintained in the glasshouse The roots from these seedlings are being used in the screening for *Phytophthora* root rot resistance

Screening work for resistance to *Phytophthora* root rot requires techniques which must not kill the plant, or must leave a clone, since the tissue culture group of this project needs susceptible plants for induction of resistance Furthermore, replicated experiments for screening works are necessary for verification The current protocol uses detached roots However, the use of intact roots for screening must be done since there may be a big difference between detached and intact roots Intact roots still have their vascular system active which could absorb the toxin Marcotting is therefore a useful technique in producing clones for screening work

Marcotting of avocado plants utilizes existing procedures The growth regulator, indole butyric acid or IBA (50 ppm) is used to induce root growth Several plants were marcotted and roots are allowed to develop which would take about three months with about 10% success To induce development of branches for marcotting, plants were planted in the field (Figs 1 1 and 1 2) These plants are to be marcotted as soon as branches become available

SUMMARY

Four out of the 34 avocado strains collected on-site were found to be promising selections in terms of fruit qualities These selections were Balete 9, Matina 1, De Leon No 1 and 227 strain They were medium- to big-fruited, thick flesh, high edible portion and had superior organoleptic characters like nutty and buttery taste, non-fibrous and absence of unpleasant flavors

Screening for *Phytophthora* root rot resistance was undertaken with the Plant Pathology group Collection trips were conducted in nearby avocado-growing areas Seeds were collected, allowed to germinate in the glasshouse and the roots from the seedlings were used to screen for *Phytophthora* root rot resistance Six avocado strains were found to be apparently resistant to *Phytophthora* root rot under field conditions These were Tranca, Jolo, De Leon, Cardinal, Quarry 5 and Semile

The promising selections as well as apparently resistant strains to Phytophthora root rot were asexually propagated by either grafting or marcotting and are being maintained in the screenhouse prior to field-planting in Pasong Kipot, Tranca, Bay, Laguna

O D1-1	O B-24	O VNV	
O Semil	O M1T2-5	O Batangas strain	
O Batangas strain	O M1T2-3	O Batangas strain	
O De Leon TS 7	O B 52	O Batangas strain	O Batangas strain
O Quarry 5	O D1-2		O Batangas strain
O Batangas strain	O B 94		O Batangas strain
O Jolo	O Semil		O Batangas strain
O Cardinal Talisay 4	O Batangas strain	O Batangas strain	O Batangas strain
O De Leon TS 7	O D1-3	O Quarry 5	O Batangas strain

Dirt Road

Guava Area (PK 1)

Figure 1 1 Layout of the avocado area in Tranca, Bay Laguna where branches are being allowed to develop for marcotting of the plants

Figure 1 2 Promising avocado strains like Batangas strain (A) and D1-1 strain (B) collected on-site and are being planted in Tranca, Bay, Laguna

2 0 Establishment of the *in vitro* culture and regeneration system for different avocado species

2.1 Introduction

Various types of avocado tissue cultures (*Persea americana* Mill) have been developed and utilized advantageously for several distinct purposes The avocado *in vitro* system, being aseptic and fully controlled, has been used to study cytokinins biosynthesis (Blumenfeld and Gazit, 1971), and the physiological response of flower buds to environmental conditions (Schroeder, 1975a) The high proliferation capacity of shoot cultures has been utilized for efficient micropropagation of horticulturally important clonal varieties of avocado and other *Persea* species (Barringer et al , 1996) *P schiedeana and P americana* cv Duke 7 were micropropagated as rootstocks resistant to the root rot disease in avocado orchards (Cooper, 1987, Gonzales-Rosas et al, 1985, Harty, 1985) Parameters such as calli viability, stem leaf and axillary buds elongation (Young, 1983), and morphogenesis capacity (Pliego-Alfaro et al , 1987) were characterized for optimization of *in vitro* shoot propagation (Schroeder, 1975b, Schall, 1987) The rapid *in vitro* propagation of the vegetative material was also utilized for elimination of viroid-like organisms causing sunblotch disease (Nel and Kotze, 1982)

Shoot cultures of *P indica*, a highly susceptible host of the root rot pathogen *P cinnamomi*, were used to study the mode of pathogenic of the indicated disease under controlled conditions (Nel et al , 1982, 1983, Nel and Kotze, 1982)

Avocado callusing and morphogenesis were done mostly by using the MS basal medium (Murashige and Skoog, 1962), enriched with sucrose, vitamins and combinations of cytokinins and auxins (Barringer et al , 1996, Gonzales-Rosas et al , 1985, Mooney and VanStaden, 1987, Nel et al, 1983, Pliego-Alfaro and Murashige, 1988, Pliego-Alfaro et al , 1987, Pruski et al , 1996, Schall, 1987) However, other media such as WPM (woody plant medium, Lloyd and McGown, 1980, Cooper, 1987), Anderson mineral salt and vitamins (Young, 1983) and Dixon and Fuller medium (Harty, 1985) have also been used for *Persea in vitro* callusing and micropropagation

Embryogenic avocado callus has been developed from immature zygotic embryos as explants, on MS medium containing the auxin picloram (Mooney and VanStaden, 1987, Pliego-Alfaro and Murashige, 1988) However, the capability for *in vitro* regeneration of many woody species, including avocado, is restricted and seems to be limited to juvenile material (Pliego-Alfaro et al, 1987) In fact, regeneration of a complete and normal plant from callus tissue of avocado on a large scale has not yet been achieved Only a small percentage of green shoots and normal leafy plantlets was regenerated (Mooney and VanStaden, 1987, Pliego-Alfaro and Murashige, 1988)

2 2 Materials and Methods

2 2 1 Explant Source and Surface Sterilization

Calli from different *Persea* species (*P americana* -avocado genotypes/cv/accessions, *P burbonia* and *P cinarescens*) were induced from mature/immature cotyledon/seed, leaves, stems, petiole, midrib or roots obtained from greenhouse or field grown plants Surface

sterilization of explants varied depending on developmental stage or plant parts Mature and immature fruits collected from field grown trees were soaked in detergent, rinsed in running tap and surface sterilized in pure bleach for 20 min Cotyledons were excised aseptically and cut into 10 mm sections The embryo portion was separated and germinated *in vitro* The leaves, stem, petiole and roots from *in vitro* germinated seedlings were used for callus induction

For stem and leaf explants, healthy shoots with fully expanded leaves were used Leaves, stem and petioles were separated, soaked in detergent, rinsed with tap water, soaked in 70% ethyl alcohol for 10 min and surface sterilized with 40% commercial bleach for 10-20 min followed by 10% for 10 min and rinsed 5x with sterile distilled water Stem, midrib and petiole and leaf explants were cut into 5 to 10 mm sections

2 2 2 Callus Induction

The medium for callus induction contained Murashige and Skoogs (MS) basal medium supplemented with 30 g/L sugar, 5 mg/L 2,4-D and 0 5 mg/L BAP (designated as M14 medium) Maintenance of callus growth was achieved using the callus induction medium (M14), MS + 2 mg/L BAP + 1 mg/L IBA (BA_2 medium) or MS + 5 mg/L NAA + 1 mg/L kinetin (Ki medium)

All cultures (callus initiation and maintenance) were maintained in an airconditioned room (25±2°C) under 16h light Callus growth was observed 4 to 8 weeks after culture Calli were subcultured onto fresh maintenance medium (M14 or BA_2, or Ki medium) every 4 to 6 weeks

2 2 3 Somatic Embryogenesis

The effect of different growth regulator combinations and the influence of different types of calli were studied to increase the efficiency of embryogenesis and somatic embryo regeneration

Yellow friable calli (about 5 mm/clump) were cultured onto MS medium with different combination of BAP (0, 1, 2 and 4 mg/L) and IBA (0, 1, 1 5 and 2 0 mg/L) The best embryogenesis medium (MS +0 1 mg/L TDZ + 0 5 mg/L GA3, designated as RA_3) developed in Activity 1 was included as the control media treatment Callus regrowth and occurrence of embryogenesis were taken after 3 weeks in culture

The response of different types of calli produced in Experiment 1 upon culture onto MS + 2 mg/L BAP + 1 mg/L IBA (best medium designated as BA_2 medium) was studied to determine the best type of callus material for embryogenesis and shoot differentiation Small pieces (5 mm) of yellow friable, green compact and yellow compact calli were cultured onto BA_2 medium Callus regrowth, embryogenesis and shoot differentiation were taken after 3 weeks of culture

The occurrence of embryogenesis, differentiation of shoots and subsequent growth of regenerated plantlets were compared using the embryogenesis protocols developed in Protocol 1 used globular to cotyledon stage embryos, transferred to maturation medium and then regeneration medium using RA_3 medium Protocol 2 used globular to cotyledon stage

embryos without undergoing maturation, transferred to RA_3 regeneration medium Protocol 3 used embryogenic calli (yellow friable to yellow compact calli) cultured onto BA_2 medium Shoot differentiation was assessed after 3 weeks in culture

2 3 Results and Discussion

2 3 1 Factors Affecting Somatic Embryogenesis and Regeneration of Avocado

The effects of different growth regulator combinations and the influence of different types of calli were studied to increase the efficiency of embryogenesis and somatic embryo regeneration

2 3 1 1 Effects of BAP and IBA combination in enhancing embryogenesis

Five different types of calli (yellow friable, white fine friable, yellow to creamy compact, green compact and brown calli) were produced in all media treatments with or without BAP and IBA (Table 2 1, Fig 2 1) The combination of 2 mg/L BAP and 1 mg/L IBA gave the highest embryogenesis (40%), higher (30%) than the control medium (RA_3) Other treatment combinations gave the same level of embryogenic calli as the MS medium alone Secondary somatic embryogenesis occurred very efficiently in avocado, as in the case of Vetis (Krull and Warley, 1977, Gray, 1987) in medium with or without auxin

2 3 1 2 Regrowth and embryogenesis initiated from different types of calli

Yellow friable calli upon culture onto BA_2 medium produced 6 types of callus regrowth, 4 types being embryogenic The most abundant and highest embryogenic regrowth was observed with yellow friable calli (Table 2 2A) This type of regrowth also gave the highest shoot differentiation The green compact calli upon culture onto BA2 medium produced 3 types of regrowth, 2 types being highly embryogenic (Table 2 2B) The yellow friable calli showed the highest embryogenesis and shoot differentiation The light yellow friable calli upon culture onto BA2 medium produced 4 types of embryogenic callus regrowth The white fine calli showed the highest embryogenesis (Table 2 2C)

Results of this experiment showed that the occurrence of embryogenesis and shoot differentiation is greatly influenced by the type of callus used in subculture The yellow friable calli gave the most embryogenic regrowth followed by the yellow compact calli and green compact calli Differentiation of shoot-like structures was also highest using the yellow friable calli These calli regenerated 1-5 shoots/callus clump

The growth of embryogenic calli was very fast and calli have to transferred onto fresh medium every 3-4 weeks Calli left in culture for more than 6 weeks turned brown and died In some instances, brown calli also produced embryogenic callus regrowth upon subculture onto fresh medium (Fig 2 1D)

Table 2 1 Effect of BAP and IBA supplemented onto MS medium on callus regrowth and embryogenesis after 3 weeks in culture

Treatment	Callus regrowth (% cultures)				
	Golden yellow friable calli	white fine friable calli	creamy compact calli	green compact calli	Embryogenic calli
MS alone	20	20	10	20	20
MS +0 BAP + 1 0 mg/L IBA	20	30	20	0	10
MS +0 BAP + 1 5 mg/L IBA	30	20	20	10	0
MS +0 BAP+2 0 mg/L IBA	10	20	10	20	10
MS +1 0 mg/L BAP + 0 mg/L IBA	10	40	0	20	20
MS +1 0 mg/L BAP + 1 0 mg/L IBA	20	0	20	20	10
MS +1 0 mg/L BAP + 1 5 mg/L IBA	10	0	10	20	0
MS +1 0 mg/L BAP+2 0 mg/L IBA	40	30	0	10	10
MS +2 0 mg/L BAP + mg/L IBA	0	20	10	0	0
MS +2 0 mg/L BAP + 1 0 mg/L IBA	30	0	10	0	40
MS +2 0 mg/L BAP + 1 5 mg/L IBA	0	40	0	30	0
MS +2 0 mg/L BAP+2 0 mg/L IBA	20	20	10	0	0
MS +4 0 mg/L BAP +0 mg/L IBA	20	10	10	0	0
MS +4 0 mg/L BAP + 1 0 mg/L IBA	10	30	10	0	20
MS +4 0 mg/L BAP + 1 5 mg/L IBA	20	30	10	0	10
MS +4 0 mg/L BAP+2 0 mg/L IBA	20	20	0	0	10
MS+0 1 mg/L TDZ+0 5 mg/L GA3 (control)	30	10	40	0	30

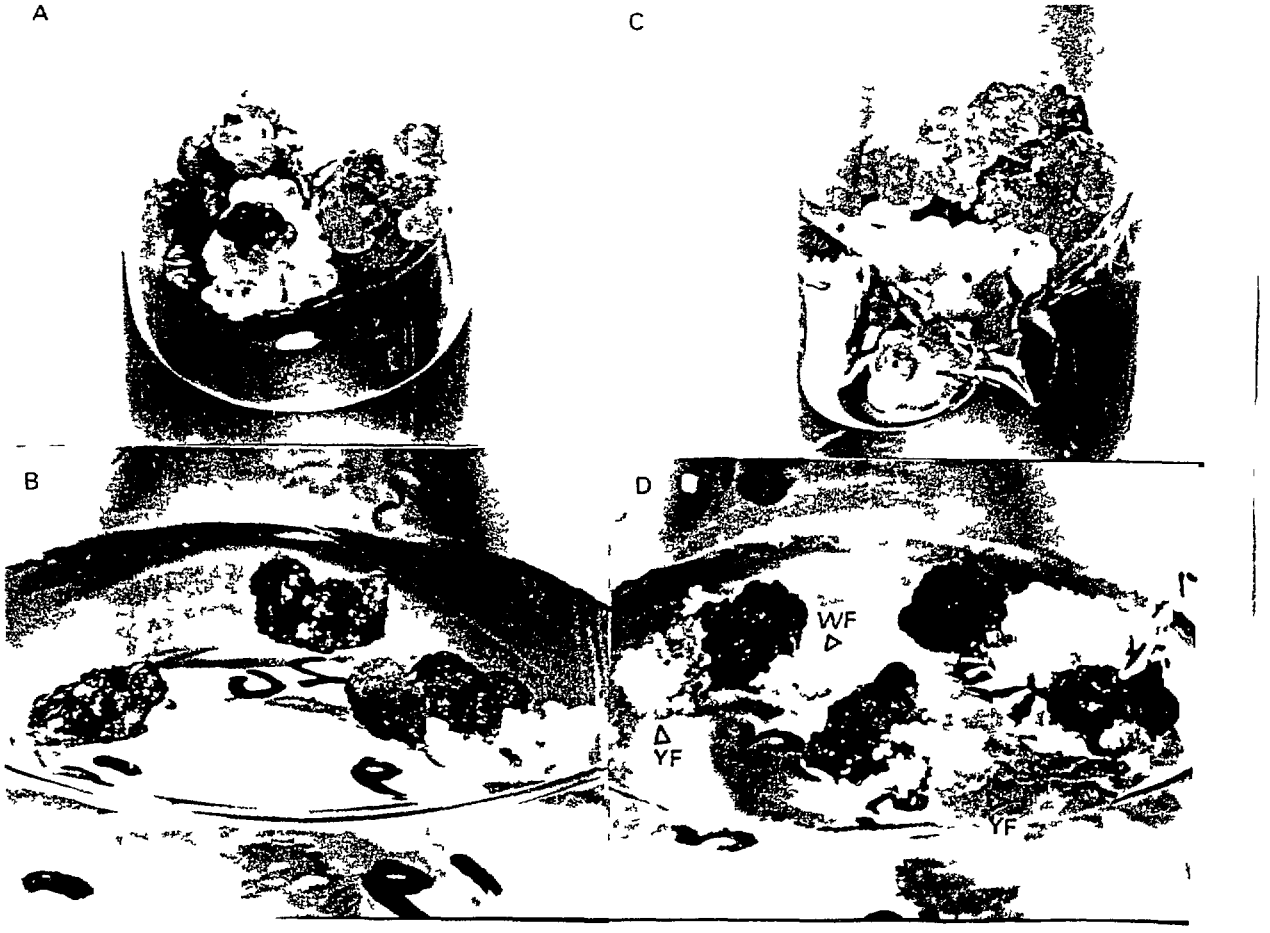


Fig 2 1 Different types of callus regrowth on BA_2 medium (A) Initiation of yellow friable (YF) and compact (YC) calli, (B) green compact (C) yellow green compact and white globular calli and (D) brown calli with yellow friable (YC) and white fine (WF) calli

Table 2 2A Response of yellow compact calli upon culture onto BA_2 medium after 3 weeks in culture

Type of callus produced	Callus regrowth (% cultures)	% Embryogenic calli	% Shoot regeneration	No of regenerants (range)
yellow compact	16	20	0	0
green compact	16	30	30	1-2
yellow friable	32	65	45	1-5
white globular	2	0	0	0
white fine	14	0	0	0
light brown	18	9	9	1

Table 2 2B Response of compact green calli upon culture onto BA_2 medium after 3 weeks in culture

Type of callus produced	Callus regrowth (% cultures)	% Embryogenic calli	% Shoot regeneration	No of regenerants (range)
green compact	65	10	10	1-3
yellow friable	24	71	28	2-6
light brown	10	0	0	0

Table 2 2C Response of yellow friable calli upon culture onto BA_2 medium after 3 weeks in culture

Type of calli produced	Callus regrowth (% cultures)	% Embryogenic calli	% Shoot regeneration	No of regenerants (range)
light yellow friable	21	42	21	2-5
light yellow compact	15	20	10	1
white fine	37	48	8	3-4
Light brown	25	29	23	1-2

2 3 1 3 Comparison of the embryogenesis and shoot regeneration response induced using the developed protocols

Protocol 1 gave 20% shoot differentiation, 50% of the cultured embryos showed secondary embryogenesis and the remaining 30% of embryos turned brown and died (Table 2 3) Protocol 2 gave 0% shoot differentiation and 40% showed secondary embryogenesis Protocol 3 gave 20% shoot differentiation with 1-3 shoots/callus clump and 70% embryogenesis

Table 2 3 Embryogenesis and shoot differentiation response obtained using developed protocols

Protocol	% Embryogenic calli	% Shoot differentiation	No of regenerants	Growth of regenerated shoot
1	50	20	1/somatic embryo	shoot elongation
2	40	10	1/somatic embryo	limited to shoot-like structures
3	70	20	1-3/callus clump	shoot elongation and limited leaf expansion

The rate of regeneration for Protocols 1 and 2 was the same, however, Protocol 3 has the advantage of regenerating plantlets directly from the embryogenic calli without undergoing embryo maturation In addition, regeneration of shoots occurred in shorter time, around 4-6 weeks as compared to 8-10 weeks for protocol 1 Shoots regenerated using protocol 3 showed shoot elongation and limited leaf expansion and absence of roots (Fig 2 2D&E)

For both systems, regeneration was mostly limited to shoot and a very low percentage (about 1%) gave normal germination of both shoot and root (observed only in protocol 1) While increasing shoot production with increasing BAP concentration (Mohamed-Yassen Y, 1994) finding shows subsequent growth (i e , root formation, shoot elongation and leaf expansion) of regenerated shoots was not easily achieved with the BA_2 and RA_3 media, suggesting different media requirements or culture conditions are needed for normal growth Absence of roots and progressive necrosis of shoots regenerated using either protocol prevented the recovery of avocado plantlets (Fig 2 2C-F)

2 3 2 Callus induction in different *Persea* species and different explants

Calli were induced from *P americana* (11 accessions/genotypes), *P cinarescens* and *P burbonica* using different explants (Table 2 4) The rate of callus induction and the type of callus produced varied with different accessions and explants Callus induction from immature seed/cotyledon ranged from 20 to 55% The calli produced were cream, white, yellow green compact calli and these turned embryogenic as early as the second subculture cycle for *P cinarescens* and after 5 or 6 subculture cycles for cv RCF Purple

Table 2 4 Callus induction in different *Persea* species and avocado genotypes using different explants

Persea species (Genotype/Cv/Acc)	Reaction to *P cinamomi* (using detached root method*)	Explant	% Callus induction	Embryogenesis/ Regeneration
P americana				
Quarry	Highly resistant	mature cotyledon	41	not observed
		leaf	43	not observed
		petiole	0	
RCF Purple	Resistant (for verification)	immature cotyledon		**embryogenesis/ regeneration**
		petiole	0	
		stem	33	not observed
U8	Resistant	petiole	50	not observed
		midrib	75	not observed
U2	Highly susceptible	midrib	42	not observed
		petiole	0	
U7	Highly susceptible	petiole	84	not observed
		leaf	0	
Sta Cruz	Highly susceptible	mature cotyledon	22	not observed
		leaf	52	not observed
		stem	0	
		petiole	73	not observed
U6	Moderately susceptible	petiole	100	not observed
		stem	100	not observed
		leaf	31	**early embryogenesis**
		midrib	100	not observed
Balete	Not tested	mature cotyledon	63	regeneration via organogenesis
		root	20	not observed
P cinarescens (from Israel)	Resistant	immature seed	20	**early embryogenesis**
		mature seed	0	
P burbonia (from Israel)	Resistant	mature seed	55	not observed
P americana G-755 (from Israel)	Moderately resistant	petiole	55	not observed

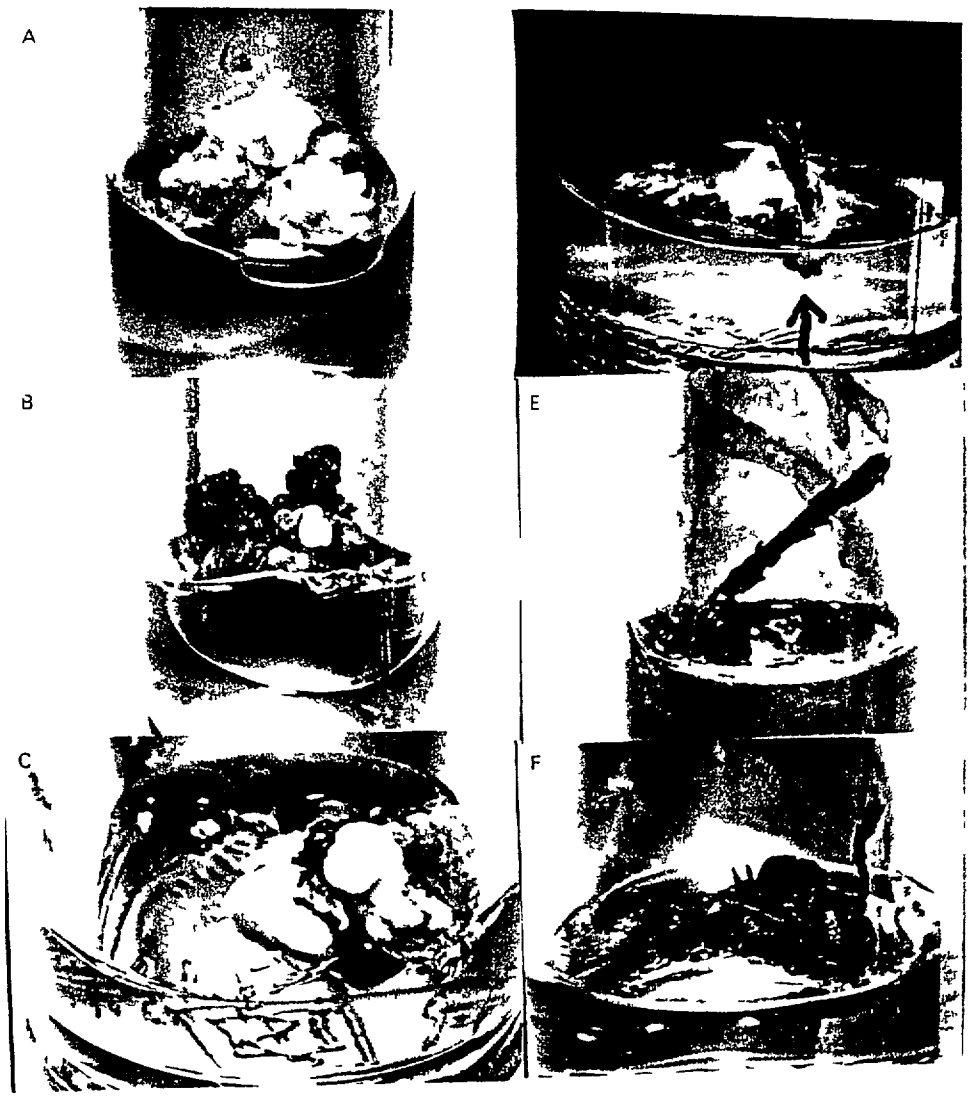


Fig 2 2 Callus initiation, embryogenesis and plantlet regeneration using Protocol 2 developed in Activity 2 (A) Compact/friable calli, (B) differentiation of shoot like structures, (c) regeneration of shoot from embryogenic calli, (D&E) elongation of regenerated shoot and (F) necrosis and death of regenerated shoot

Callusing was easily achieved using mature cotyledon explants (22 to 63% callus induction) and the calli produced were brown, white, creamy or green in color and compact soft, loose or watery texture and appearance (Fig 2 4) Somatic embryogenesis was not observed in calli initiated from mature cotyledon even after more than 5 subculture cycles However, regeneration of plants via organogenesis was observed from compact white to yellow callus produced in avocado acc Balete (Fig 2 4F)

Callus initiation from stem, midrib and petiole and leaf explants was genotype dependent (Table 2 4) Callus initiation ranged from 0 to 52% for leaf explants, 0 to 100% for stem explants, 0 to 100% for petiole explants, and 42 to 100% for midrib explants The type of callus produced was compact, soft or loose in texture and cream, brown or white in color (Fig 2 5) Calli initiated from petiole, stem and midrib explants did not show embryogenesis even after more than 5 subculture cycles while calli initiated from leaf tissues showed some early embryogenesis after 6 subculture cycles (Fig 2 5B)

The occurrence of somatic embryogenesis in *Persea* species is influenced primarily by the kind of explant used in initiating callus From the calli initiated from 13 genotypes using 7 different explants, embryogenesis was observed only from immature seed/cotyledon and leaf explants Regeneration of plantlets from avocado calli occurred through somatic embryogenesis or organogenesis

Conclusion

Callus induction, somatic embryogenesis, shoot differentiation and plantlet regeneration were developed for avocado cv RCF Purple Callus initiation was easily achieved in avocado *(P americana*, 11 accessions/genotypes), *P burbonia* and *P cinarescens* using different explants Initiation of embryogenesis was obtained only from immature cotyledon/seed and leaf explants At this point, the best conditions which give 70% embryogenesis and 20% shoot differentiation in protocol 3 Further studies are needed to determine the culture requirements for subsequent plantlet development

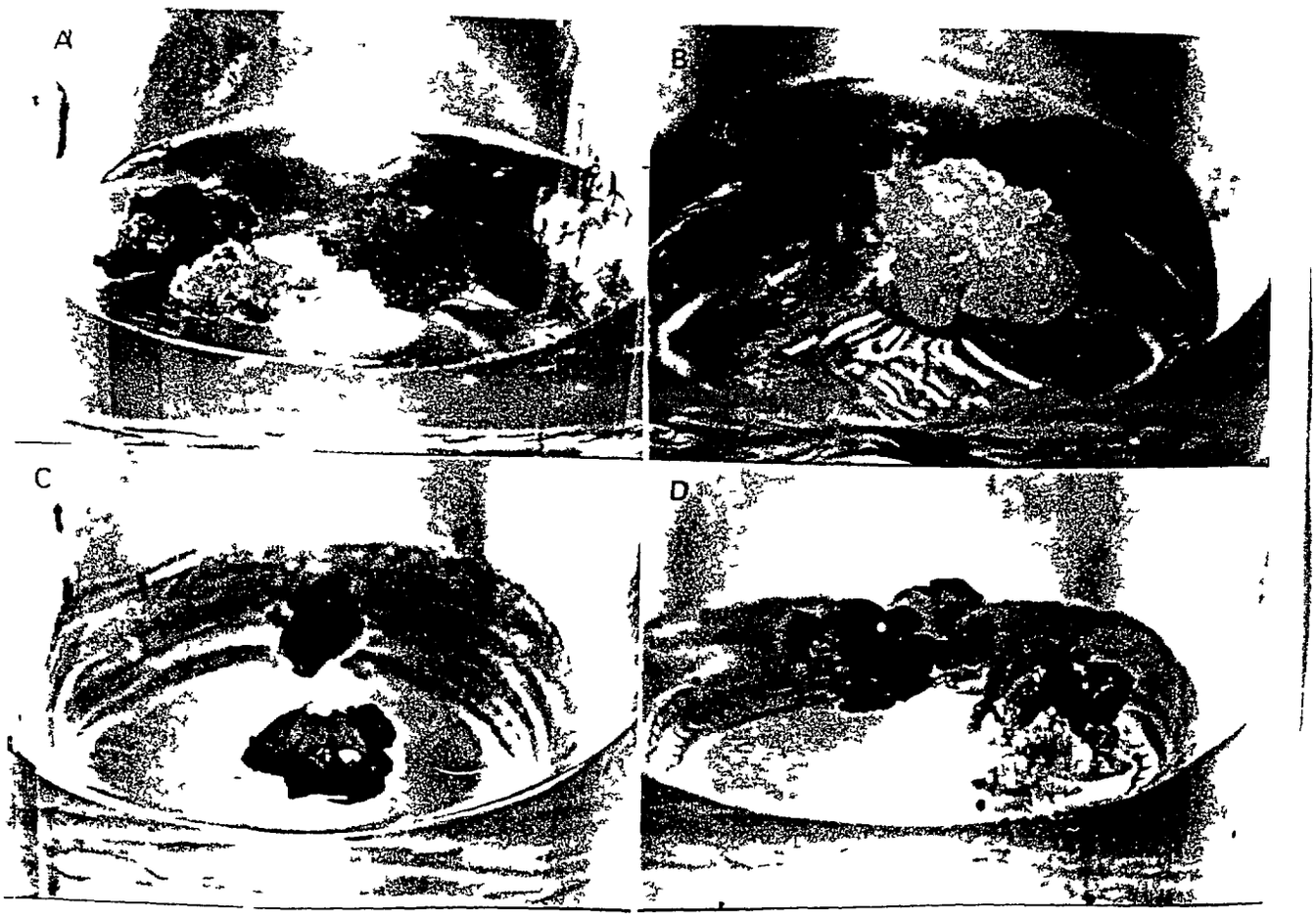


Fig 2 3 Callus induction from immature seed/cotyledon Callus induced (A) and embryogenesis after 5 or 6 subculture cycles(B) from immature cotyledon of cv RCF Purple, (C) immature seed explant and (D) callus and early embryogenesis after 2 subculture cycles induced from immature seed of *P cinarescens*

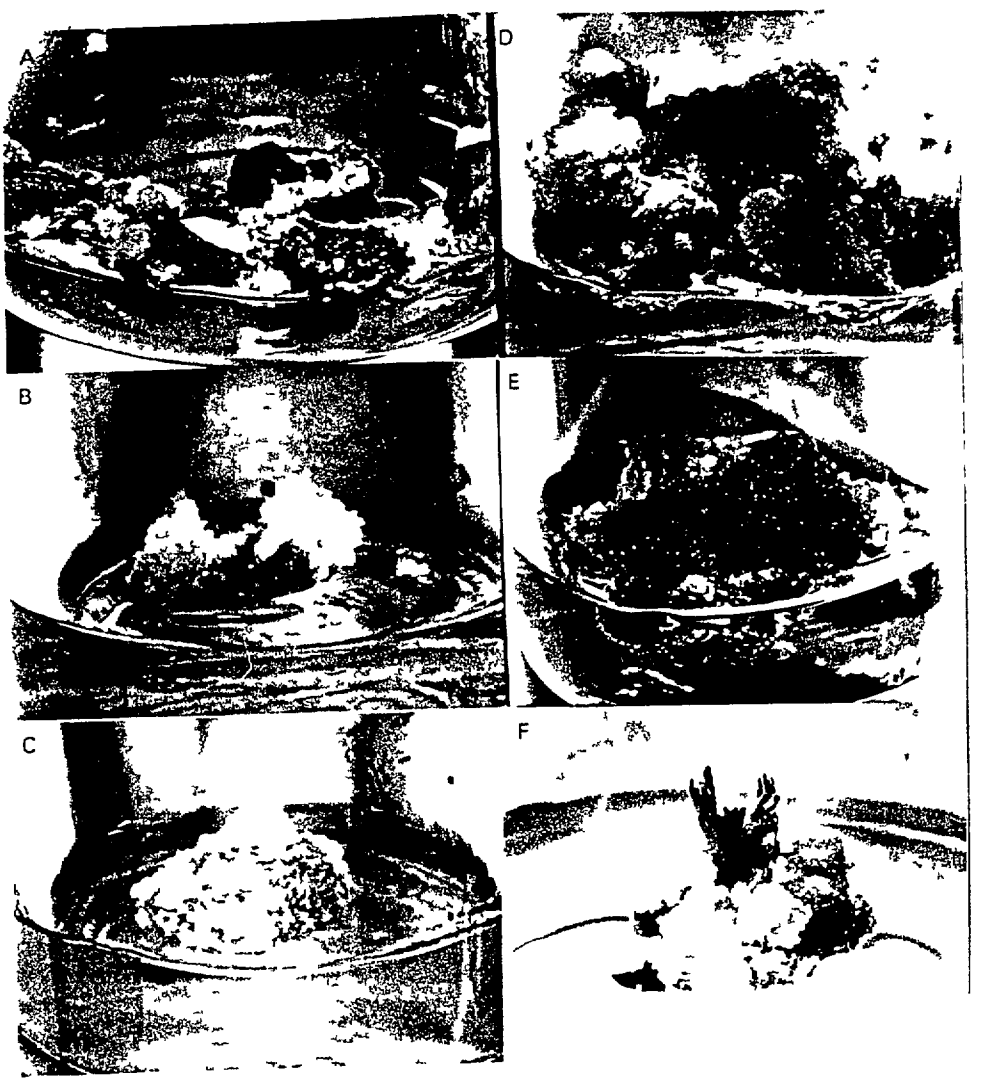


Fig 2 4 Callus initiated from mature cotyledon explants (A) Cotyledon explant showing callus initiation, (B) cream compact calli (C) green compact calli (D) white to light brown, loose and watery calli (E) brown soft watery calli and (F) regeneration from cream to yellow compact calli

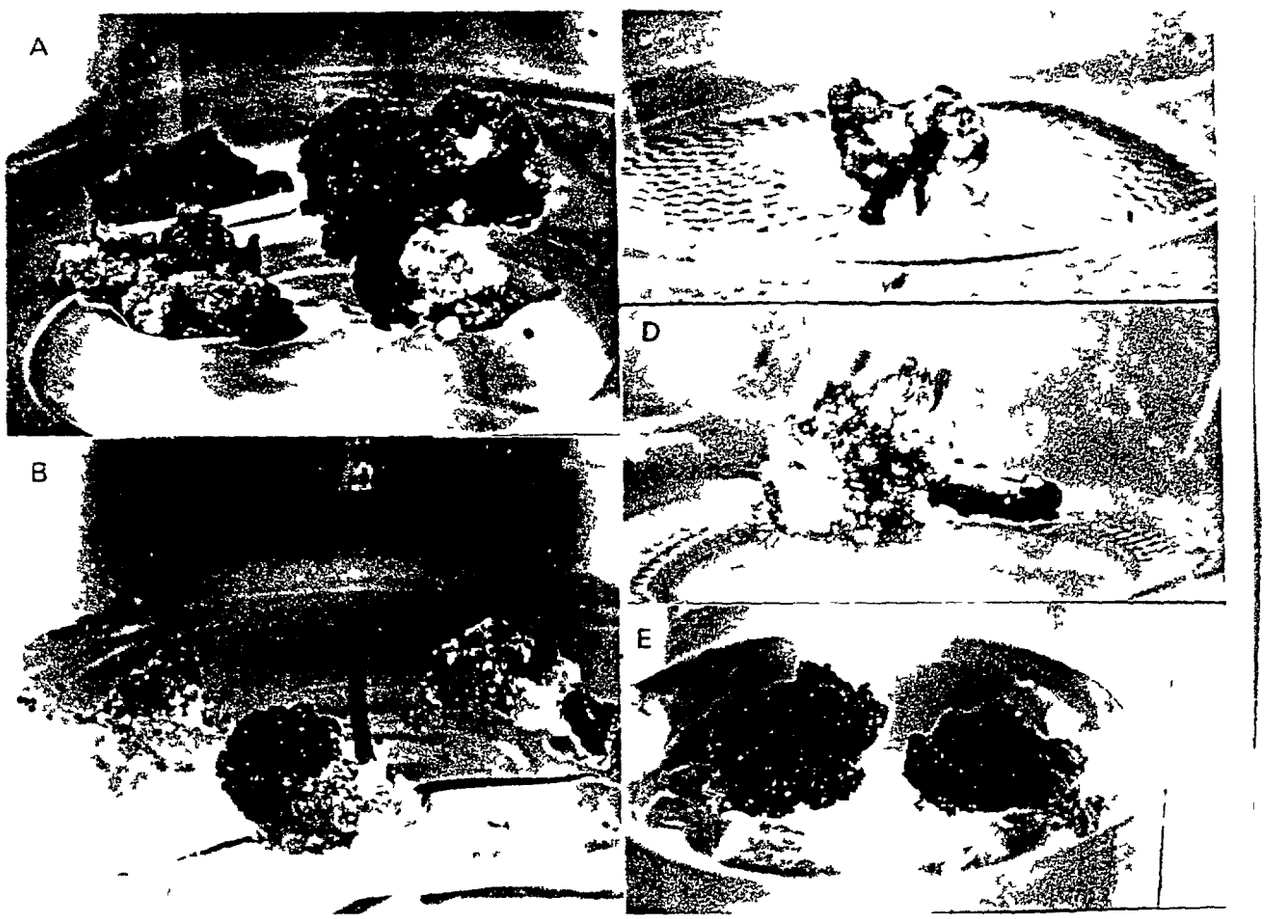


Fig 2 5 Calli initiated from stem, petiole midrib and leaf explants (A) Green and brown calli initiated from leaf explants, (B) embryogenic calli from leaf calli, (C) creamy compact callus from midrib explant (D) light brown loose and soft calli initiated from petiole explant and (E) brown soft calli from stem tissues

3 0 Development of Appropriate Screening Methods that will Correlate *In Vitro* Resistance with the Intact Plant Response

3 1 Collection, isolation, purification and identification of the causal organism

Root rot caused by *Phytophthora cinnamomi* is one of the most serious diseases of avocado in the Philippines There has been report of root rot disease caused by *Pythium debaryanum* Hesse (Roldan and Raktakanishta, 1934) but root rot caused by *P cinnamomi* has not yet been reported Although, many avocado trees along peripheral roads, orchards and backyards are dying, the cause or causes of their death has not been ascertained In other countries like Israel, Australia, Mexico, USA and New Zealand, root rot due to *P cinnamomi* is wide-spread and causing a significant loss/damage to avocado industries (Wager, 1942, Chee and Newhook, 1965, Zentmyer, 1970, Pegg and White, 1987, Pinkas, 1989)

3 1 1 Objective

To collect, isolate and identify the pathogen causing root rot disease in avocado

3 1 2 Methodology

Feeder roots of infected abocado were collected from Pasong Kipot, Tranca, Bay, Laguna Roots were dipped in 70% (v/v) ethyl alcohol for 2 min and rinsed with sterile distilled water One to two cm root segments were planted onto potato dextrose agar (PDA) and incubated at 24 C Similar decontamination procedure was used in tissues planted onto corn meal agar (CMA) containing pimaricin (10 mg), ampicillin (125 mg), rifampicin (10 mg) and pentachloronitrobenzene (100 mg) per liter (CMA+PARP) (Solel and Pinkas, 1984) Purification of the isolated fungus from bacterial contamination was done using pancake method (Sleeth, 1945) Mycelial strips growing from 24 hr incubated tissue at 28-30 C were picked and planted onto agar blocks containing 10 g dextrose, 2 g NH_4 H_2 PO_4, 1 g KNO_3 and 1 g $MgSO_4$ per liter of distilled water Inoculated agar blocks were inverted in a sepparate Petri dish and incubated at 20-25 C Mycelia which grew up through the agar block were picked aseptically and transferred to PDA The conventional method of subsequent plating was also used Identification od the causal organis, was done using available literature/references Pure cultures were being maintained in tes tube containing sterile mineral oil and were used for subsequent studies

3 1 3 Results and Discussion

The pathogen was isolated with difficulty due to bacterial contamination Bacterial contamination was commonly encountered using PDA However, the use of CMA+PARP medium and pancake method were successful in eliminating bacterial contamination Growth of the fungus was observed 3 to 4 days after seeding on PDA Based on the distinct characteristics of the colony growth on PDA and morphology of the

hyphae and sporangium as seen under the microscope, the isolate has been identified *as Phytophthora cinnamomi* Rands (Ribeiro, 1978)

3 2 Pathogenicity Test

This was conducted to determine whether the isolated fungus is the pathogen causing the root rot disease in avocado

3 2 1 Methodology

Detached rootlets of greenhouse-grown avocado seedlings were surface sterilized by submerging in 20% (v/v) sodium hypochlorite (Zonrox, 5 25% a i) for 20 min and were then rinsed with sterile distilled water Excess tissues were trimmed-off and rootlets were placed in sterile Petri plates bottom-lined with sterile filter paper Mycelial strips of 5-day-old agar growth of the fungus were planted onto rootlet tips Inoculated roots were incubated for 5 days at 25-27 C

For intact roots, mycelial fragments of 5-day-old culture of the fungus were placed at the base of the roots of 6-mo-old avocado plant using the method of Crandall (1948) The fragments were covered with moist cotton wad to prevent dehydration Appearance of symptoms was observed Re-isolation from inoculated roots was done

3 2 2 Results

Using detached root inoculation, the rootlets turned black after 2 days Unfortunately, the inoculated fungus was not recovered upon reisolation For intact roots inoculation, the inoculated seedling started showing symptoms of wilting followed by drying of leaves The base of the inoculated plant turned brown after a month from inoculation and the plant beecame completely dead after 2 months The plant failed to produce feeder roots The inoculated fungus was recovered upon isolation of the affected roots

3 2 3 Conclusion

Symptoms produced on inoculated roots and the recovery of the pathogen from the infected roots revealed that *Phytophthora cinnamomi* is indeed the pathogen of root rot in avocado

3 3 Morphological and Cultural Characteristics of *P cinnamomi*

3 3 1 Introduction

Prior to the implementation of the project, *P cinnamomi* has not yet been reported in the Philippines Although this pathogen is known to infect about 1000 crop species including fruits such as pineapple, which is commonly grown in our country, no attempt

has been made to isolate and characterize this pathogen Hence, description of the pathogen was based only on the available literatures abroad

3 3 2 Objectives

To provide description of the Philippine isolate of *P cinnamomi*, to compare it with isolates reported abroad and to determine its growth response on different agar media

3 3 3 Methodology

P cinnamomi was grown in nine agar media, viz PDA, CMA, yeast extract agar (YEA), V-8 juice agar (V8JA), modified carrot agar (MCA), potato carrot agar (PCA), soybean seed decoction agar (SSDA), malt extract agar (MEA) and oatmeal agar (OMA) The seeded agar plates were incubated at 25-27 C for 5 days under continuous light The chaacteristics of the colony and mycelial growth and diameter of the colony were taken On PDA and V-8JA, the morphology of the hyphae, chlamydospores, sporangium and zoospores were described as seen under the microscope

3 3 4 Results and Discussion

Young hyphae of *P cinnamomi* were distinctly coralloid, hyaline, slender becoming thick-walled or tough and septate with age Chlamydospores were thick-walled, globose to pyriform, terminal or on short lateral branches (Fig 3 1a) Sporangia were borne terminally, ellipsoid to ovoid, hyaline, thin-walled, with flat inconspicuous papilla on end opposite point of attachment Spore case partially collapsed after zoospore release (Fig 3 1b) Zoospores were bean- or kidney-shaped with two flagella of unequal length attached to concave side, hardly seen

Of the nine media used, MCA gave the largest mean colony diameter (59 7 mm), while PDA had the shortest mean colony diameter (32 2 mm) (Table 3 1, Fig 3 2) The mycelia grew slowly in SSDA and PDA Distinct camelloid or rosette pattern of colony growth was observed in PDA (Fig 3 3) In PCA, the camelloid pattern was noted on the center of the colony only This is due to the presence of potato in PCA The mycelial growth was slightly dense in PCA, MCA, and V-8JA The mycelia were hardly seen in other media Under the microscope, the distinct coralloid type of hyphal growth was observed on all media used, but the density and size of hyphal swelling varied (Table 3 2) According to Ribeiro (1978), the type of hyphal swelling and camelloid or rosette pattern colony growth on PDA were the distinctive characters that differentiate *P cinnamomi* from other *Phytophthora* species Hence, the use of PDA for routine isolation of this fungus is highly recommended

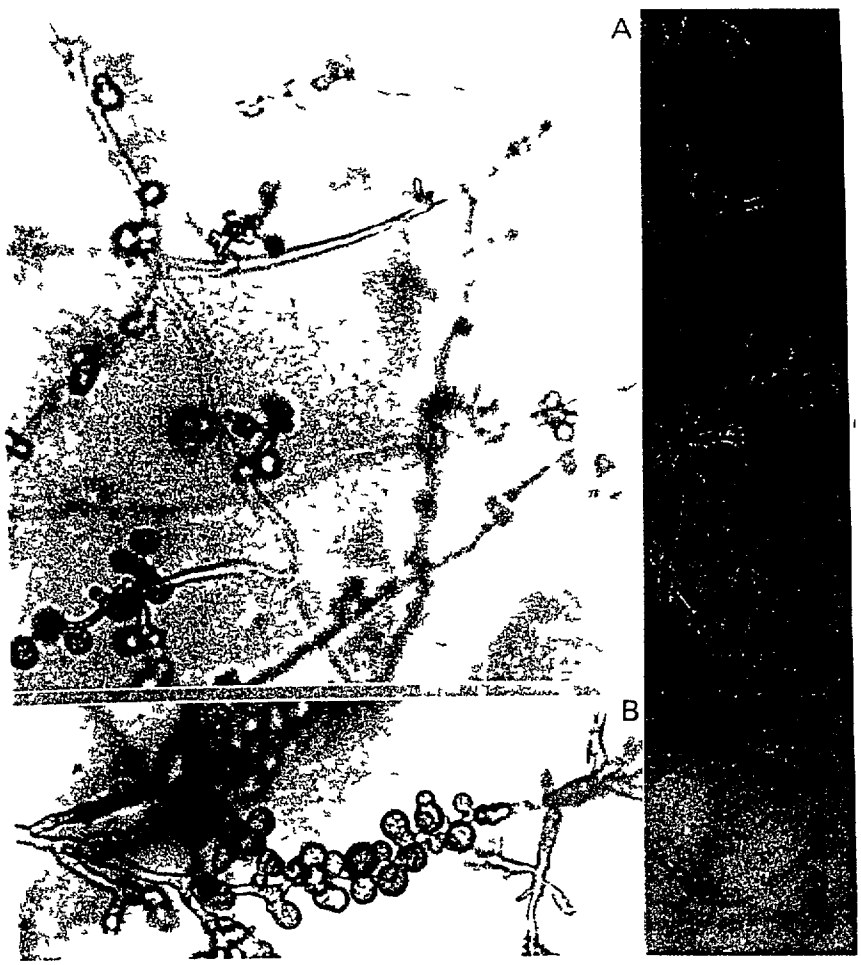


Fig 3 1a *Phytophthora cinnamomi* Rands Young (a) and mature (b) hypha Note the distinct coralloid (rounded vesicles or hyphal swellings) type of hypha, secondary conidia in successive sympodial fashion stained with lactophenol cotton blue (c), chlamydospores (d) (x400)

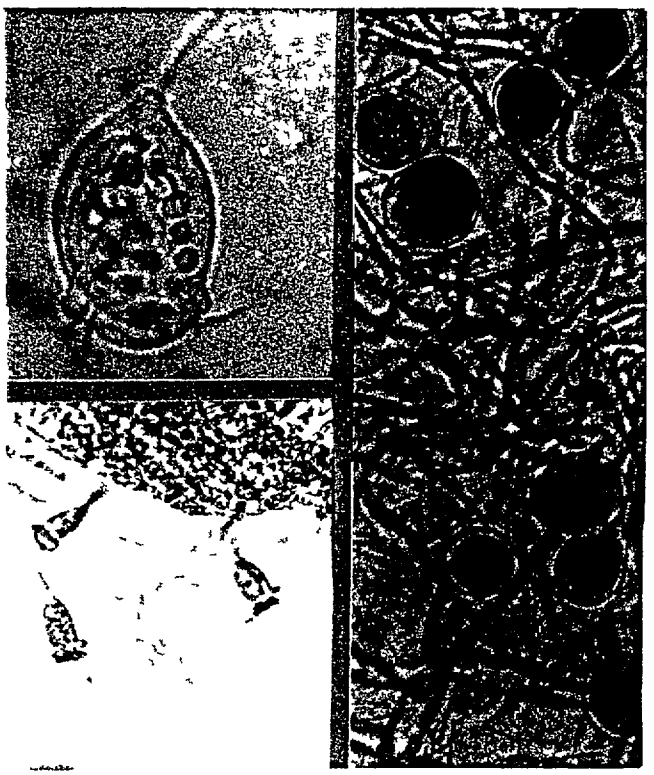


Fig 3 1b *Phytophthora cinnamomi* Rands Sporangia stained with lactophenol cotton blue (a), zoosporangia arising from unbanched hypha (b) (x400), close-up of a zoosporangium releasing zoospores (c) (x1000)

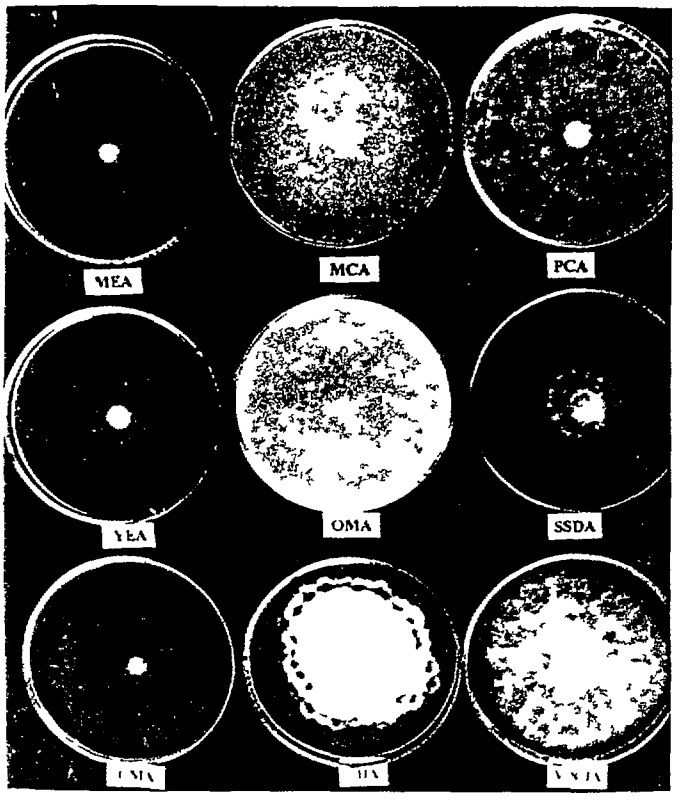


Fig 3 2 Colony morphology of *Phytophthora cinnamomi* on different agar media after 5 days of incubation at 28-30 C MEA-malt extract agar MCA-modified carrot agar, PCA-potato carrot agar, YEA-yeast extract agar OMA-oatmeal agar, SSDA-soybean seed decoction agar, CMA-corn meal agar PDA-potato dextrose agar, V-8JA-V-8 juice agar

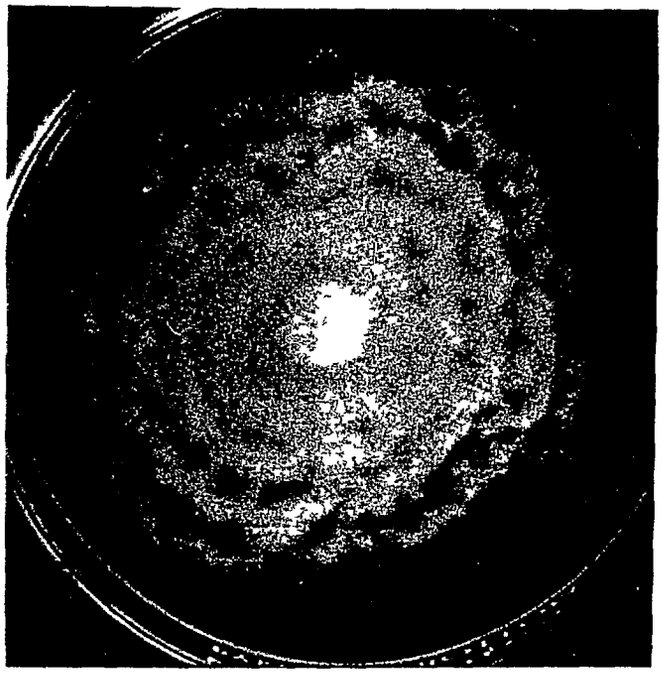


Fig 3 3 Mycelial growth of *Phytophthora cinnamomi* on potato dextrose agar
Note the camelloid or rosette pattern of colony growth

Table 3 1 Mycelial growth of *Phytophthora cinnamomi* in different agar media

Agar Medium	Colony Diameter (mm)					
	24 hr	48 hr	72 hr	96 hr	120 hr	Mean
modified carrot	23 5	43 2	62 3	24 hr	88 80	59 7
malt extract	19 9	42 6	54 0	80 5	88 0	57 0
potato carrot	19 7	38 0	56 8	73 0	88 0	55 1
oatmeal	15 4	28 5	39 0	49 0	88 0	44 0
V-8 juice	18 2	30 5	42 0	55 8	70 5	43 3
yeast extract	18 1	30 6	42 9	50 0	62 4	37 4
soybean seed decoction	16 3	23 8	37 5	47 0	62 2	36 0
potato dextrose	13 1	23 5	33 7	35 2	55 6	32 2

Incubated at 25-27 C under continuous light

Table 3 2 Morphological characteristics of the colony and hypha of *Phytophthora cinnamomi* grown in different media, five days after incubation under continuous light

Agar Medium	Colony Morphology	Hyphal Growth[a]
modified carrot	slightly dense	very few, distinct and big hyphal swelling
malt extract	very scarse	few and small hyphal swelling
potato carrot	slightly dense, slightly camelloid at the center	few hyphal swelling
oatmeal	very dense	very few hyphal swelling
V-8 juice	slightly dense, fluffy	profuse and big hyphal swelling
yeast extract	scarse	ffew hyphal swelling
corn meal	very scarse	few hyphal swelling
soybean seed decoction	very scarse at periphery, dense at center	profuse and big hyphal swelling
potato dextrose	very dense with distinct camelloid or rosette pattern of growth	profure and big hyphal swelling

[a]As seen under the microscope

3 3 5 Conclusion

Based on morphological and cultural studies, the Philippine isolate resembles that of the *Phytophthora cinnamomi* Rands isolated from the bark of *Cinnamomum burmani* infected with stripe canker in West Coast Highland of Sumatra PDA is an appropriate medium for isolation of this pathogen

3 4 Sporangial and Zoospore Production

Sporangia are necessary for zoospore production Zoospores, on the other hand, are important as they are the structures necessary for infection To produce sporangia and zoospores among *Phytophthora* species, several conditions should be met A specific species may require specific conditions for sporangial or zoospore production

3 4 2 Objectives

To determine the optimum conditions for sporangial and zoospore production of the Philippine isolate of *P cinnamomi* and to compare two procedures for inducing sporangial and zoospore production

3 4 3 Methodology

Two procedures were tried The first procedure was a modification of the method developed by de Zoeten et al (1982) Agar pieces from 7-day-old cultures on V-8JA were plated onto 5 % OMA and incubated for 10 days in complete darkness at 28-30 °C Zoospore release was induced by stripping off the mycelial mat from agar plate and floated in 2 ml sterile distilled water in Petri dish, chilled for 30 min followed by a 7 hr incubation at 24 °C The second procedure was carried out as followed *P cinnamomi* was grown in V8JA for 5 days under continuous light Ten to 15 pieces of mycelial agar disc were transfer to diluted V8 juice broth (1 10) and incubated for 16 hr at 24 °C under continuous darkness The broth was withdrawn and mycelial pieces were washed with sterile mineral salt solution on an hourly basis for 7 hr Afterwhich the mycelial pieces were incubated in mineral salt solution for 18 hr under continuous light The mineral salt solution was withdrawn, replaced with sterile distilled water and incubated for 15-20 min at 4-5 °C Then they were shifted at room temperature under continuous light The sporangial production and zoospore release were observed under the microscope

3 4 4 Results and Discussion

In both procedures, numerous sporangia were produced Zoospores were released followed by a 15-30 min incubation at 4 to 5 °C followed at 24 to 28 °C under continuous light It was observed that in the first procedure, zoospore release started on the 7th hr of incubation, whereas in the second procedure, release of zoospores started after the second and third hour of incubation The zoospore remained motile for few hour after which they encyst Exposure to complete darkness inhibited the mycelial growth of the fungus At 21-24 C, the fungus is capable of producing good yield of zoospores is induced upon exposing the fungus at very low temperature preferably 5 C (Chen and Zentmyer, 1970) and returning to 24 C According to Palzer (1976), the small drop in temperature could induce zoospore release in *P cinnamomi*

3 4 5 Conclusion

P cinnamomi requires initial incubation at low temperature (4 to 5 C) followed at higher temperature (24 to 28 C) under continuous light for sporangial and zoospore production Comparing the two procedures, it took shorter time for sporangial prodution and zoospore release using the second procedure than the first procedure

3 5 Development of Screening Methods Against *Phytophthora* root rot

The success of any disease resistance breeding work depends on the proper identification of materials that possess some levels of resistance to a particular disease This can be achieved if screening procedure is reliable, efficient and effective

Different techniques for use in disease screening work are available However, their effectiveness varies with the plant and particular disease These techniques may involve whole plant or plant part In recent years, the use of callus in screening work is becoming popular as calli are readily available and can be handled in large volume Screening using calli are also less expensive, less tedious and results can be obtained at the earliest possible time However, correlation of resistance using *in vivo* (detached or live plants) and *in vitro* (callus) selection should first be established in order to validate the use of calli in screening work in avocado

3 5 2 Objectives

To develop screening procedures that are reliablefor use in identifying resistant source, to assess/evaluate the effectiveness of existing screening procedures for *Phytophthora* root rot disease in avocado, and to correlate their effectiveness

3 5 3 Methodology

A Root Inoculation Using Mycelia

Detached root inoculation method developed by Botha et al (1989) and van der Merwe et al (1990) was employed with some modifications Roots of 3 to 4 mo-old avocado plants were detached, cut into 40 mm long and washed thoroughly with water Roots were then aseptically placed perpendicularly onto two sterile rods (7mm diameter, 60 mm) in Petri dish with water agar (Fig 3 4) Each root tip was inoculated with 10 ul of mycelial fragment of *P cinnamomi* To produce the mycelial fragments, mycelial disc of the fungus were grown in pea broth for 4 days in rotary shaker The mycelial pellets were fragmented in a waring blendor Inoculated roots were placed in dark condition at 24-25 C for 48 hr

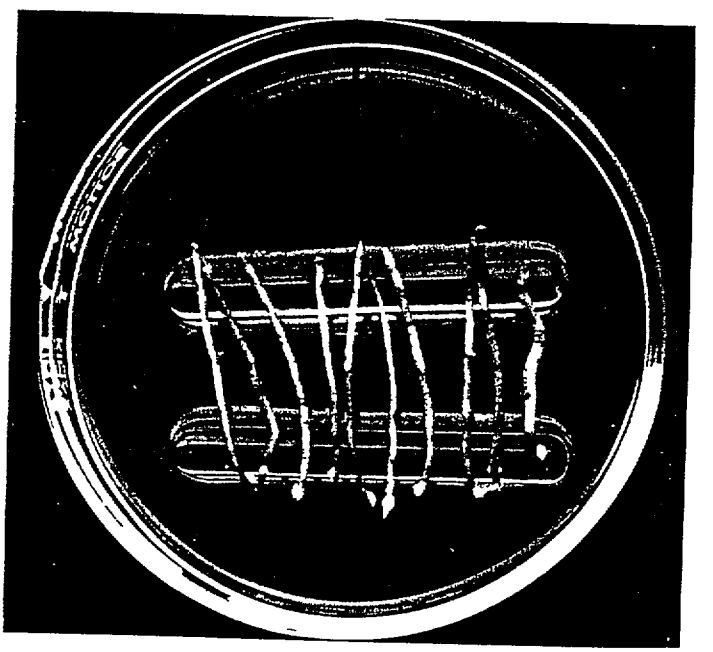


Fig 3 4 Set up for evaluating the resistance of avocado to *Phytophthora* root rot using detached root inoculation method

The roots were cut into 4 segments, 10 mm per segment, surface sterilized for 5 sec in 70 % ethanol, plated in potato dextrose agar (PDA) and incubated at 24-25°C for 12 to 14 hr under dark condition The total length of root colonization was determined by counting the number of segments from which *P cinnamomi* developed divided by the total number of segments multiplied by 100

Results and Discussion

Using mycelial fragments of *P cinnamomi*, browning of the roots was observed 24 hr after inoculation Browning moved to the upper portion of the roots as the fungus continued to grow

Potato dextrose agar was used instead of PARP medium as mentioned in the methods of Botha et al (1989) and van der Merwe et al (1990) since PDA is readily available and less expensive Length of incubation of inoculated roots was shorten to 12 to 14 hr using PDA in contrast to the 72 hr using PARP medium as stated in the studies of the preceding workers It was observed that if the length of incubation is extended beyond 14 hr, counting of root segments with mycelial growth become difficult since after 14 hr, the plate was entirely covered with the growth of the fungus

One of the parameters used by the authors in order to evaluate resistance of avocado is the length of the lesion after incubation at 24 to 25°C for 48 hr In this study, difficulty was encountered in taking the lesion length since roots of some varieties were brown This parameter is applicable only for some varieties/selections that produce white root tips

B Direct Inoculation of Callus Using Mycelia

Methodology

Calli of three avocado species and selection, namely, *Persea americana* var Topa Topa (susceptible), *P cinerascens* (resistant) and RCF, were used in the experiment The first two cultivars came from Dr Zilkah of the Agricultural Research Organization, The Volcani Center, Israel, while the latter is a local selection which was found to be resistant in the screening work conducted

P cinnamomi was plated in PDA and after 3 to 4 days, 2 x 2 mm sterile miracloth was laid over the mycelial growth for 7 day Calli of the three cultivars were plated in MS medium and the miracloth was put on the top of each callus Three types of calli from RCF were used Type 4 - loose, Type 5 - moderately loose and Type 7 - compact, hard Calli of RCF were white For other cultivars, loose type of callus were used Calli of *P americana* var Topa Topa and *P cinerascens* were light brown to black Necrosis or browning of the callus and characteristic of the hyphal growth on the callus were noted for 4 days of incubation at the dark, 25°C

Results and Discussion

Difficulty was encountered in determining necrosis/browning of *P americana* var Topa Topa and *P cinerascens* due to the color of the callus *P cinerascens* has indistinct necrosis of the callus but the necrosis at the margin was distinct With regards to the three types of callus of RCF, browning of the center and margin of the callus was observed on Type 5 and Type7, 48 hr after incubation (Fig 3 5) Hyphal growth on the callus was slow on three type of callus, while fast on the two cultivars (Table 3 3) Surface hyphal growth was sparse to moderate on Type 7 but dense on the rest Aerial hyphal growth was sparse on the three types of callus On the materials tested, the fungus produced the typical coralloid type of hypha However, stunting of hyphal branches was observed on Type 5 and Type 7 of RCF

Table 3 3 Response of callus of different species and selection of avocado (*Persea* sp) to inoculation of *Phytophthora cinnamomi*, after 48 hr incubation at 25°C, under dark condition

Criterion	*P americana* var Topa Topa (susceptible)[a]	*P cinerascens* (resistant)[a]	RCF (resistant)[b]		
			Type 4	Type 5	Type 7
rapid necrosis	indistinct	distinct	indistinct	distinct	distinct
necrotic margin	indistict	distinct	indistinct	distinct	distinct
hyphal growth on callus	fast	fast	slow	slow	slow
aerial hyphal growth	dense	dense to moderate	sparse	sparse	sparse
surface hyphal growth	dense	dense	dense	dense	dense to moderate
type and characteristic of hypha	coralloid	coralloid	coralloid	coralloid, stunted branching	coralloid, stunted branchin g

[a] As evaluated by Dr Zilkah of Agricultural Research Organization, Volcani Center, Israel

[b] As evaluated by the author using detached inoculation root method

Conclusion

There is no clear correlation between resistance level of plant source and the effect of direct inoculation of callus with mycelia To assess resistance against *Phytophthora* root rot, detached root inoculation is considered a better screening procedure than direct callus inoculation This could be attributed to the difference in the type of callus derived from the source

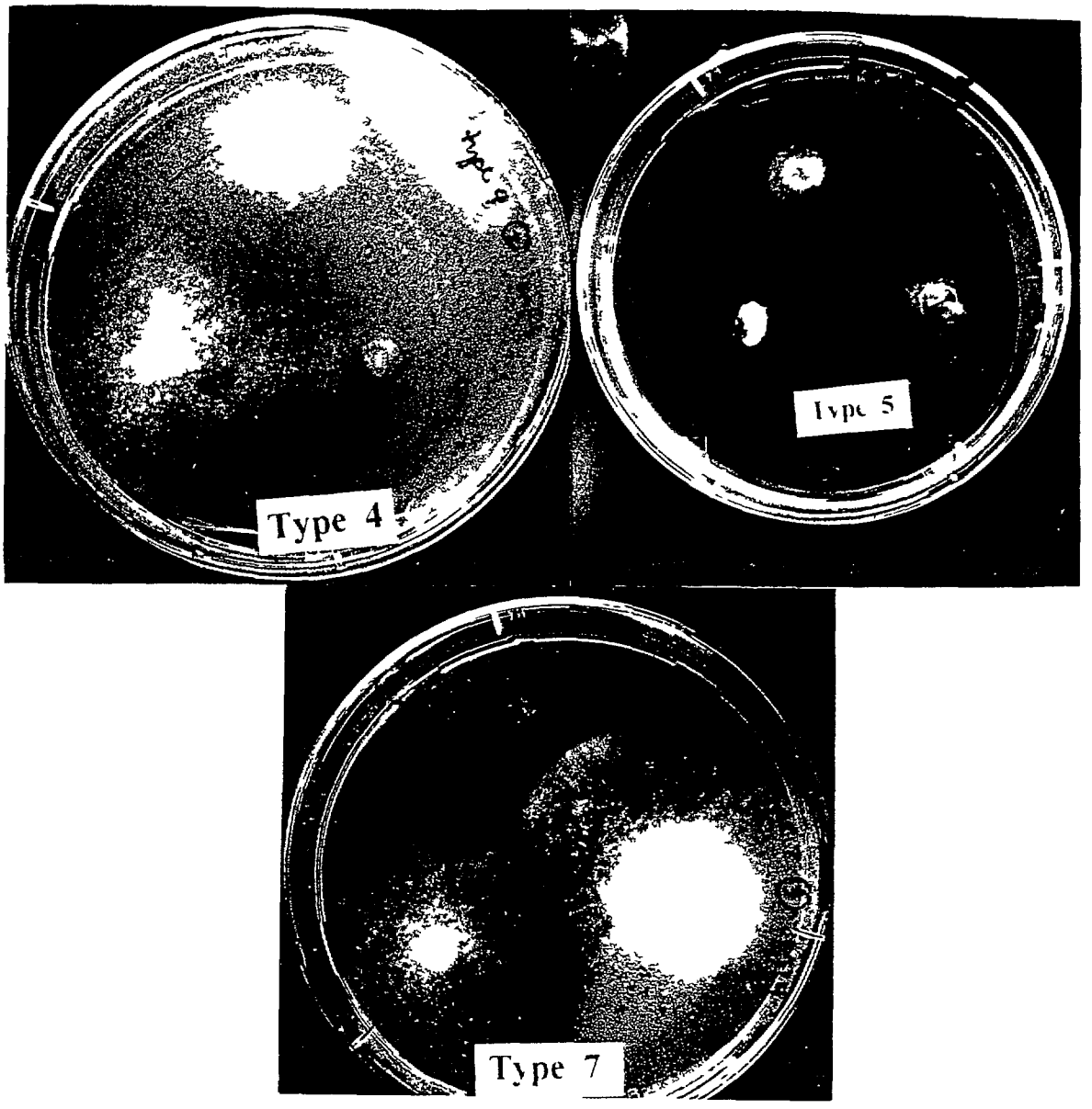


Fig 3 5 Response of three types of callus of RCF selection to direct inoculation of the mycelia of *Phytophthora cinnamomi* 48 hr after inoculation (Type 4 upper - inoculated callus, left - mycelial disc, right - uninoculated callus, Type 5 - upper - mycelial disc, right - inoculated callus, left - uninoculated callus, Type 7 - upper - uninoculated callus, right - inoculated callus, left - mycelial disc)

C Mycelial attraction to callus

Methodology

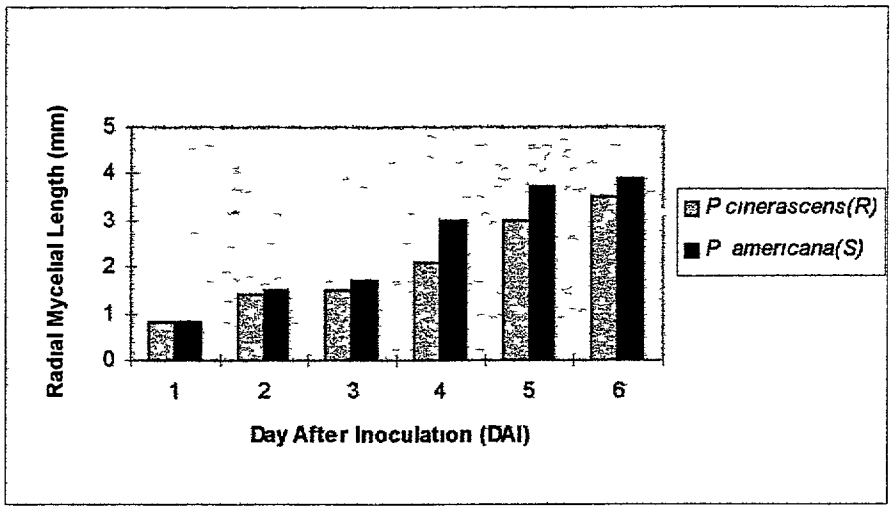
Calli of resistant, *P cinerascens,* and susceptible, *P americana* avocado species from Israel and a local selection, U7 which was found highly susceptible in the screening work, were used Two tests were conducted, *P cinerascens* versus *P americana* and *P cinerascens* versus U7 Callus of each test material was plated on opposite side of a Petri dish containing water agar A 7 mm diameter mycelial agar disc of *P cinnamomi* was seeded at the center of the plate, equidistant from each callus Inoculated plate was incubated at 28-30°C under dark condition The radial length of the colony towards each callus was measured daily for 6 days Furthermore, the morphology of the mycelial growth on the callus was described on the 5th, 6th and 14th day of incubation

Results and Discussion

Generally, the fungus grew faster towards the susceptible species/selection than the resistant species (Fig 3 6a and 3 6b, Fig 3 7) In both tests, mycelia of the fungus started to move towards the susceptible side on the second day of incubation Greater differences on mycelial radial length were observed on the fourth day (0 9 mm on *P americana* versus *P cinerascens*), and on the fifth day (0 8 mm on *P cinerascens* versus U7) However, on the sixth day, the difference was similar in both tests (0 4 mm)

On the fifth day, the mycelia of *P cinnamomi* reached the susceptible callus than the resistant callus for both tests (Fig 3 7a) *In P cinerascens* versus *P americana*, the mycelia were scarse and limited and concentrated at the bottom of the callus of *P cinerascens*, while in *P americana*, the mycelia were dense partially covering the callus and the growth extended beyond In *P cinerascens* versus U7, the mycelia were scarse at the bottom and limited covering about 25 percent of the callus of *P cinerascens* while the mycelia were dense at the bottom and the whole callus of U7 Likewise, the mycelia grew beyond the callus of U7 After the 14th day of incubation, the calli of susceptible, *P americana* and U7, were toally covered with the mycelia, whereas, callus of resistant, *P cinerascens*, were partially covered (Fig 3 7c) The results obtained agreed with the findings of Philipps and co-workers (1991)

Based on the rate of mycelial growth, higher rate was observed towards the susceptible, 0 67 for *P americana* and 0 69 for U7, than the resistant species, *P cinerascens*, 0 54 for first test and 0 56 for second test (Fig 3 8) These results could provide a useful alternative and relatively fast assay technique for selection for resistance in *Phytophthora* root rot in avocado



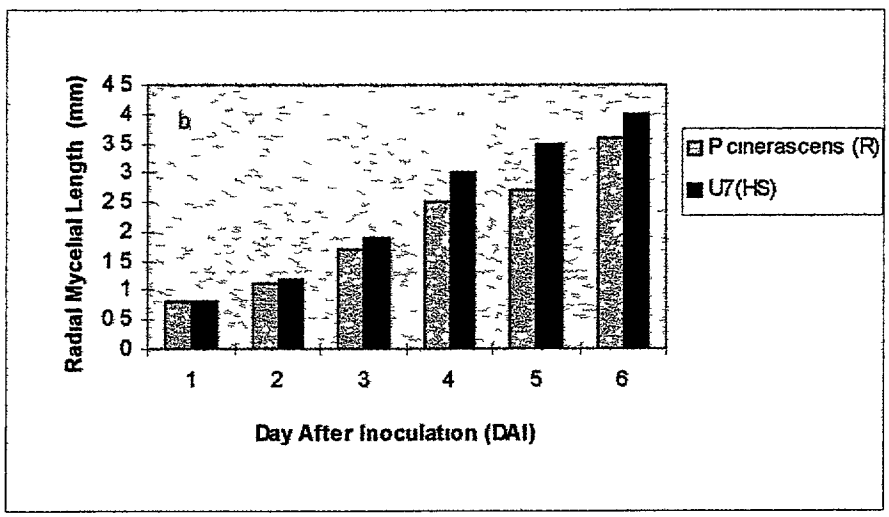
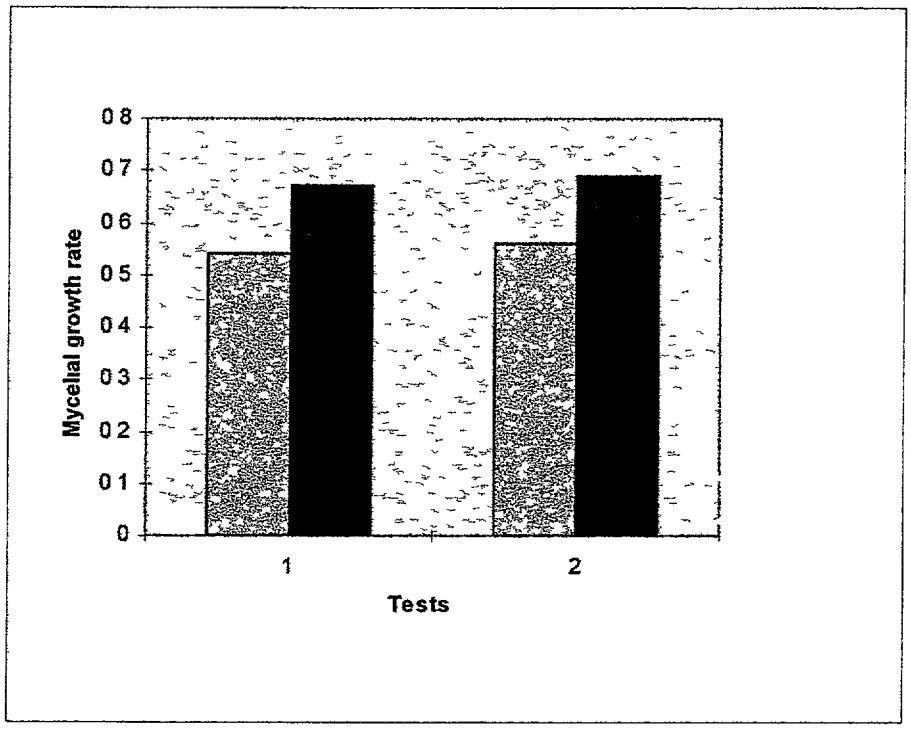


Figure 3 6 Mycelial growth of Phytophthora cinnamomi towards callus of resistant (R) and a susceptible (S), (a) higly susceptible (HS), (b) species of avocado (Persea sp)



Legend

P cinerascens (R) *P cinerascens* (R)

P americana (S) 1 U7(HS) 2

Figure 3 7 Rate of mycelial growth of *Phytophthora cinnamomi* towards callus of a resistant (R) and susceptible species/selection (S/HS) of avocado (Persea sp)

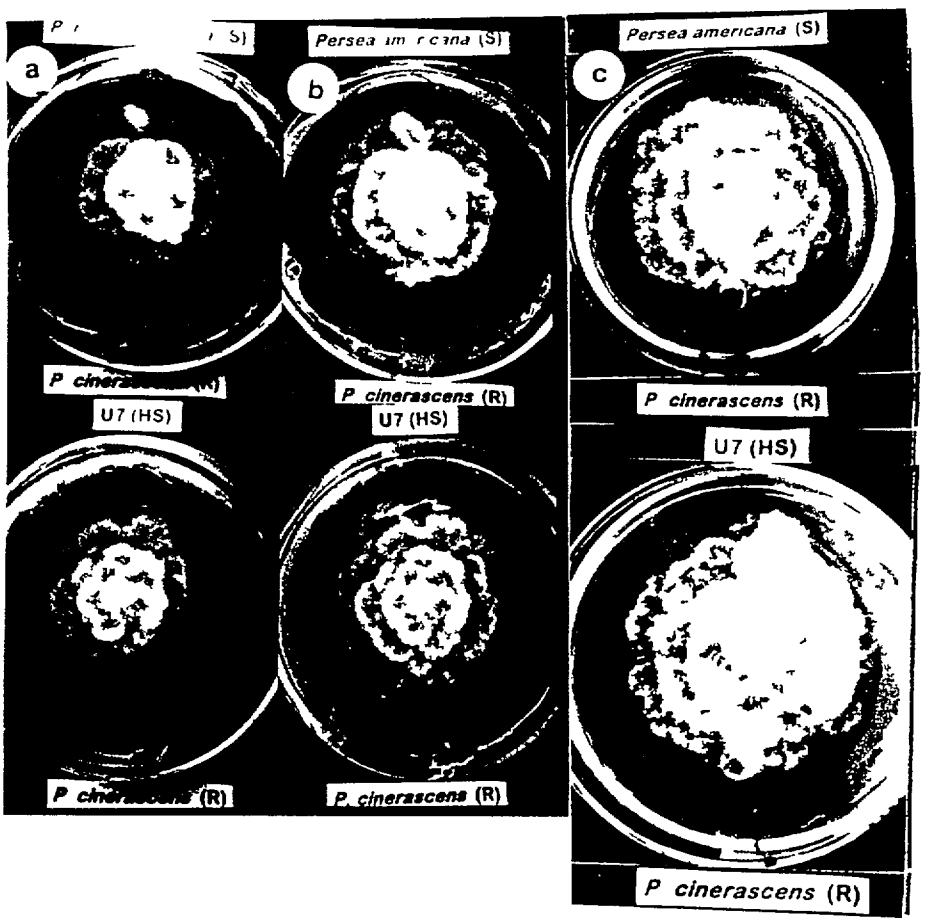


Fig 3 8 Mycelial growth of *Phytophthora cinnamomi* on calli of avocado (*Persea*) species/selection resistant (R), *P cinerascens* and susceptible (S/HS), *P americana* and U7, to *Phytophthora* root rot after 5 (a), 6 (b) and 14 (c) days of incubation at 25 C, dark condition

Conclusion

Based on the results obtained, a correlation of resistance between *in vivo* and *in-vitro*-based avocado varieties/selections exists using the mycelial attraction to the callus Mycelial attraction to the callus could be an alternative technique in screening for *Phytophthora* root rot resistance in avocado

3 6 Screening of Avocado for Resistance to Root Rot

Several approaches to control root rot disease in avocado have already been established These include use of mounds or ridges for planting new trees (Coffey, 1987), addition to soil of microbial antagonist such as *Myrothecium roridum* Tode ex Fr (Gees and Coffey, 1989), foliar spray (Coffey et al , 1984), or trunk injection of fosetyl-aluminum (Darvas et al, 1984) and the use of tolerant rootstocks (Zentmyer, 1980, Coffey, 1989) Although the cultural, biological and chemical controls are potential methods of eliminating or reducing the incidence of the disease, they have limitations In which case, the use of tolerant or resistant rootstock still offers the best opportunity for effective, economical, non-health hazardous and environmentally safe, and sustained control of root rot on avocado

3 6 1 Objective

To identify avocado cultivars or selections that possess resistance to *Phytophthora* root rot

3 6 2 Methodology

A total of 48 selections of avocado collected from various areas of the country were evaluated for their reaction to the disease using the modified detached root inoculation method The inoculation procedure mentioned in Section 3 5 A was followed

The following disease rating scale was used

Length of Colonization	Percent Recovery	Reaction
0 - 2 0	0 - 12 0	HR
2 1 - 4 0	12 1 - 25 0	R
4 1 - 8 0	25 1 - 50 0	MS
8 1 -12 0	50 1 - 75 0	S
12 1 - 16 0	75 1 - 100 0	HS

* HR - highly resistant, R - resistant, MS - moderately susceptible, S - susceptible, HS - highly susceptible

3 6 3 Results

Two replications were conducted on 23 selection and one on 17 Ten roots were used for each replication On 5 selections, less than 10 roots were used From the two selectiond, 5 were found highly resistant and 3 resistant to the disease (Table 3 4) From one replication, 8 were highly resistant and 4 resistant From less than 10 roots evaluated, all were highly resistant All other selections were found either moderately susceptible, susceptible or highly susceptible to root rot

3 6 4 Conclusion

Several local strains or selections of avocado possess resistance to *Phytophthora* root rot in avocado

Table 3 4 Reaction of avocado roots to *Phytophthora* root rot using detached root inoculation method

Selection	Linear Colonization	Per cent Recovery	Reaction[b]
Two replications			
B22	0 20	1 25	HR
B17	0 72	5 40	HR
B31	0 75	5 40	HR
U9	0 80	5 00	HR
B52	1 35	12 50	HR
U8	3 40	23 75	R
B11	3 90	25 85	R
B12	3 90	24 60	R
ST	4 55	32 50	MS
B16	4 60	28 75	MS
B13	4 65	31 25	MS
F1	5 10	33 30	MS
U4	5 55	45 00	MS
B18	5 70	37 10	MS
B14	5 90	41 25	MS
B15	6 00	40 00	MS
U6	6 00	43 30	MS
F2	6 40	46 70	MS
U11	7 50	68 65	S
B2	9 60	56 70	S
U7	8 40	88 80	HS
U5	8 75	73 40	HS
U2	11 7	83 75	HS
One replication			
U12	0	0	HR
B23	0	0	HR
B72	0	0	HR
B41	0 30	3 30	HR
B42	0 80	5 00	HR
B62	0 80	5 00	HR
B79	1 60	10 00	HR
U10	2 00	12 50	HR
U3	2 00	18 30	R
B44	2 80	12 75	R
B78	2 80	17 50	R
B43	3 60	22 50	R
B1	7 00	46 70	MS
T2	7 70	58 40	MS
Y1	8 10	60 90	S
T1	9 93	65 60	S
B3	9 90	69 20	S
Less than 10 roots			
B53	0	0	HR
Q1	0 20	1 25	HR
B77	0 57	3 57	HR
B75	0 80	5 00	HR
B61	1 14	7 14	HR

[a] After 48 hr incubation at 25 C dark condition

b HR - highly resistant R - resistant, MS - moderately susceptible S - susceptible HS - highly susceptible

4 0 Isolation, purification and partial characterization of *P cinnamomi* necrosis-inducing factor

4.1 Introduction

Phytophthora root rot is the most damaging disease of avocado and causes significant yield loss It is caused by the soil-borne pathogen *P cinnamomi* that infects roots, causes necrosis, and eventually death of host tissues (Davison et al, 1994) Necrotrophic fungi are believed to produce non-host specific toxins and cell wall degrading enzymes that aid in disease infection (Oliver and Osbourne, 1995)

In 1980, Woodward et al reported that polysaccharides isolated from *P cinnamomi*, *P cryptogea* and *P nicotinae* caused wilting in Eucalyptus The secretion of toxins by other *Phytophthora sp* such as *P drechsleri*, *P citrophthora*, P fragariae and *P cactorum* have been reported (Strange et al , 1989, Breiman and Barash, 1981, Breiman and Galun, 1981, Rowland et al , 1993 and Plich and Rudnicki, 1979) However, the chemical nature and mechanism of the toxin have not been fully described

Crude filtrates from *Phytophthora* were tried as tools for *in vitro* selection Behnke (1979) successfully used crude filtrates of *P infestans* to select resistant calli and regenerate resistant potato plants On the other hand, Vardi and co-workers (1986) found that crude filtrates of *P citrophthora* to be ineffectual in *in vitro* selection of citrus variants

The best examples of fungal toxins are host specific (Walton, 1996) and of low molecular weight that functions as positive agents of virulence or pathogenecity However, the reported fungal toxins reported from *Phytophthora* spp are not host specific in nature The term fungal toxins will not be used in this study and instead, the term "necrosis-inducing factor" will be used to denote substances produced by the pathogen that will aid in the penetration and colonization of susceptible host tissues The objective of this study is to isolate, purify and characterize necrosis-inducing factors from *P cinnamomi*

4 2 Materials and Methods

4 2 1 Production of *P cinnamomi* culture filtrate

Fifty ml of basal synthetic medium (Maas, 1973) in a 125 ml flask was inoculated with a single *P cinnamomi* agar plug taken from the edge of 5-7 day-old culture grown in potato-dextrose agar (PDA) plate Cultures were incubated at 25°C with shaking (150 rpm) under darkness After 60 days, culture filtrates (CF) were filtered (Whatman No 1) to remove the mycelia

4 2 2 Fractionation of *P cinnamomi* culture filtrates

The filtrates were fractionated into high and low MW components using an ultrafiltration A pressurized vessel (Amicon) with an integral magnetic stirring bar was used to pass low MW components through a YM1 membrane This membrane retains solutes above 1000

4 2 3 Enzyme activity assays

Polygalacturonase assay and cellulase assays were done following the procedure of Ofuya and Wood (1981) as modified by Flor Cruz (1995) For the polygalacturonase assay, 0 1

ml of crude filtrate was mixed with 0 9 ml of 0 1 M acetate buffer pH 5 5 and 1 ml of 1% polygalacturonic acid in buffer The solution was incubated at 30°C for 1 hour after which 0 5 ml DNS reagent was added The solution was vortexed then incubated in boiling water bath for 10 min Five ml water was added, the mixture was allowed to cool and absorbance was read at 546 nm Basically, the same procedure was used for the cellulase assay using 0 1 M citrate buffer pH 5 0, 1% carboxymethylcellulase as substrate and 24 hours incubation time

Protease assay was based on the method by Drapeau (1978) as modified by Lapis (1993) Crude filtrate was added to a pre-incubated (37°C, 5 min) mixture of azocasein solution and 0 02 M Tris-HCl pH 7 5 After incubating the samples for 24 hrs, TCA was added to stop the reaction Tubes were left undisturbed for 10 min then filtered using Whatman No 1 Equal volumes (2 5 ml each) of the supernate and 0 5 N NaOH were mixed and the absorbance was read at 440 nm

4 2 4 Thin Layer Chromatography

One hundred ml each of *P cinnamomi* CF, high MW and low MW fractions were mixed with an equal volume of ethyl acetate to extract polyphenols The mixture was placed in a separatory funnel, shaken and allowed to stand at room temperature After two hours, the organic layer was collected and concentrated to dryness using a rotary evaporator The phenolics were dissolved in minimum amount of petroleum ether/methanol and a single spot of extract was applied on a TLC plate (plastic sheets of silica gel 60 F254) Plates were developed in ethyl acetate petroleum ether (3 1 v/v)/ chloroform acetic acid (9 1 v/v) and viewed using a UV light

4 2 5 Determination of Phenolic Content

Total polyphenol content was determined using the total phenol analysis by Folin and Ciocalteau (1927) The protein precipitable method (Hagerman and Butler, 1978) was used to estimate condensed and hydrolyzable tannins and the acidified vanillin assay (Broadhurst and Jones, 1978) was followed to determine the amount of flavan type phenolics

4 2 6 Bioassay of fractions

Fungal fractions were incorporated into MS media to test if they could be used for *in vitro* disease resistance screening and/or induction of resistance (see Study 5) Briefly, *P cinnamomi* CF, high and low MW fractions were incorporated into tissue culture media to obtain 0, 20, 40, 60 and 80% concentration Calli were placed in the medium and observed for response To test for heat stability of the necrosis-inducing factor, *P cinnamomi* CF and fractions were autoclaved at 121°C for 20 minutes

4.3 Results and Discussion

4 3 1 Optimization of culture medium

To be able to isolate and purify the necrosis-inducing factor from *P cinnamomi*, it was necessary to determine in which media fungal production would be optimum Seven different media were tested V8 Juice, pea broth (PB), mineral salts medium (MS), avocado root decoction (RD) medium, basal synthetic medium (BSM), BSM plus chopped avocado roots (BSM^{+}) and glucose-asparagine medium (GAM) Cultures were incubated at 25°C with shaking under darkness

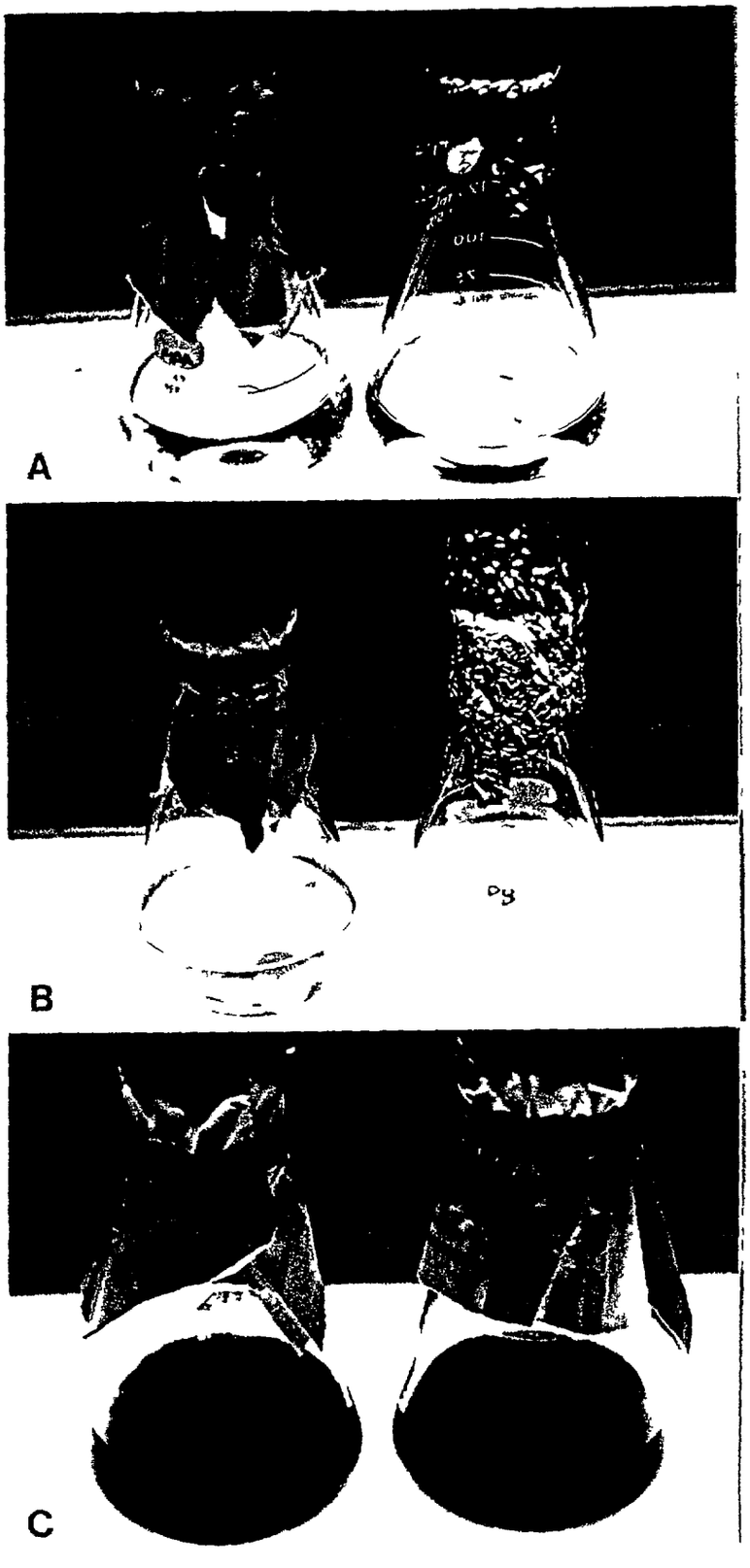


Figure 4 1 Growth of *P cinnamomi* in different media (A) Mineral salts medium, (B) pea broth and (C) root decoction method Left flask is inoculated with the fungus, right flask is uninoculated (control)

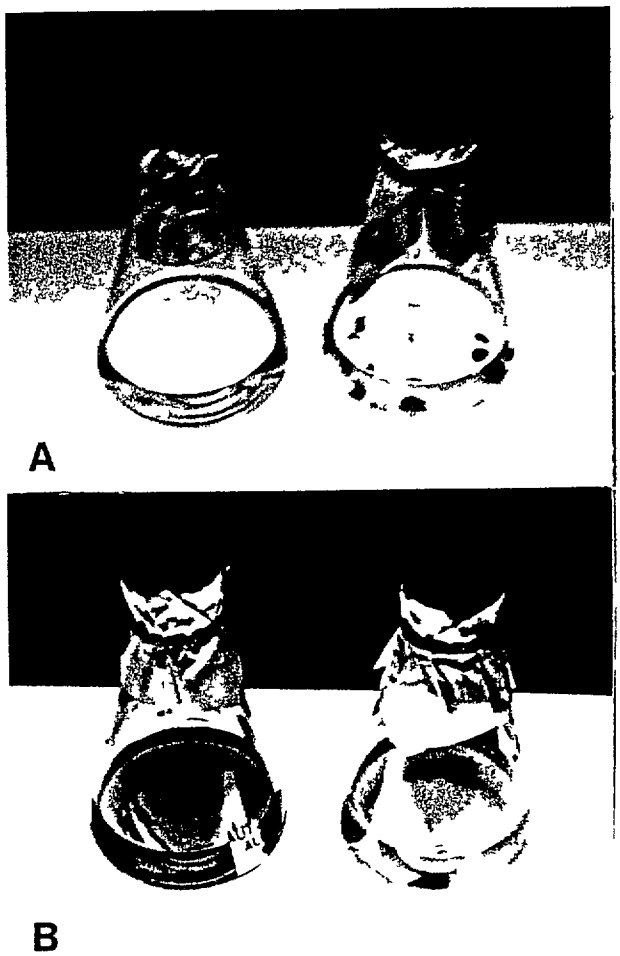


Figure 4 2 Growth of *P cinnamomi* in bazal synthetic medium (A) and in bazal synthetic medium with chopped avocado roots Left flask is uninoculated (control), right flask is inoculated with the fungus

The first media tested was V8 juice, the media most commonly used to grow *P cinnamomi* The CF grown in V8 Juice was fractionated into high and low MW components using ultrafiltration with a YM1 membrane RCF Purple calli inoculated with at least 20 µL high MW fraction and low MW fraction showed some degree of browning compared to the control (uninoculated) However, callus treated with the same amount of media (V8 juice) had the same color change implying that a component of V8 juice also causes necrosis Since the bioassay is based on the browning effect of the necrosis-inducing factor, V8 juice is therefore not a good media

After trying V8 juice, five other media were tested *P cinnamomi* grown in MS was white and strand-like in appearance while that from BSM, BSM$^+$, PB and RD where globular However, the size, color and number of globules differed in the three latter media 1-2 relatively small dark red globules in RD and BSM$^+$, 5-6 big yellow globular mass in PB and 5-7 big yellow globules in BSM Based on these observations, it is clear that BSM is the best media for growing *P cinnamomi* This was also the media used in growing *P fragariae* by Rowland et al (1993)

4 3 2 Production of Polysaccharide

To check if *P cinnamomi* produces a wilt-inducing polysaccharide (Woodward et al, 1980) the fungus was grown in GAM No slimy material was observed in the culture visually However, it is possible that *P cinnamomi* produces only small amounts of the polysaccharide *in vitro* compared to *in vivo* conditions

4 3 3 Synthesis of cell wall degrading enzymes

The production of cell wall degrading enzymes may play a role in disease infection Polygalacturonase, cellulase and protease activity were determined to check if *P cinnamomi* secretes cell wall degrading enzymes

After two days of culture, polygalacturonase (PG) activity was already detected Results show a relatively high PG activity (20X) in BSM than in other media tested (Table 4 1) PG activity was higher in BSM than in BSM$^+$ (Table 4 2) The inclusion of roots in the media did not seem to stimulate the production of cell wall degrading enzymes Activity for this enzyme increased with prolonged culture of the fungus in BSM and was highest after 8 weeks of culture Glucose-asparagine medium (GAM) was also tried but no enzyme activity was detected

Data show that there is positive correlation between PG activity and long term mycelial growth of the fungal pathogen No induction or stimulation of PG activity in the presence of susceptible root tissues suggests that PG is not involved directly or indirectly in host penetration through the roots However, PG might be involved in host colonization once the pathogen is inside the susceptible host tissue

Studies by Le Cam et al (1994) show that *Mycocentrospora acerina*, the causal agent of root rot in carrots, secretes polygalacturonase, pectin methylesterase, pectate lyase and endoglucanase enzymes *in vitro* The secretion of pectate lyase and endoglucanase were observed but only after one week of culture High PG was observed *in vitro* but in a follow-up study (Le Cam et al, 1997), weak PG activity was observed in infected carrot roots

For the two other cell wall degrading enzymes tested, less than 1 nanomole/ml/min of cellulase activity was obtained and protease activity was negligible

Table 4 1 Polygalacturonase activity of *P cinnamomiamomi* grown in four different media

LENGHT OF CULTURE (Weeks)	POLYGALACTURONASE ACTIVITY (nm/ml/min)			
	BSM	PB	MS	RD
1	228	6	0	4
2	-	8	0	3
3	260	10	0	0
4	-	-	0	0
5	587	11	0	<1
6	561	0	0	0
7	602	1	0	0
8	792	27	11	38

Table 4 2 Polygalacturonase activity of *P cinnamomiamomi* (nm/ml/min)in Basal Synthetic Medium (BSM) and BSM plus chopped avocado roots

LENGTH OF CULTURE (Weeks)	POLYGALACTURONASE ACTIVITY	
	BSM	BSM^{+}
1	37	15
2	78	8
3	156	44
4	217	44
5	225	37
6	310	87
7	330	172
8	435	258

4 3 4 Low molecular weight compounds

Initial results from TLC showed a similar chromatographic profile for the crude filtrate, high MW and low MW fractions There were two spots observed in all three samples one that co-migrated with the ethyl acetate petroleum ether solvent and another at the point of origin These observations imply that the some of the low molecular weight compounds are very hydrophobic while the others are hydrophilic

The hydrophilic bands were scraped off the plates and extracted using ethyl acetate Extracts were evaporated to dryness, dissolved in methanol and run on silica gel plates with chloroform-acetic acid as solvent system Two bands were observed for the crude filtrate while both high and low MW samples had four co-migrating bands

The hydrophobic bands will also be extracted and run in TLC plates using a different solvent system All unique bands present in the samples will be tested using the detached root method developed by the pathology group

4 3 5 Phenolic content

To determine whether the low MW fraction is phenolic in nature, it was subjected to total phenol and precipitable protein analyses The first assay estimates the amount of simple phenols, non-tannin flavans and tannins while the second determines the amount of protein-precipitating tannins Both assays gave negative results However, using the vanillin assay, 0 093 mg catechin equivalent /ml was obtained from the low MW fraction

Data suggest that the majority of the components of the low MW fraction may not be phenolic in nature However, a positive test using the vanillin assay (specific for flavan-type phenolics) might also suggest that one of the minor components of the low MW fraction is flavan phenolic Because of the heterogeneity of the low MW fraction, it is important to fractionate it further by using different solvent systems of TLC One probable reason for the negative tests of phenolic compounds is that the other components might be interfering in the colorimetric assays

4 3 6 Effect of *P cinnamomi* filtrate on avocado callus

Table 4 3 summarizes the effect of *P cinnamomi* CF and fractions on avocado calli The CF was autoclaved to test the heat stability of the necrosis-inducing factor and to determine its nature At temperatures more than 100°C, polysaccharides are inactivated and most proteins are denatured Since the autoclaved filtrate (AF) still caused necrosis in avocado calli, then the necrosis-inducing factor is not polysaccharide in nature but could be a heat-stable protein In contrast, the toxic activity of *P drechsleri* on protoplasts was completely lost upon heat treatment (Shohet and Strange, 1989) Partial purification of the toxin revealed three major bands of 31, 36 and 48 kD after polyacrylamide gel electrophoresis The bands stained positive for both protein and carbohydrates implying that the toxin molecule has a peptide and a carbohydrate part Studies by Breiman and Barash (1981) also report a toxin from *P citrophthora* as glycopeptide

The ability of low MW fraction to cause browning suggests that the necrosis-inducing factor could be a polyphenol A study by Breiman and Galun (1981) revealed that the phytotoxic components in *P citrophthora* were of high and low molecular weights It was further reported that the low molecular weight fraction was acidic and hydrophilic Experiments are underway to further purify and characterize the necrosis-inducing factor

Table 4 3 Browning effect of P cinnamomi on avocado calli

P cinnamomi	Necrosis of calli	Possible nature of VF
Crude filtrate (CF)	Yes	
Autoclaved filtrate (AF)	Yes	Heat-stable protein
High MW fraction	Yes	Heat-stable protein
High MW fraction (autoclaved)	Yes	
Low MW fraction	Yes	Phenolic
Low MW fraction (autoclaved)	Yes	

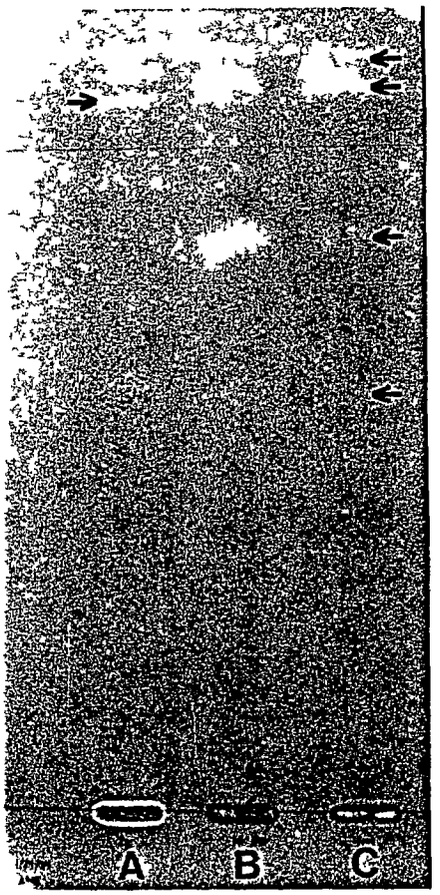


Figure 4 3 Chromatogram of *P cinnamomi* samples using chloroform-acetic acid (9 1) as eluent (A) crude filtrate, (B) high MW and (C) low MW

4 4 Conclusion

Two major components of the necrosis-inducing factor in the crude filtrates of *P cinnamomi* are the cell wall degrading enzyme polygalacturonase and low MW compounds High activity of polygalacturonase was evident as early as two days after start of culture and increased afterwards This suggests an important role for polygalacturonase in the pathogenesis of avocado by *P cinnamomi*

We are still in the process of isolating the components of the low MW fraction that causes tissue necrosis This component is also heat stable as heat-treated fractions still cause necrosis of callus tissues

5 0 Induction of Root Rot Resistance in Avocado by Somaclonal Variation and *in Vitro* Directed Selection and Analysis of Putative Variants

Objectives

1. To induce root rot resistance through somaclonal variation without using *P cinamomi* crude filtrate/toxin
2. To induce root rot resistance through *in vitro* directed somaclonal variation using *P cinamomi*
3. To induce root rot resistance using directed somaclonal variation using *P cinamomi* culture filtrate/toxin
4. To analyze putative variants

5 1 *In vitro* selection of calli for resistance to *P cinamomi* crude filtrate

To induce resistance in cell cultures, embryogenic calli of avocado cv RCF Purple were cultured on MS medium + 2mg/L BAP + 1 mg/L IBA (standard callus maintenance medium) supplemented with 20, 40, 60 and 80% of the following BSM (Basal salt medium, Maas, 1973) alone, BSM + avocado roots uninoculated, BSM + roots inoculated with *P cinamomi*, and crude filtrate of *P cinamomi* The addtional components were either filter sterilized or autoclaved Cultures were maintained in an airconditoned growth room (25±2°C) under 16h light Callus regrowth was assessed after 15 days of culture Surviving calli from all treatments were subcultured onto fresh standard maintenance medium every 4-6 weeks for 3 subculture cycles

Addition of *P cinamomi* either as inoculated roots or crude filtrate to standard medium inhibited callus regrowth by about 20% compared with treatments containing BSM alone or BSM + uninoculated roots (Table 5 1) The regrowth of callus was lower when *P cinamomi* components were filter sterilized than when autoclaved Some components of BSM and avocado root tissues were also inhibitory to callus regrowth as indicated by 31 to 63% regrowth compared to 80% regrowth observed on calli grown on standard medium The reduced callus regrowth with treatments containing *P cinamomi* indicates that some substances produced by the organism were inhibitory to callusing (Fig 5 1)

Surviving calli from different treatments were excised and cultured on standard callus maintenance medium for 3 subculture cycles Embryogenesis ranging from 33 to 80% was observed on calli initially cultured on media with BSM +inoculated roots while calli obtained from treatments with crude filtrate showed embryogenesis of 33 to 76% (Table 5 2) Some embryogenic calli obtained from treatments with *P cinamomi* showed continuous growth as indicated by the increase in the number of callus cultures Most embryogenic calli showed differentiation of shoot-like structures Other surviving calli turned brown and died during subsequent subculturing The reaction of these calli and shoot-like structures to *P cinamomi* will be determined using the method developed in Section 2

Table 5 1 Regrowth of RCF calli cultured on medium with or without *P cinamomi* taken after 15 days of culture

Media Treatment	% Callus regrowth	
	filter sterilized	Autoclaved
MS + 2 mg/L BAP + 1 mg/L IBA (standard medium)		80
20% BSM + standard medium	33	75
40% BSM + standard medium	50	50
60% BSM + standard medium	50	75
80% BSM + standard medium	33	50
Mean response	**42**	**63**
20% BSM + root uninoculated +standard medium	50	25
40% BSM + root uninoculated +standard medium	100	25
60% BSM + root uninoculated +standard medium	100	50
80% BSM + root uninoculated +standard medium	75	25
Mean response	**81**	**31**
20% BSM +root inoculated with P Cinamomi + standard medium	40	50
40% BSM +root inoculated with P Cinamomi + standard medium	25	75
60% BSM +root inoculated with P Cinamomi + standard medium	25	25
80% BSM +root inoculated with P Cinamomi + standard medium	0	0
Mean response	**23**	**38**
20% P Cinamomi crude filtrate + standard medium	20	25
40% P Cinamomi crude filtrate + standard medium	25	25
60%P Cinamomi crude filtrate + standard medium	16	25
80% P Cinamomi crude filtrate + standard medium	25	50
Mean response	**22**	**32**

Table 5 2 Regrowth of surviving RCF calli obtained from different media treatments containing *P cinamomi* after 3 subculture cycles on standard callus medium

Media Treatment Source	No of surviving callus cultures obtained from *P cinamomi* media treatments	No of callus cultures after 3 cycles on standard medium	% Cultures showing embryogenesis
20% BSM + standard medium	2	-	-
40% BSM + standard medium	3	3	33
60% BSM + standard medium	4	11	63*
80% BSM + standard medium	6	9	100*
20% BSM + root uninoculated +standard medium	2	4	75
40% BSM + root uninoculated +standard medium	10	12	75*
60% BSM + root uninoculated +standard medium	3	6	83
80% BSM + root uninoculated +standard medium	3	4	100*
20% BSM +root inoculated with P Cinamomi + standard medium	3	5	80*
40% BSM +root inoculated with P Cinamomi + standard medium	7	3	33
60% BSM +root inoculated with P Cinamomi + standard medium	2	3	66
80% BSM +root inoculated with P Cinamomi + standard medium	4	7	71*
20% P Cinamomi crude filtrate + standard medium	4	-	-
40% P Cinamomi crude filtrate + standard medium	2	3	33
60%P Cinamomi crude filtrate + standard medium	9	13	76*
80% P Cinamomi crude filtrate + standard medium	2	4	75

*Cultures showing differentiation of shoot-like structures

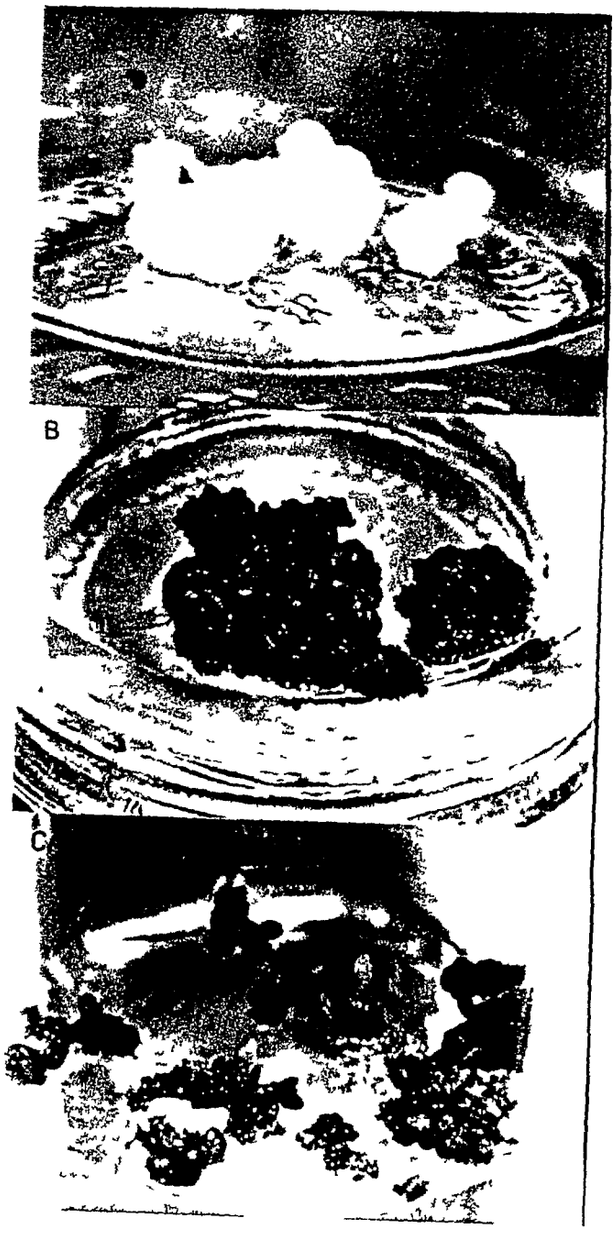


Fig 5 1 Response of RCF calli to medium containing *P cinamomi* crude filtrate (A) Initial callus explant used for *in vitro* selection (B) Browning of explant on medium with P *cinamomi* (C) Regrowth and occurrence of embryogenesis from calli initially grown on medium with *P cinamomi* upon culture on standard callus medium

5 1 1 Conclusion

Embryogenic calli from treatments with *P cinnamomi* showed continuous growth, most embryogenic calli showed differentiation of shoot-like structures The reaction of these calli and shoot-like structures to *P cinnamomi* will be determined using the developed method

5 2 Testing resistance of calli to *P cinnamomi* by mycelial attraction method

Long term callus cultures of avocado genotypes/accessions RCF Purple, Sta Cruz and U2, U6, U7 and U8 will be tested for reaction to *P cinamomi* using the method developed in Study 2

The Israeli team

1 Tissue culturing of *Persea* species

1 1 Background

Tissue culture system being aseptic and fully controlled have been advantageously used for studying several physiological aspects of avocado (*Persea americana* Mill), such as cytokinin biosynthesis (Blumenfeld and Gazit, 1971), and response of flower buds to environmental conditions (Schroeder, 1975a) Shoot cultures have been used for an efficient proliferation of horticulturally important clonal varieties of avocado and other *Persea* species (Barringer et al , 1996) *P schiedeana* and *P americana* cv Duke 7 were clonally micropropagated as rootstocks resistant to the root rot disease in avocado orchards (Cooper, 1987, Gonzales-Rosas et al , 1985, Harty, 1985) Parameters such as calli viability, elongation of stem, leaf and axillary bud (Young, 1983), and the morphogenesis capacity (Pliego-Alfaro et al , 1987) were characterized for optimization of *in vitro* shoot propagation (Schroeder, 1975b, Schall, 1987) The rapid *in vitro* propagation of the vegetative material was utilized for elimination of viroid-like organisms causing sunblotch disease (Nel and Kotze, 1982)

Shoot cultures of *P indica*, a highly susceptible host of the root rot pathogen *P cinnamomi*, were used to study the mode of pathogenesity of the indicated disease under controlled conditions (Nel and Kotze, 1982, Nel et al , 1982, 1983)

Avocado callusing and morphogenesis were done mostly by using the MS basal medium (Murashige and Skoog, 1962), supplemented with sucrose, vitamins and combinations of cytokinins and auxins (Barringer et al , 1996, Gonzales-Rosas et al , 1985, Mooney and VanStaden 1987, Nel et al , 1983, Pliego -Alfaro and Murashige, 1988, Pliego-Alfaro et al , 1987, †Pruski et al , 1996, Schall, 1987) However, other media such as WPM (woody plant medium, Lloyd and McGown, 1980, Cooper, 1987), Anderson mineral salts and vitamins (Young, 1983) and Dixon and Fuller medium (Harty, 1985) have also been used for *Persea*††*in vitro* callusing and micropropagation

Embryogenic avocado callus was developed from immature zygotic embryos explants on MS medium containing the auxin picloram (Mooney and VanStaden, 1987, Pliego Alfaro and Murashige, 1988) However, the capability for *in vitro* regeneration of many woody species including avocado, is restricted and seems to be limited to juvenile material (Pliego-Alfaro et al , 1987) A procedure of repetative somatic embryogenesis from cell suspension cultures of avocado was used (Cruz-Hernandez et al , 1998) for genetic transformation However, in fact, regeneration of a complete and normal plant from *in vitro* cultures of avocado on a large scale has not yet been achieved Only a small percentage of green shoots and normal leafy plantlets were regenerated (Mooney and VanStaden, 1987, Pliego-Alfaro and Murashige, 1988)

1.2. Plant sources for tissue culturing

Calli have been derived from 8 *Persea* species and varieties that have experienced varying levels of resistance to *P cinnamomi* (Table 1) *P cinerascens* (Zentmyer 1980) and *P borbonia* (Zentmyer and Schroeder 1954) were considered highly resistant to *P cinnamomiÆ†P indica* (Nel et al 1983) and the avocado (*P americana*) cv Topa topa were considered susceptible *P schiedeana* var G755 was considered moderately resistant (Zentmyer and Schieber 1992) Avocado vars VC 6, VC 51 and VC 66 are West-Indian vegetative clones, which were originally selected as rootstocks tolerant to soil stress (Ben-Ya'acov 1993) Under heavily infested soil conditions, VC 6 and VC 51 were†found to be susceptible and VC 66 found to be relatively tolerant to *P cinnamomi* (Zilberstaine et al 1992, Ben-Ya'acov and Zilberstaine, unpublished data)

The *P borbonia* †*P cinerascens* and *P schiedeana* var G755 used in the present study were 4-7-year-old trees that were grown outdoors in the germplasm collection plot at the Volcani Center, Bet Dagan, Israel The *P indica, P americana* cv Topa topa and *P americana* vars VC 6, VC51 and VC66 were seedlings that were grown in 2-4-l pots of soil, under growth chamber conditions of 25^0C, 12-h photoperiod provided by cool white fluorescent lamp (13-15 µmol m^{-2} s^{-1}light intensity) and 60% relative humidity

1 3 Effect of explant source on callus induction

Several plant parts of *Persea* species and varieties were tested for their callus induction potential (Table 2) All varieties were cultured on medium (i), except for RCF Purple, which was cultured on medium (iv) The axillary bud and the leaf petiole were found to be the best for inducing calli for all varieties except for *P borbonia* calli which were better

induced from mature seed Consequently, petioles were used routinely for callus induction

Using a common explant tissue for culturing of five different *Persea* species, gives us a good chance to use the same explant successively for culturing other *Persea* species and varieties necessary for studying of their *in vitro* response to *P cinnamomi* inoculation

Table 1 The response of *Persea* species to *Phytophthora cinnamomi* †

Plant	Response
P cinerascence	resistant
P borbonia	resistant
P schiedeana x *P americana* G755	moderate resistant
P americana Topa topa	susceptible
P indica	susceptible
P americana VC 6	susceptible
P *americana* VC 51	susceptible
P americana C 66	moderate resistant

1 4 Effect of medium composition on callus induction and development

Four different compositions of callus induction medium were examined (i) MS basal medium (Murashige and Skoog, 1962, Sigma) 4 4 g, 30 g sucrose, 0 1 g myo-inositol, 100 µg each of thiamine -HCl, pyridoxine-HCl, nicotinic acid-HCl and 4-amino-3,5,6 trichloropicolinic acid (picloram, Aldrich) (per liter) Vitamins were filter-sterilized separately and added to previously autoclaved medium (Prusky et al , 1996) (ii) MS basal medium 4 4 g, supplemented with 30 g sucrose, 0 1 g myo-inositol, 400 µg thiamine-HCL ,1 mg 6-benzyl aminopurine (BAP), 2 mg naphthalene acetic acid (NAA), 10 mg 2,4-dichloroacetic acid (2-4, D), 2 5 g charcoal per liter (iii) WPM basal medium (Sigma), 30 g sucrose, 0 1 g myo-inositol and 50 µg 2,4-D (per liter) (iv) MS basal medium with macro-nutrients at half-strength, sucrose 30 g, myo-inositol 0 1 g (per liter) and various concentrations of 2,4-D and BAP

The pH of all media was adjusted to 5 7 before autoclaving Medium (i) was solidified with Phytagel (3 g/l, Sigma), the other media were solidified with Bacto-agar (4 2 g/l Difco) Cultures were incubated at 25-28^{0}C under light (17-35 µmol m^{-2} s^{-1}, 16 h

photoperiod) or dark conditions, at 60% r h The experiments were set up in a completely randomized design arranged in factorial treatments Subculturing was performed every 3-4 weeks

The effect of medium composition on callus initiation showed that medium (i) was the most suitable and yielded approximately 100% callusing of the explants in the varieties examined White undifferentiated tissue developed 7-14 days after placement on the medium, at the edges of the cut borders of the explant, whether petiole or seed Some 2-3 weeks later when calli reached 0 5-1 0 cm diameter, they were cut from the explant and subcultured on medium (i) The developed yellowish-white calli were grown for at least 4 weeks, before they turned brown and ceased to develop Media (ii) and (iii) gave very poor callus induction, and browning occurred within 2 weeks In medium (i), some of the varieties (*P cinerascens, P schiedeana* var G755, *P americana* cv Topa topa, *P indica* and *P americana* VC 66) developed white, hard compact calli after a few months of subculturing, and had to be cut with a scalpel for the continued routine subculturing *P borbonia* calli were compact and soft, *P americana* vars V C6 and V C51 calli were friable and breakable

Subculturing on medium (i) was done by transferring 6-10 mm diameter pieces of callus onto fresh medium, although for most species (all except VC 6), smaller calli pieces of 3-5 mm diameter developed into calli as well

Callus growth was measured as added fresh weight (Fig 1) The generation time was calculated as the time elapsing until double weight attainment at the log growth phase According to the growth rate data, the proper subculturing intervals and timing for establishment of suspension cultures were determined for the different species and varieties

The most rapidly growing calli were vars VC 66,Topa topa and G755 No relationship was found between callus growth rate and either callus color or plant resistance to *P cinnamomi* However, there was a positive correlation between callus growth rate and the ability to grow as suspension cultures Calli with rapid exponential growth phase were subcultured at shorter time intervals

1 4 Development of cell suspension

†Cell suspensioning was performed on liquid medium (i) All varieties except *P americana* var VC6 developed suspensions At 2 weeks after subculturing, the translucent liquid phase turned opaque gradually Cell concentration increased in 4 weeks from approximately 3-8x10^2 cells/ml to app 5-15x10^4 cells/ml (packed volume of 2-5%) At that stage, cell suspension was spread over solid medium (i) (0 1 ml of suspension, 5-15x10^3 cells per petri dish of 90x15 mm) Cells growth for 4-5 weeks on solidified medium yielded a visible layer of cells In some parts of the cell layer, cell clusters developed more than in others, and small calli appeared after additional 4-6 weeks

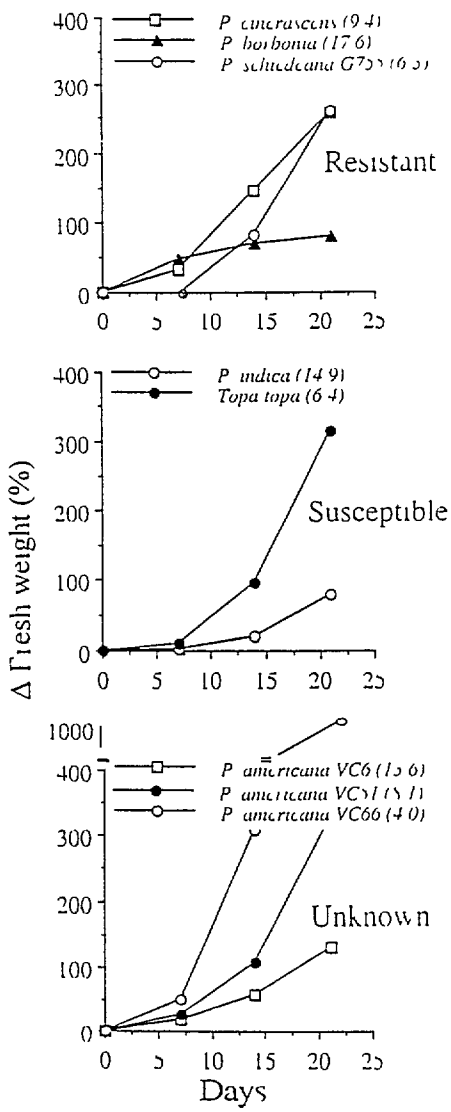


Fig 1 Growth of Persea calli Growth is expressed as percent added fresh weight over initial weight Numbers in parentheses designate the generation time in days as calculated at the exponential phase of the culture growth

Table 2 Effect of plant source explants from various *Persea* species and varieties on callus induction

Plant source	Bud	Petiole	Stem	Seed	Blade
P cinerascens	++	++	+	*	+
P borbonia	+	+	-	++	-
P schiedeana var G755	++	++	*	*	-
P americana cv Topa topa	++	++	-	*	-
P indica	++	++	-	*	-
P americana cv RCF Purple	*	*	*	++	*
P americana var VC6	++	++	*	*	*
P americana var VC51	++	++	*	*	*
P americana var VC66	++	++	*	*	*

*, not tested, +, ++, relative size of healthy callus which developed, -, no callus developed

2 Pathogen culture preparation

2 1 Pathogen isolates

Two isolates of *P cinnamomi* were used for inoculation a high-virulence isolate (HVI), collected at Mishmar-Hae'meq, Israel, and a low-virulence isolate (LVI), collected at Bareqet, Israel (Zilberstaine and Pinkas 1988) Both isolates were grown on a V8 medium One can of commercial V8 juice (295 ml) was mixed with 2 95 g $CaCO_3$ and centrifuged (7000 *g*) for 20 min The supernatant was brought to a final volume of 1475 ml by adding distilled H_2O Medium solidification was done by adding agar 2% The pH was adjusted to 6 4 before sterilization in an autoclave Fungal mycelium was inoculated either directly onto the V8 solidified medium or onto a sterile piece of miracloth, which had been laid over the V8 medium The cultures were incubated at 25°C in darkness

2 2 Production of sterile *P cinnamomi* zoospores

Sterile *P cinnamomi* zoospores were produced in order to examine their attraction and their growth rate onto different tissue cultures and roots of *Persea* species Zoospores

were also used for studying electrical changes and membrane leakage of suspended cell subjected to zoospore inoculation

Procedure for zoospore production

1 Fresh (4 days) culture of the fungus was used as a source for zoospores Few (10-20) pieces (0 5 cm diam in size) of agar V8 with fungal hyphae were cut from the outer edges of the fungal culture and transferred into dishes containing liquid V8 medium diluted 1 9 They were incubated for 16-19 hrs at 25^0C in the dark for mycelial growth

2 Agar pieces were washed 6 times hourly, in mineral solution

Mineral solution Ca(NO3)2 0 01M, KNO3 0 005M, MgSO4 0 004M, Chelated iron 1 ml, in 1000 ml deionized water

Chelated iron EDTA 13 05 g, KOH 7 5 g, Fe SO_4 $7H_2O$ 24 9 g, in 1000 ml of deionized water

3 Fungal mycelium was incubated in mineral solution at room temperature under 2X40 w fluorescent lamps for at least 18 hours

4 Mineral solution was discarded and replaced by sterile dH2O After incubation at 4^0C for 20 min , dishes containing mycelium were put in room temperature Zoospores release started within 30 min , and they were motile for 30-90 min until encysted

3. Inoculation of *Persea* cultures with *P cinnamomi*

Calli were studied for their response to inoculation with *P cinnamomi* Their resistance response was compared to that of the originating source plant Inoculation was done in 4 ways

3 1 Direct inoculation

Calli of diameter of 1 5-2 0 cm were inoculated with the fungal mycelium immediately after being transferred to medium The composition of the callus medium may interfere in the interaction between the host callus and the pathogen fungus To avoid such interference the direct callus inoculation was performed onto a water agar medium

A small piece (1x1 mm) of miracloth supporting 10-12-day-old fungal mycelium was laid directly in the center of each callus Inoculated calli were incubated at 25°C and 60% relative humidity, either in light (12-h photoperiod, 17-35 μmol m^{-2} s^{-1} light intensity) or in darkness

3 2. Indirect inoculation of calli through filter membrane

For indirect inoculation through a membrane,a wet Whatman paper no 1 (90 mm in diameter) was laid in an empty sterile Petri dish A round piece (65 mm in diameter) of miracloth supporting fungal mycelium was laid over the paper and a sterile polycarbonate

membrane (0 4 µm pores, 76 mm in diameter, by Poretics) was laid on top of the miracloth Above the membrane, three or four calli of one *Persea* species were laid Inoculated calli were incubated at 25^{0}C, 60% relative humidity, either in light (12-h photoperiod, 17-35 µmol m^{-2} s^{-1} light intensity) or in darkness

A scheme of indirect inoculation procedure

---------- calli
---------- filter membrane
---------- miracloth with mycelium
---------- wet Whatman paper
_____ dish

Each experiment was performed with all Persea species and the 2 isolated of *P cinnamomi* Results are shown in Fig 7 Results show no clear correlation between the resistance level of callus plant source and calli response to the indirect inoculation, either by HVI or by LVI

3 3. Indirect inoculation: inoculum laid in a distance from calli

Indirect inoculation of the calli was done in order to study the co-influence of vegetative and pathogenic substances on the fungal - calli interaction, and compare it to the resistance reaction of the originating plant The indirect inoculation also enabled us to compare the rate of mycelial colony growth in a petri dish, as influenced by 2 different calli that were laid in its opposite direction and maximal distance from the inoculum

For inoculation, 2 calli were transferred into poor agar medium and laid at the edge of a petri dishes, opposing each other A small (1x1mm) section of miracloth supporting fungal mycelium of 10-12 days old was laid in the center of the dish, at a distance of 4 5 cm from each callus

The fungal colony was followed daily to measure its growth rate in terms of colony diameter, thickness and growth pattern Preliminary results indicated on more vigorous growth of thefungal mycelium toward the resistant callus (see Figs 9,10 in the Intermediate Report for the period of 1 1 96-31 5 97) However, this experiments repeated many time and the results were not reproducible

3 4. Inoculation of calli with zoospores

As inoculation in nature occured by zoospores, we studied whether this was possible also *in vitro* Inoculation was done by introducing zoospores to calli either directly or via glass tube through which we could follow microscopically the zoospore movement

When Persea calli inoculated with zoospores, massive hyphae were developed in them Initial results demonstrated zoospores movement preferably towards resistant derived calli More experiments regarding zoospores movement towards different calli are now in process

4. Quantitative response of necrosis and peroxidase activity for expressing resistance to *Phytophthora cinnamomi* in *Persea* callus cultures

4 1 Background

It has been shown that plant resistance to *P cinnamomi* can be expressed at the callus level in avocado (Phillips et al 1991) and several other native and horticultural species (McComb et al 1987) The rate of hyphal extension across the callus surface was correlated with the susceptibility of the plant from which the callus was derived (McComb et al 1987, Phillips et al 1991) The callus response indicated that the resistance level is determined by physiological or biochemical factors, rather than anatomical ones (Phillips et al 1991) There is evidences that browning is involved in resistance of intact tissue the browning response of roots to inoculation with *P cinnamomi* zoospores was greater in the more tolerant *Eucalyptus* host (Byrt and Holland 1978) Phenolic compounds in mature leaves of several avocado selections were associated with tolerance of these selections to *P cinnamomi* (Brune and van Lelyveld 1982) It has been qualitatively observed that callus cultures derived from resistant species responded to *P cinnamomi* inoculation with fast (Phillips et al 1991) and more intense (McComb et al 1987) browning The browning mechanism and peroxidase activity have been associated with resistance to insects in callus tissue (Dowd and Norton 1995) Systemic increase of peroxidase resulted from *P cinnamomi* infection of avocado roots in tolerant plants (van Lelyveld and Brodrick 1975), suggesting that peroxidases contribute to the browning response through oxidation of phenolic acids, and thereby enhance resistance Low-virulence isolate (LVI) of *P cinnamomi* has been found in Israel (Zilberstaine and Pinkas 1988) by using this and high-virulence isolate (HVI), in a comparative manner it will be possible to improve the characterization of the resistance reaction in the in vitro system

The objective of the present study was to characterize and quantify the responses of necrosis development and peroxidase activity to inoculation with *P cinnamomi* on calli derived from eight *Persea* varieties and species which expressed varying degrees of

resistance to the pathogen The expected results would be integrated into the in vitro selection procedure for new genotypes resistant to *P cinnamomi*

4 2 The evaluation of callus necrosis

The callus color change resulting from the inoculation was evaluated in three ways 1 Visual evaluation callus color intensity was ranked from 0 to 5, with 0 representing non-inoculated control callus and 5 the most necrotic one The necrosis index was calculated by multiplying the color rank by the percentage of callus surface having that color 2 Colorimetric analysis a 3-g of callus tissue was ground in 15 ml of phosphate buffer (0 05 M, pH 7 2), centrifuged (48,000 *g*, 10 min) and vacuum filtered through Whatman no 1 paper The optical density of the filtrate was measured by spectrophotometer at 510 nm 3 Chromameter analysis calli were color analyzed with a Minolta C-200 chromameter, which measures the color reflectance of surfaces Callus color was measured on an 'L' (lightness / darkness) scale, on which a higher value indicates a lower color intensity

4 3 The measurement of peroxidase specific activity in callus

The peroxidase specific activity was determined according to Hammerschmidt*et al* (1982) with modification Two grams of *Persea* calli (14 days after subculturing) were ground with 2 ml of sodium phosphate buffer (0 05 M, pH 6 0) at 4°C The extract was centrifuged (31000 *g*, 20 min) and the supernatant was used as the enzyme fraction

Protein concentration was measured according to Bradford (1976), with Coomassie Brilliant Blue G-250 (Sigma) as a color reagent Protein solution (5-10 µl) was mixed with 3 0 ml of the diluted (x 5) color reagent, and the optical density was determined by spectrophotometer at 595 nm Bovine serum albumin (1-10 µg) was used as a standard A substrate mixture comprising of 100 ml sodium phosphate buffer (0 01 M, pH 6 0), 10 ml guaiacol 1% in ethanol 50%, and 10 ml H_2O_2 0 03% was freshly prepared, and was incubated for 30 min at 25°C A reaction mixture comprising of 1350 µl substrate mixture, 50 µl sodium phosphate buffer (0 05 M, pH 6 0) and 3-5 µg of enzyme fraction, was prepared in disposable spectrophotometer quivettes The reaction was started by addition of the enzyme fraction to the mixture, and the reaction was followed in the spectrophotometer (470 nm) for 3 min Specific activity of Prx was defined as $\Delta OD\ min^{-1}\ mg\text{-}protein^{-1}$, in a range of constant colour change with time

4 4 Effect of *P cinnamomi* on necrosis development in resistant and susceptible *Persea* calli

The objective of the present work was to examine whether the trait of resistance to *P cinnamomi* expressed in intact avocado plants could also be expressed in the derived calli Up to now, no resistant rootstock has been efficient enough to be generally introduced in commercial avocado orchards Therefore, it was necessary to include in our present experiments varieties from other *Persea* species than avocado, that had been considered highly resistant to *P cinnamomi* Such species are *P cinerascens* (Zentmyer 1980) and *P borbonia* (Zentmyer and Schroeder 1954), although they are both graft-incompatible with the commercial fruiting avocado

Previous studies were undertaken to examine the in vitro / in vivo relationship with regard to resistance to *P cinnamomi* in avocado (Phillips et al 1991) and other species (McComb et al 1987) The resistance was assessed by quantitative measurement of the hyphal extension rates across the callus surface In the present in vitro / in vivo comparison system, involving a relatively large sample of eight *Persea* varieties, such measurements were difficult to apply and did not discriminate adequately among the tested varieties (data not presented) Development of necrosis in response to the pathogen inoculation seemed to be easier to detect and to quantify, which is important for a rapid selection procedure This†approach was evidently justified since browning development and increased peroxidase activity have been found to be associated with the resistance response (Brune and van Lelyveld 1982, Byrt and Holland 1978, Dowd and Norton 1995 McComb et al 1987, Phillips et al 1991, van Lelyveld and Brodrick 1975)

Calli derived from plants expressed varied degrees of resistance to *P cinnamomi*, developed necrosis at differing speeds and color intensities in response to direct inoculation with the HVI of the pathogen (Figs 2, 3) Calli that were derived from resistant plants (*P cinerascens* and *P borbonia*) developed necrosis that started within the first day of incubation in darkness By 1 day after inoculation, the necrosis spots were 1-2 mm in diameter, and their brown-black color sharply contrasted with the white surroundings tissue Within the next 2 days the necrosis borders spread rapidly (3 1-4 5 necrosis index units per day) towards the edges of the calli The fungal hyphal growth was not confined within the necrotic surface area, and it reached the agar medium within approximately 3 days after inoculation The necroses at the inoculation sites of the intermediately resistant species (*P shiedeana* var G755) were light brown, with no sharp margins between necrotic and viable tissues (Fig 2) The experimental rootstock VC 66 reacted similarly to G755, as intermediately resistant, with reddish-brown necroses (Fig 2) No necroses developed within the first 2 days after inoculation in calli derived from the susceptible experimental variants VC 6 and VC 51, the color remained unchanged, similarly to the non-inoculated control calli (Fig 2) However, some necroses in VC 51 started to become visible within the next 2 days (Fig 3) Calli derived from the susceptible plants (avocado cv Topa topa and *P indica*) started to develop necroses at low rates (0 9-1 3 necrosis index units per day, Fig

3) within the second day The necrosis on Topa topa had a dark brown-black color and its spread in the callus was very limited (Fig 2) The time in which 50% of the callus volume turned necrotic (NI 50) was 2 2-3 6 days for the calli of resistant and moderately resistant origin and about 5 5 days for the susceptible ones (Table 3)

It can be concluded that plant-derived calli can express different levels of plant resistance following direct inoculation with the common HVI isolate of *P cinnamomi* (Figs 2 3 Table 3) Calli of resistant sources *P cinerascens* and *P borbonia*) reacted rapidly with tissue necrosis following the inoculation while the necrosis reaction of calli derived from moderately resistant sources (*P shiedeana* G 755 and *P americana* VC 66)

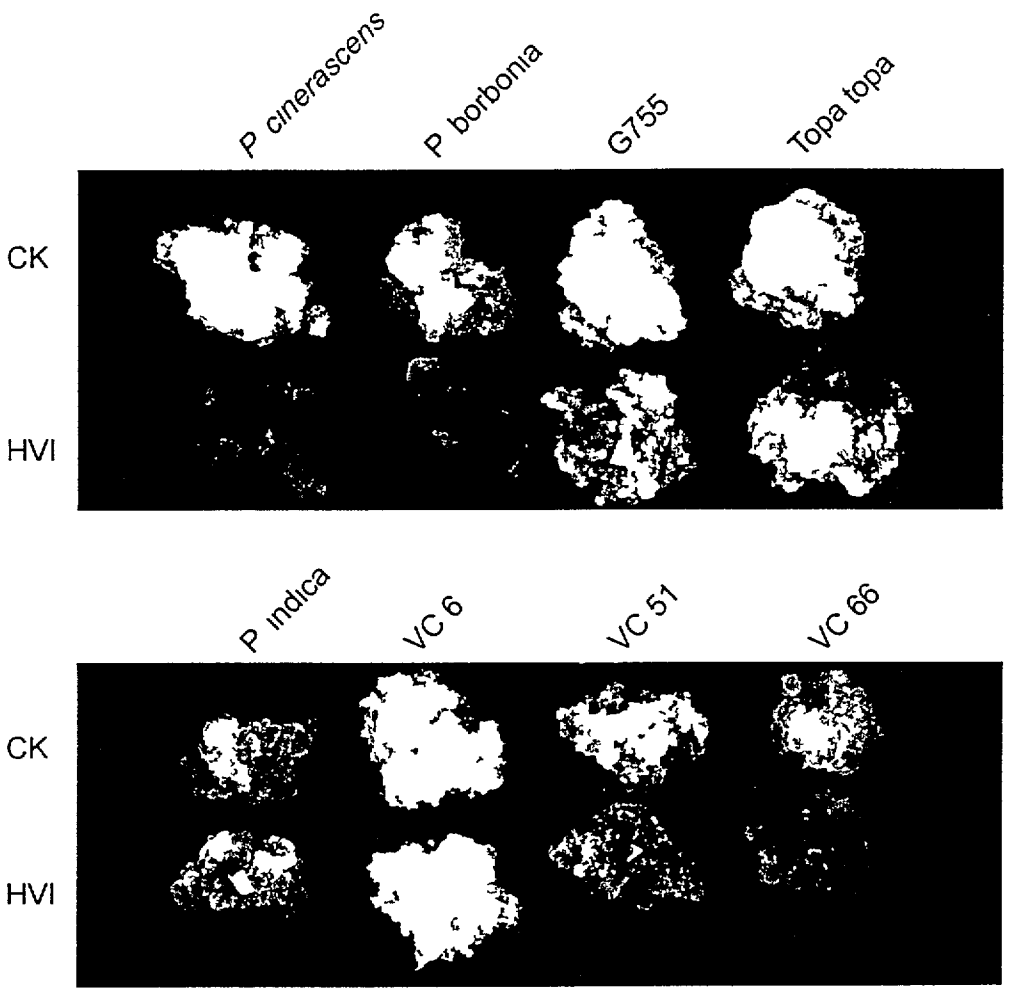


Fig 2 Necrosis in *Persea* calli as developed in response to inoculation with high-virulence isolate (HVI) of *Phytophthora cinnamomi* Calli cultures of eight *Persea* species and varieties were directly inoculated with 1x1-mm piece of miracloth supporting the pathogen mycelium The calli were cultured during the inoculation over water-agar medium and incubated in the darkness Picture was taken 2 days after inoculation

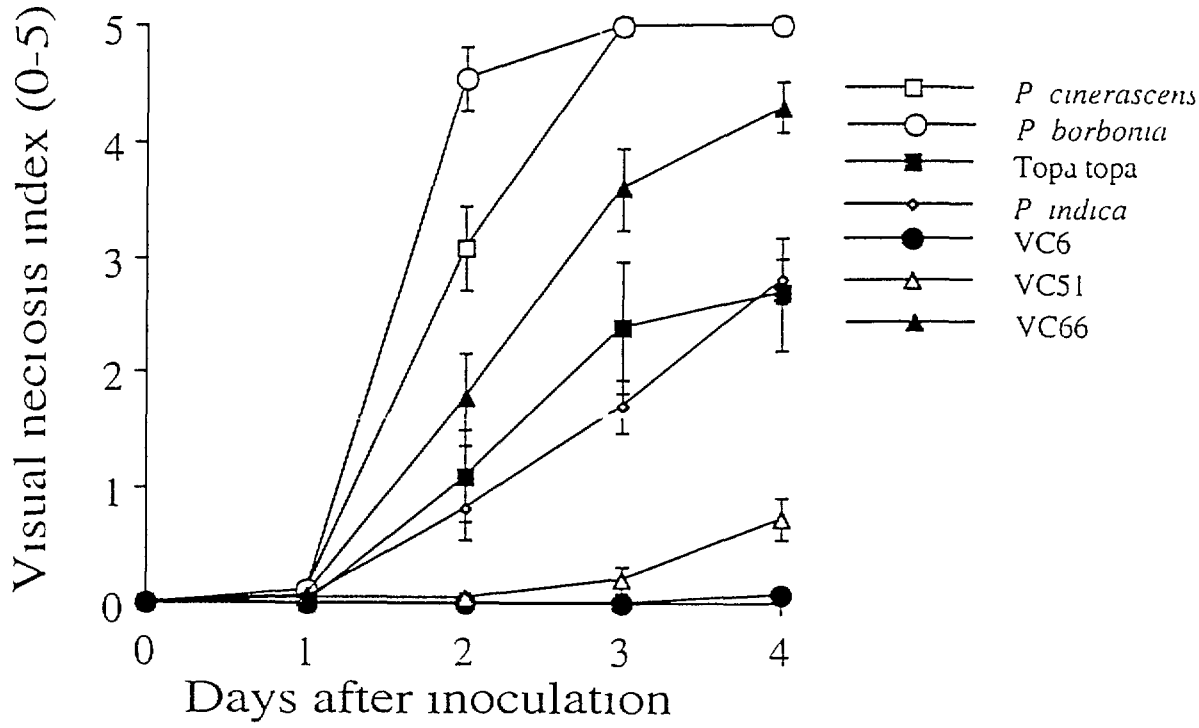


Fig 3 The development of necrosis on *Persea* calli in response to inoculation with high-virulence isolate (HVI) of *P cinnamomi* Calli of *Persea* species were cultured in light over a water-agar medium Necrosis index was determined by multiplication of the visual assessment of color intensity (ranked on a 0-5 scale) by the percentage of callus surface that turned necrotic Each point represents an average (±S E) of 10 replicates of callus that were cultured in separate Petri dishes

Table 3 Visual assessment of necrosis developed on calli derived from *Persea* species and varieties revealed varying degrees of resistance to *Phytophthora cinnamomi* The values are number of days after inoculation with the high-virulence isolate of *P cinnamomi* when necrosis reached 50% of the maximal values of Necrosis Index (NI 50) The incubation was in darkness

	Persea species and varieties	NI 50	Average
Resistant	P cinerascens	A 2 18	
	P borbonia	A 1 96	2 07
Moderately resistant	G 755	AB 3 61	
	VC 66	A 2 62	3 12
Susceptible	Topa topa	AB 5 48	
	P indicia	BC 7 67	
	VC 6	BC 6 37	
	VC 51	C 10 0	7 38

Means with the same letter do not differ significantly at P=0 05, by Duncan's multiple range test

was delayed Calli derived from varieties that were considered susceptible, seemed to be divided between those that developed partial and delayed necrosis (*P indica* and *P americana* var Topa topa), and those that showed hardly any visible changes (*Persea americana* VC 6 and VC 51) By using the NI 50 index it was possible clearly to distinguish the difference in time response between the susceptible- and the resistant-originated groups of calli This difference in necrosis development responses makes it possible to discriminate among calli having graded levels of resistance It seemed that the proper time for performing such an assessment is after around 48 h in darkness, following inoculation

Previous data obtained by inoculating calli with *P cinnamomi* (Phillips et al 1991) suggested that the *P shiedeana* variety G755 ('Martin Grande') was moderately resistant, although it had been selected as highly resistant Our results (Fig 2, Table 3) support this suggestion, as calli of G755 turned necrotic brown later than the resistant calli, with an NI 50 around 3 6 days It is suggested here that the experimental *P americana* VC 66 is a moderately resistant clone, with NI 50 showing a delay relative to the resistant species, although it did not differ statistically from them (Figs 2, 3, Table 3) Intermediate field data (Zilberstaine et al 1992) support this suggestion

4 5 Effect of light on necrosis development

P cinnamomi is a soil pathogen which is naturally active in roots in darkness, which was the reason for examining the effect of light on the host-pathogen (HVI) interaction (Fig 4) The results showed a delay in necrosis development under light conditions, particularly for the fast-responding calli of the resistant species (*P cinerascens* and *P borbonia*, Fig 4) and for Topa topa Nevertheless, the quantitative differences in necrosis development, between the resistant and susceptible species, persisted under the light conditions as well (Figs 3, 4) It is therefore proposed that the resistance response of plant roots to the pathogen is likely to be more intensive under darkness in comparison to light

4.6. The response of *Persea* calli to inoculation with HVI and LVI

HVI and LVI differ significantly in their virulence against roots of intact avocado plants (Zilberstaine and Pinkas 1988), and comparison between the two isolates enables better characterization of the resistance response Calli were therefore inoculated with the two isolates under light and dark conditions (Fig 5)

Except for *P cinerascens* in darkness and VC 51, which did not develop a significant level of necrosis under either lighting condition, the necroses developed in response to LVI were greater than or equal to those developed in response to HVI inoculation (Fig 5A, B) This difference between the isolates may support the suggestion that the resistance response to LVI is closely related to the more rapid and more intense necrosis development

The comparisons between the light and dark conditions showed that the LVI / HVI ratio of necrosis elicitation was higher under light than in darkness (Fig 5C), probably because of a lower necrosis elicitation of HVI in light However, the inter-relationships among the tested varieties in their ratios of LVI to HVI- induced necroses were similar under both conditions Topa topa showed the highest, *P cinerascens* the lowest, and VC 66 and *P borbonia* intermediate values of LVI / HVI ratio (Fig 5C) It means that the discrimination in necrosis elicitation between LVI and HVI is mainly influenced by the inoculated varieties and less by the lighting conditions that had a similar effect on the both isolates

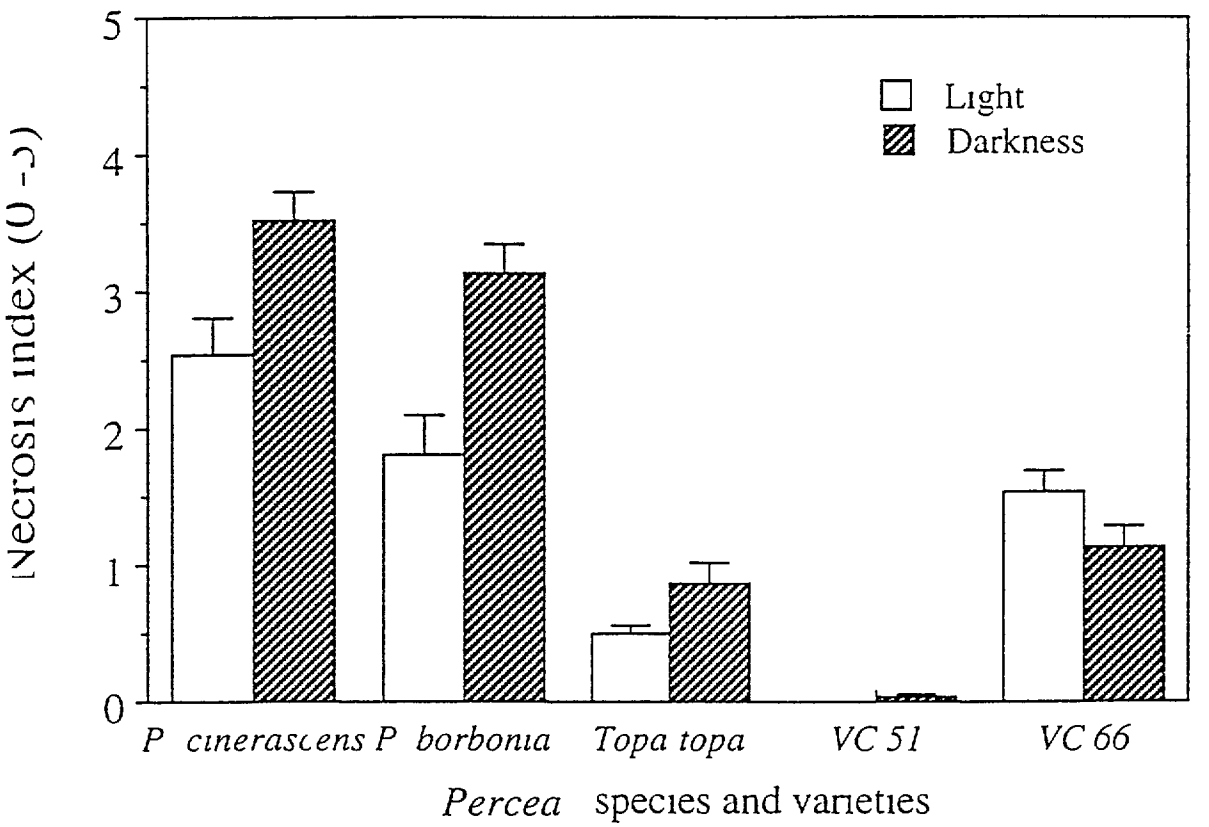


Fig 4 The effect of lightning conditions on necrosis response of *Persea* calli to inoculation with high virulence isolate (HVI) of *P cinnamomi* The values are averages (±S E) of 10 calli that were cultured on a water-agar medium in separate dishes Necrosis index was assessed visually 2 days after inoculation as described in Fig 3

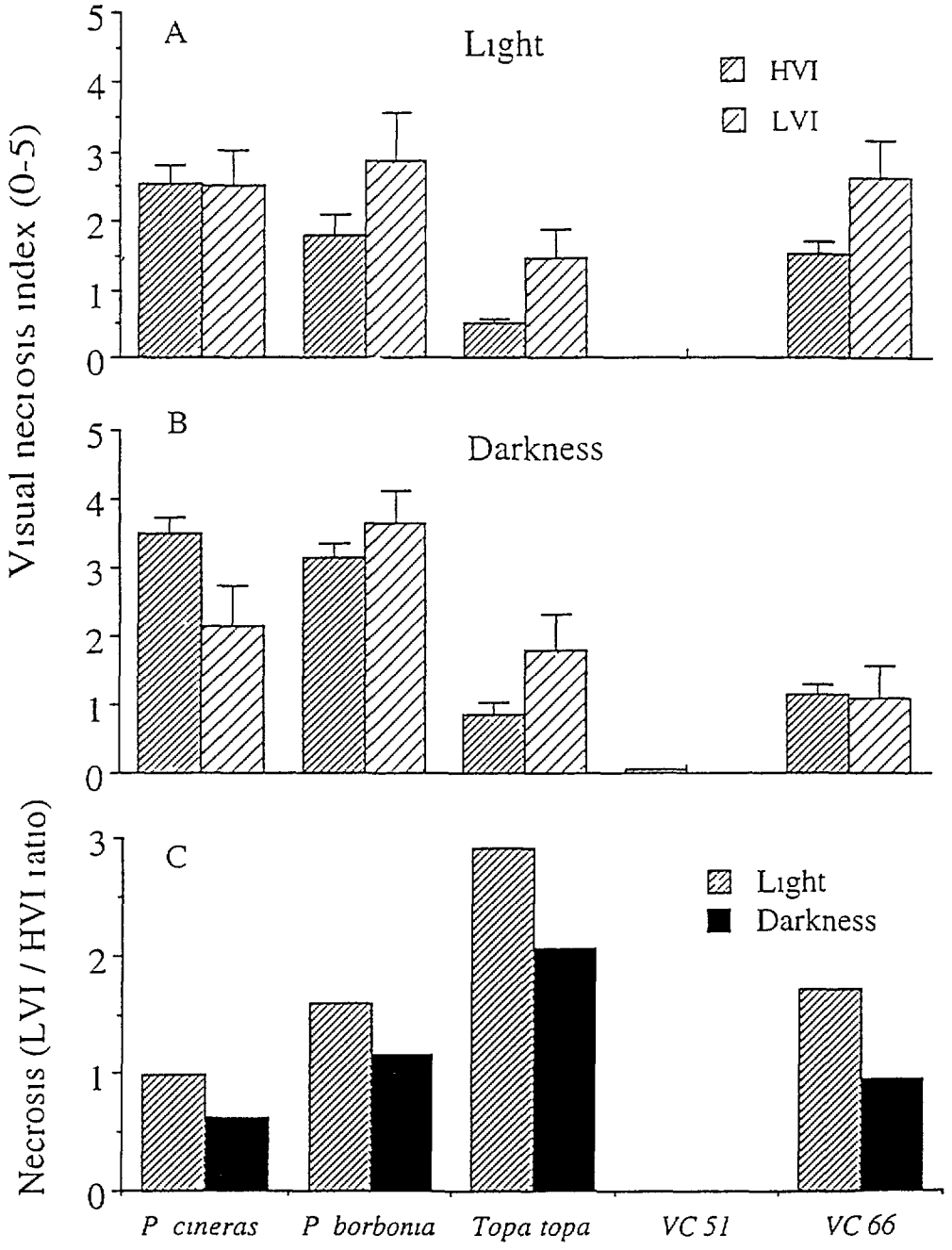


Fig 5 Comparison of necrosis development in *Persea* calli between high-virulence isolate (HVI) and low-virulence isolate (LVI) of *P cinnamomi* The values are averages (±S E) of 10 calli that were cultured under either light or darkness, on a water-agar medium in separate dishes Necrosis index was assessed visually 2 days after inoculation as described in Fig 3

4 7 Effect of HVI on specific activity of peroxidase in *Persea* calli

The peroxidase specific activity was higher in calli of resistant origin than in of non-resistant origin (Fig 6) The experimental var VC 51 calli expressed low peroxidase specific activity, while the enzyme aetivity in VC 66 calli resembled those in calli derived from the resistant *P cinerascens* and *P borbonia*

This reaction can be used as an another indicator for the resistance level of an unknown variety of *Persea* By this reaction it was possible to reconfirm the resistant trait of the experimental avocado VC 66 clone in contrast to the extreme susceptibility of the VC 6 and VC 51 clones (Zilberstaine et al 1992, Ben-Ya'acov and Zilberstaine, unpublished data) The response of†the peroxidase specific activity correlated significantly with the necrosis development as indexed visually (Fig 7A) This correlation might support the previous work concerning the expression of insect resistance in calli, in which enhanced peroxidase activity was associated with brown callus and a hypersensitive-like reaction (Dowd and Norton 1995) The elevated peroxidase activity in calli originated from resistant sources (Fig 6) is in consistent with the systemic increase of peroxidase, which resulted from infection of avocado roots in tolerant plants by *P cinnamomi* (van Lelyveld and Brodrick 1975) van Lelyveld and Brodrick (1975) suggested that peroxidases contribute to the browning through oxidation of phenolic acids, thereby enhancing the resistance of the surroundings of the necrotic tissue, and our present results could be consistent with this suggestion However, VC 66 seemed to be significantly distant from the regression line (Fig 7A), indicating that this clone showed a high level of peroxidase specific activity, which was not fully expressed in necrosis development

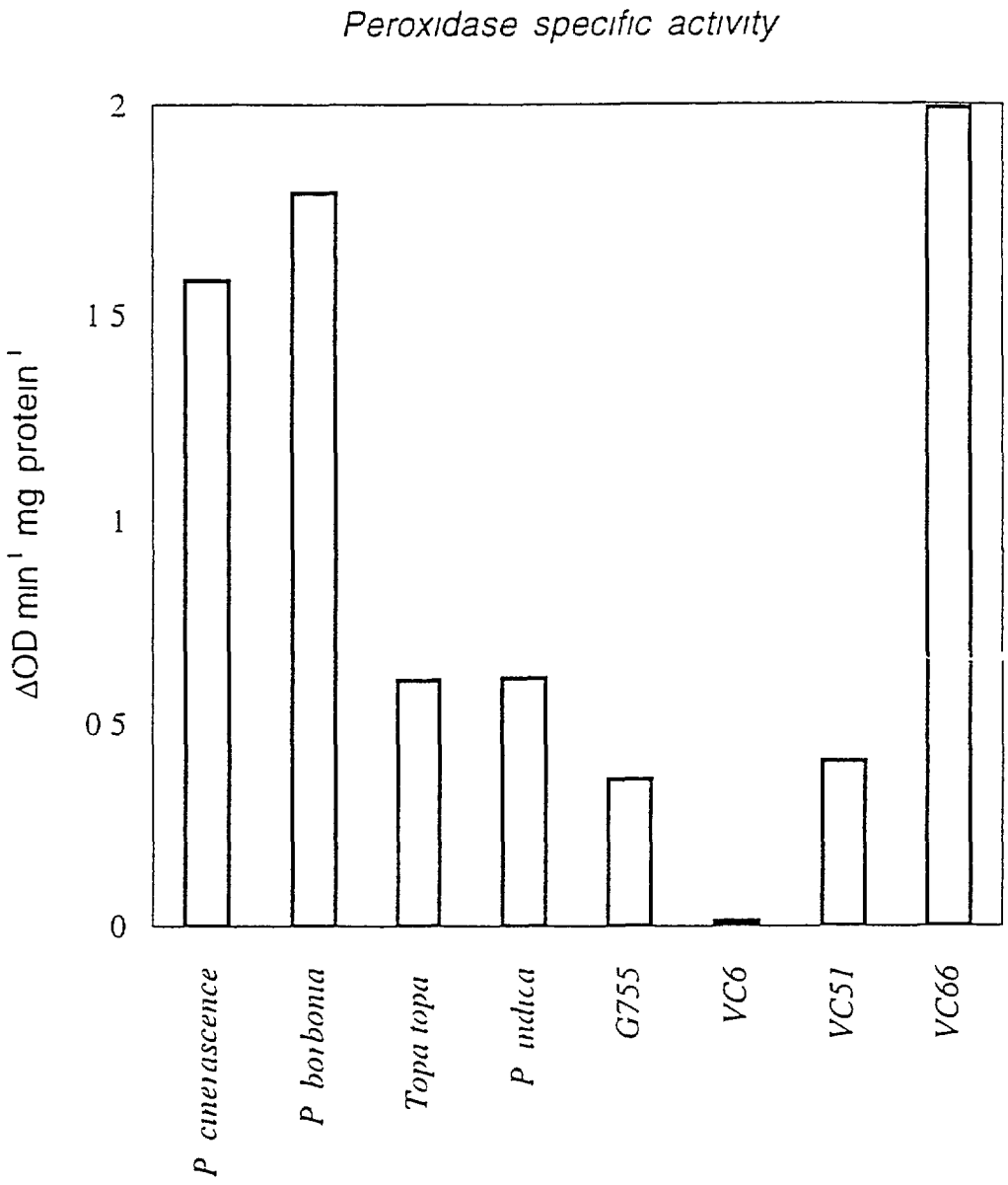


Fig 6 The effect of inoculation with high-virulence isolate of *P cinnamomi* on specific activity of peroxidase in *Persea* calli The values are averages of 10 calli that were incubated in darkness Peroxidase enzymes were extracted from the calli 2 days after inoculation and evaluated colorimetrically for specific activity

4.8 Correlation of visual necrosis index with peroxidase specific activity, Minolta chromameter and spectroscopic measurements of necrosis

In addition to the visual evaluation, two other procedures were used for quantitative evaluation of necrosis intensity Callus surface color measurement with the Minolta chromameter, and spectroscopic measurement of the color of the liquid extracted from the inoculated callus tissue These two procedures should provide more objective evaluation of the necrosis response than visual evaluation Both of the measurement techniques were in a significant correlation with the visual assessment (Fig 7 B, C) The correlation of the visual necrosis†index with the 'L' scale values of the Minolta chromameter was more significant (Fig 6B) than that with the spectroscopy procedure (Fig 7C), probably because the visual and the Minolta chromameter methods are both assessing the surface color For limited necrotic area the use of Chromameter is advantageous Eeach necrosis assessment technique, which was examined in the present study, could potentially be used for detecting and selecting necrotic variants

The effect of the pathogen–callus interaction on the hyphal extension, which could be a clear indication of the extent of the resistance of the callus itself, was not addressed in detail in the present work However, limitation of hyphal growth on calli has been found to correlate with the resistance of†the plant sources in previous studies (McComb et al 1987, Phillips et al 1991), in which the browning was observed more extensively on calli from resistant than from susceptible plant sources It appears, therefore, that necrosis in callus cultures is probably a hypersensitive response reaction (HR) HR has been defined as the death of host cells within a few hours of pathogen contact, which could be caused by various mechanisms (Hammond–Kosack and Jones 1966) In the present study, the average necrosis index was calculated separately for each of the resistant, moderately resistant and susceptible groups of varieties The average necrosis index of the resistant or the moderately resistant groups of varieties, 1 day after inoculation with HVI, was 12-15 times greater than that of the susceptible group of varieties, this difference gradually decreased with time (fig 8) In response to LVI the resistant/susceptible ratio was lower immediately after inoculation and remained at the same level This appears to provide an explanation for the difference in virulence between HVI and LVI, since the susceptible calli responded to HVI inoculation with a lower intensity of HR than the resistant calli, while they exhibited almost the same level of HR in response to LVI

It can be concluded that the necrosis response to *P cinnamomi* inoculation in callus cultures could potentially be applied as an indicator for resistance in intact plants of avocado and related *Persea* species This *in vitro* system might be helpful for fast diagnosis and selection of new clones resistant to the root rot pathogen

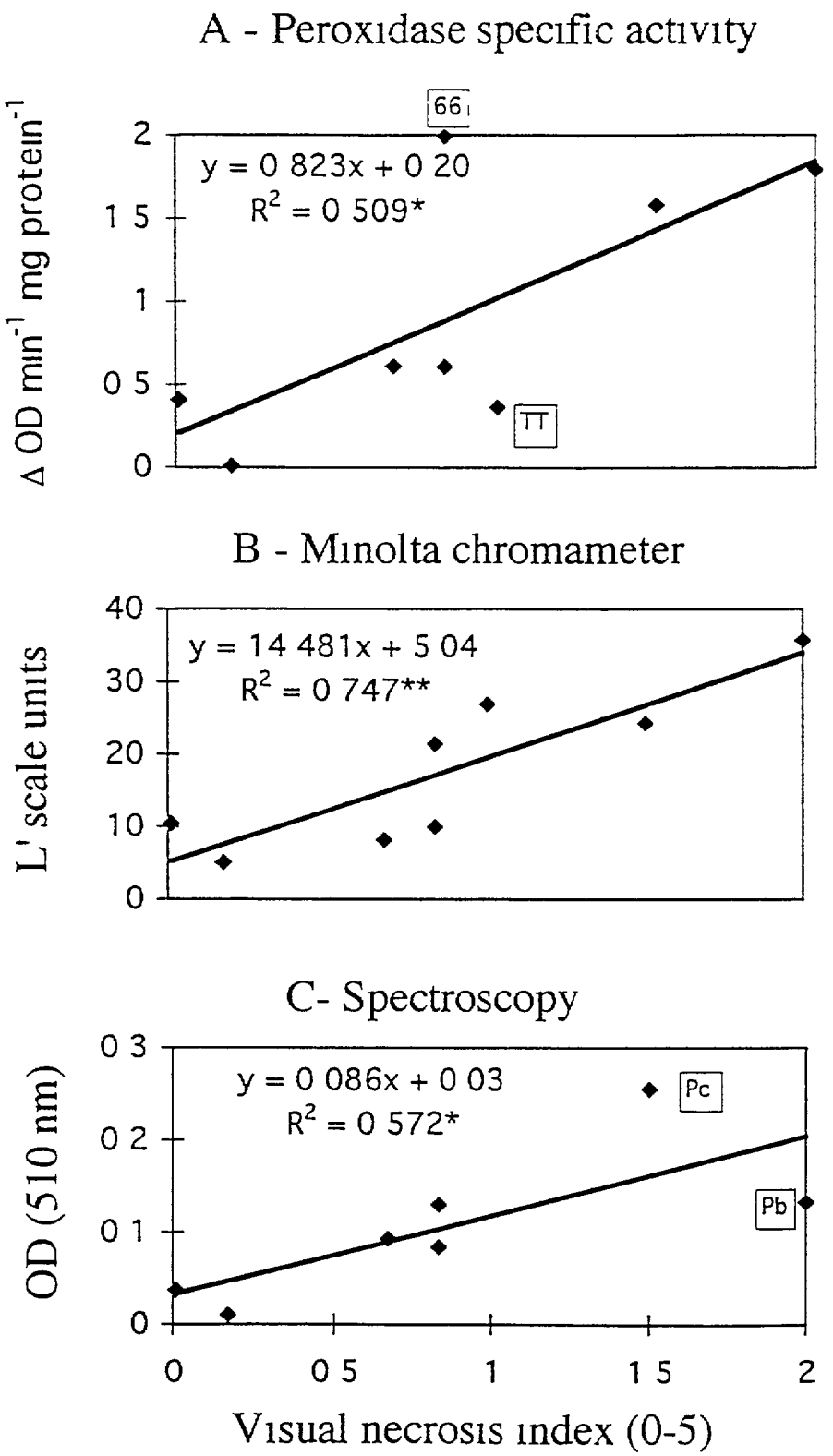


Fig 7 Correlation of visual necrosis index with peroxidase specific activity, Minolta chromameter and spectroscopic measurements of necrosis *Persea* calli were inoculated with high virulence isolate of *P cinnamomi* and incubated in darkness All measurements were done 2 days after inoculation Each point is the mean of 10 replicates of the same variety of *Persea* callus Visual necrosis index was evaluated as described in Fig 2 Color of calli surface was quantified by Minolta chromameter on an 'L' (lightness / darkness) scale Color intensity of the liquid extract from the inoculated calli was determined spectroscopically at 510 nm

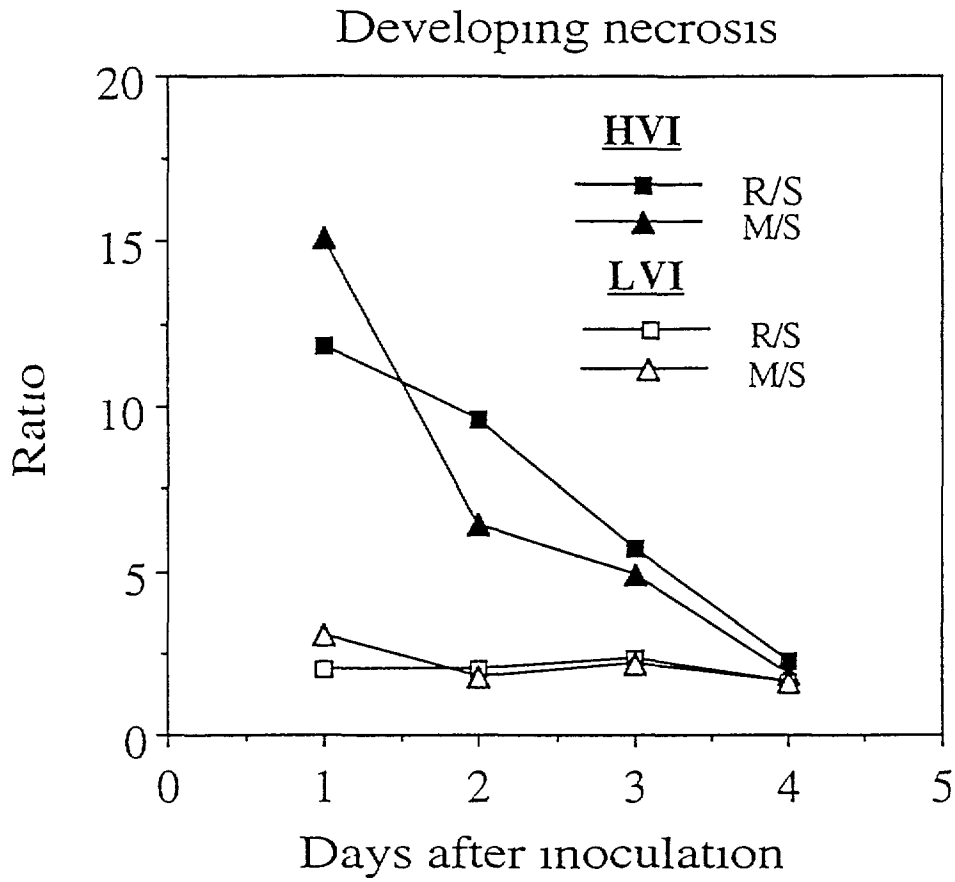


Fig 8 Ratios of index necrosis between resistant (R)/susceptible (S) and moderately resistant (M)/susceptible calli in response to high-virulence isolate (HVI) and low-virulence isolate (LVI) of *P cinnamomi* R= average of *P cinerascens* and *P borbonia*, M= average of G 755 and *P americana* var VC 66, S= average of Topa topa, *P indicia* and *P americana* vars VC 6 and VC 51 The necrosis indexes were determined 2 days after inoculation, in darkness

4 7 Conclusion

The objective was to characterize and quantify the responses of necrosis development and peroxidase activity to inoculation with *Phytophthora cinnamomi* Rands (the soil-borne pathogen causing the root rot disease in avocado)*f*on calli derived from plants of eight *Persea* varieties and species Calli originated from resistant *(P cinerascens P borbonia)* moderately resistant (*P schiedeana* var G755), and susceptible (*P indica P americana* var Topa topa) and vegetative clones of experimental avocado (*P americana*) rootstocks (VC 6 VC 51, VC 66) were inoculated directly with fungal mycelium of either a high-virulence isolate (HVI) or a low-virulence isolate (LVI) of the pathogen Calli originated from resistant plants developed dark brown necroses within 1-2 d of being directly inoculated with HVI, while susceptible calli reacted less rapidly, if at all Such a correlation was not significant in interactions with LVI inoculum The average necrosis index of the calli from the resistant or moderately resistant plant sources was 12-15 times higher than that of the susceptible group of species, on the first day after inoculation with HVI, it then decreased gradually The resistant ∫ susceptible ratio in response to LVI inoculation remained constant at about 2 5 The resistance necrosis response developed more quickly in darkness than under light The visual assessment of necrosis was significant correlated with surface colour detection of callus as defined by the 'L' parameter with a Minolta chromameter, and with spectroscopic determination of the colour of the extract The resistant originated calli expressed higher peroxidase specific activity than the susceptible ones, which was in significant correlation with the necrosis development The ability to identify and quantify resistance response in tissue cultures might be utilized for an in vitro screening and selection of new resistant avocado clones

Impact, Relevance and Technology Transfer

The findings of the present project be useful in the Philippines is several areas This project is actually the first intensive research done on the avocado crop which is actually is not intensively grown in big size orchards, but mainly grown on small plots in backyards This project enabled to develop diagnostic method for *P cinnamomi* and to screen for resistance out of avocado trees that survived in heavy infected soils or in regenerated plantlets This approach will be probably continued unless it will be ceased because of financial budget restrictions The research staff has been experienced the work with tissue cultures and plantlets' regeneration New equipment has to be purchased and used by trained individuals Labs of different disciplines such as phytopathology, biochemistry, tissue culture, genetics and extension services have been activated during the project's period, and accordingly were more capable for caring out more an integrating scientific projects like the present one

Project Activities/Outputs

Meetings attended and list of publications

Dizon, TO, LF Tisalona and EMT Mendoza 1996 Isolation growth and sporangial production of *Phytophthora cinnamomi* from avocado 27th PMCP Sci Meeting, Davao City, May 7-10, 1996

Dizon, TO, LFA Tisalona and EMT Mendoza 1997 Root rot avocado caused by *Phytophthora cinnamomi* rands ind the Philippines Phytopath 33 122-129

Raviv, A, RA Avenido, LF Tisalona, OP Damasco, EMT Mendoza, A Zveibil Y Pinkas and S Zilkah 1998 Cell culturing of *Persea* species and their in vitro interaction with *Phytophthora cinnamomi* and somatic embryogenesis IX International Congress on Plant Tissue and Cell Culture, June 14-19, 1998

Raviv, A , Avenido, R A , Tisalona, L F , Damasco, O P , Mendoza, E M T , Pinkas,Y and Zilkah, S 1998 Tissue culturing and somatic embryogenesis of *Persea* species †Plant Tissue Culture and Biotechnology, 4 196-206

Raviv, A , Zveibil, A , Pinkas, Y , Faingersh, E , Rotbaum, A and Zilkah, S 1999 Hypersensitive-like response of resistant *Percea* species to inoculation with *Phytophthora cinnamomi* in callus cultures In Doyle, B M , Curry, R F and Cassels, A C (Eds) Abstracts of the ISHS Conference on Methods and Markers for Quality Assurance in Micropropagation, (pp 212-213) National University of Ireland

Dizon, TO, OP Damasco, MJB Estrella and EMT Mendoza 1999 Resistance of detached FCSSP Annual Scientific Conference, Gen Santos City, April 1999

Dizon, TO, PM Magdalita and SB Jamias 1998 IPB-Israeli government research on avocado root rot UPLB Newsletter 17(5) 1-2, Feb 2, 1998

Bernardo, AEN, TO Dizon and EMT Mendoza 1998 *Phytophthora* root rot in avocado a biochemical and pathological evaluation Phil Journal of Crop Science, Volume 23, Supplement No 1

Zilkah, S , Raviv, A , Zveibil,A , Faingersh,E , Rotbaum,A and Pinkas, Y 1999 Quantitative response of necrosis and peroxidase activity for expressing resistance to *Phytophthora cinnamomi* in *Persea* callus cultures (Submitted for publication to a reviewed journal)

Project Productivity

The project did not accomplish the ultimate goal of selecting resistant rootstock ready to introduction to commercial avocado plantation However, 7 clones were found to be more resistant than the common avocado sources This project has to start from the beginning for†establishing the *in vitro* selection system A lot of work has been invested for

establishing the broad infrastructure of the project, which was inevitable for the project's project In the event, it was actually expected that in 3 years it was impossible to achieve a commercial product However, the developed infrastructure in the present research brought the project to an advanced level for next stage

Future Work

Future work, if it will be financially feasible, will be focused on carrying out in a large scale the in vitro screening and selection by using the methods that developed in the present project The *in vitro* output of the project could be exploit for bio-technical and genetic engineering approaches These approaches were proposed by the present project's investigators and did not approved

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