CHAPTER 4

Optimisation of the screening programme: 1998 - 2000

4.1. SUITABILITY OF VARIOUS MEDIA FOR THE SCREENING OF AVOCADO BREEDING MATERIAL WITH REGARD TO *PHYTOPHTHORA CINNAMOMI* TOLERANCE

4.1.1. INTRODUCTION

The ultimate solution to the negative financial impact that avocado (*Persea Americana* Mill.) root rot has on the South African avocado industry, is tolerant rootstocks. The development of tolerant rootstocks is usually done by screening seedlings resulting from controlled pollination (Bijzet *et al.,* 1993; Koekemoer *et al.,* 1994; Breedt *et al.*, 1995.)

The prerequisite for success, with regard to a rootstock breeding programme, is mass screening in excess of 10 000 seedlings per annum (Bergh, 1969; Poehlman, 1987). The screening should be fast, reliable, stern, cost-effective and consistent. Screening has widely been discussed as far as laboratory techniques are concerned and include colonisation of excised root tips (Kellam & Coffey, 1985), lesion development on etiolated shoots (Kellam & Coffey, 1985; Dolan & Coffey, 1986), electrolyte leakage (Zilberstein & Pinkas, 1987) and the detached root inoculation method (Botha *et al.*, 1989). Other methods include the screening of candidate rootstocks in naturally-infested soil (Zentmyer & Richards, 1952), infesting soil or sand by adding ground mycelium (Tsao & Garber, 1960), dipping intact roots in a spore or mycelium suspension (Klotz & DeWolfe, 1960) and growing infected rootstock seedlings in a nutrient solution. The nutrient solution test is very severe and provides results in a short time; in susceptible plants 90 – 95 % of the roots are rotted within 12-days at 24 °C (Zentmyer, 1982).

Due to the bulkiness of avocado seed and seedlings, *in vitro* screening methods have proved impractical and too costly for the mass screening of 10000+ seedlings. In the period 1991 to 1997, the rootstock breeding programme of the ARC-ITSC thus reverted to screening in naturally-infested soil (Zentmyer & Richards, 1952, Bijzet *et al.*, 1993) with the exception of one season when the soil was substituted with sterile vermiculite. It was found

that germination was low in naturally-infested soil relative to germination in sterile vermiculite (Chapter 3). These methods lacked scientific merit and a suitable screening medium had to be identified.

The present trial has its origin in the insufficient comparison of the suitability of different growth media with regard to both the host and the pathogen. The objective was to identify a medium which would be conducive to the development of optimal *Phytophthora cinnamomi* Rands inoculum whilst at the same time would not impair the development of the plant. The physical requirements for both *P. cinnamomi* and the avocado host are given in Table 4.1.

Table 4.1 Physical requirements for *Phytophthora cinnamomi* and the avocado host (Zentmyer, 1980; Wolstenholme, 2001).

Characteristic	***P. cinnamomi***	**Avocado**
Temperature	24	24-30
PH	6.5	5
Aeration	6.8 – 7.2 ppm O_2	60-80% Air filled porosity
Moisture	Saturated to flooded	Field (container) capacity
Light	Dark	Light
Substrate-texture	Moderate to heavy	Light to moderate

Media can be divided into two different groups, namely aggregate cultures and water cultures (Mason,1990). In water culture, nutrients are dissolved in water, which is brought in contact with the roots. The water is either aerated or roots are allowed to be in contact with air as well as with the nutrient solution. Aggregates consist of small particles of chemically inert substances providing a suitable environment for the plant roots to grow. A suitable aggregate holds sufficient moisture but drains off the excess, allowing adequate aeration (Mason, 1990). Aggregate media include vermiculite, perlite, sand, gravel, scoria, pumice, diatomite, rockwool, expanded clay and expanded plastics. Choice of media is based on suitability for the pathogen as well as the host, availability, consistency and cost.

4.1.2. MATERIALS AND METHODS

4.1.2.1. Media

Five media (Figures 4.1, 4.2) were selected, namely 100 % silica sand (used in swimming pool filters), 100 % vermiculite, soil, a water culture based on the Zentmyer tank (Zentmyer, 1960) and a mist spray based on the Schwalbach system (Mason, 1990). The soil used in this study was never fertilised or cultivated before, had a clay content of 35 %, and was collected from virgin land in the Nelspruit area.

Figure 4.1 Different growth media for the screening of *Phytophthora cinnamomi.* From left to right: soil, sand and vermiculite

Figure 4.2 Different water cultures for the screening of *Phytophthora cinnamomi.* Left an aerated water tank and right a mist system.

Media temperatures were maintained at 24 ± 2 °C. Containers used for the trial were plastic baths 485 x 350 mm with a capacity of 15 l and were filled

with medium to a depth 120 mm. Air-filled porosity (AFP) and water-holding capacity (WHC) of the soil, sand and vermiculite are given in Table 4.2

Table 4.2 Air-filled porosity and water-holding capacity of the media

Medium	Air-filled porosity (Volume %)	Water-holding capacity (Volume %)
Sand	38.10	28.12
Soil	10.41	42.32
Vermiculite	15.20	37.48

The water culture consisted of water-filled containers in which the roots were also in contact with air provided by aerating the water in the containers with a 50 Hz pump. Water for the mist spray system was delivered by a mono-phase 0.74 kW Electro pump through a 20 mm PVC pipe (Figure 4.3). Water was fed to the mist chamber (container) via spaghetti tubing connected to a DAN mist spreader with nozzle size: violet 35 l hr^{-1} (200 kPa). Mist was applied for two minutes with a seven-minute interval. Seedling trays were modified to support the plants for the water culture as well as the Schwalbach system (Figure 4.3). All the equipment and media were steam sterilised (Baker & Roistacher, 1957) prior to commencement of the trial.

Figure 4.3 An improvised Schwalbach system: Water was fed from the bottom by means of spaghetti tubes (left) and the plants were surported by a modified seedling tray (right)

4.1.2.2. Plants

Commercially produced, 18-month-old rooted Duke 7 plants were used. Plants were received in wedge-shaped micro-holders from Allesbeste nursery, Tzaneen. Plant height (root tip to growth point) ranged from 190 to 320 mm and the mass from 5.1 to 8.75 g. The root systems consisted of primary roots and structural and feeder roots could thus not be distinguished at this early stage.

Plants were transplanted into the sterile media and were left to stabilise and grow in the media for four weeks. During the stabilising period, the media were kept at container capacity (±1 kPa) that was determined for the soil, silica sand and vermiculite as described by Handreck & Black (1984). After stabilising in the media and before inoculation the plants were carefully removed from the media, washed, weighed and replanted.

4.1.2.3. Inoculation

A life culture of a *P. cinnamomi* isolate (PREM 50801) was provided by J.A. Duvenhage of Merensky Technological Services, Tzaneen. A mycelium inoculum was prepared according to the method of Duvenhage & Maas (1990).

The inoculum was added to the planting media and water culture at 100 ml l^{-1} (Duvenhage & Maas, 1990). A medium similar to that of Duvenhage & Maas (1990), but without *P. cinnamomi,* was added to the control treatments at 100 ml l^{-1} planting medium. The sand, soil and vermiculite were flooded for 48 hours after inoculation whilst the water culture was aerated as usual. The roots of the plants grown in the Schwalbach system were dipped for 48 hours in an inoculum suspension equal to the concentration that was added to the other media and then placed back in the mist system. After 48 hours of flooding the soil, sand and vermiculite, the water was drained to container capacity. Flooding and draining were repeated twice with a 48-hour interval, after which the media were maintained at container capacity. The culture of *P. cinnamomi* that was used in this study was identified by W.J Botha of the Plant Protection Research Institute, Pretoria.

4.1.2.4. Trial layout and statistics

The trial layout was a randomised block design in a greenhouse with natural light and a temperature range of 18 to 30 °C. Each of the three aggregate planting media consisted of an uninfected control (-P-F), an uninfected flooded control (-P;+F) and an infected, flooded treatment (+P;+F). The water culture and Schwalbach system consisted of an uninfected control (-P) and an infected treatment (+P), thus 13 treatment combinations in total. These 13 treatment combinations were replicated five times and each treatment combination consisted of 10 observation points (plants), thus 50 plants per treatment (Figure 4.4).

Figure 4.4 A randomised block design with 13 treatments, replicated five times, each replicate consisting of 10 plants, thus totalling 650 plants.

Data were tested for normality with a Proc Univariate and analysis of variance was done with the GLM procedure of SAS. Means were also compared with a protected Fisher test at 95 % level of significance.

4.1.2.5. Assessing the effect of flooding and *Phytophthora cinnamomi* on the plants in the various media

4.1.2.5.1. Intact root system screening

Four weeks after inoculation the area of each container was divided into 10 blocks with each block containing a plant. Each block containing a plant was then carefully removed, placed in a wire screen cylinder (mesh size = 0.5 mm^2) and immersed in sterile water (Figure 4.5) until the medium was separated from the roots (Bloodworth *et al.*, 1958). The plants were removed from the cylinder and the fresh mass of each plant was determined.

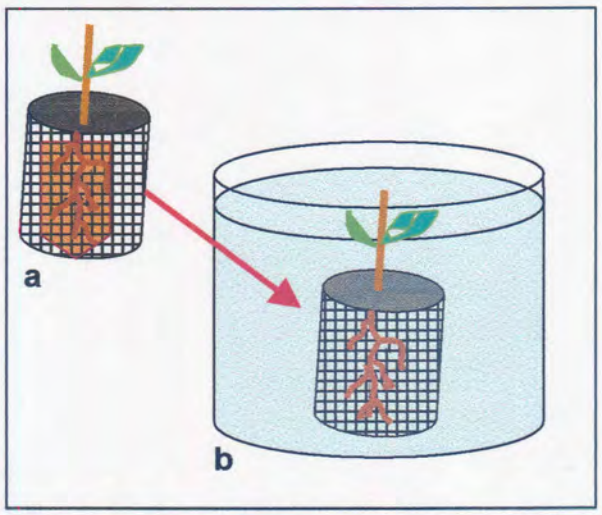

Figure 4.5 **a)** Roots enclosed in a wire screen cylinder **b)** Cylinder immersed in water to remove the medium

The above-ground part of the plants were then separated from the roots. The roots were separated into structural roots and feeder roots according to the method of Van Vuuren (1997). The fresh mass of the structural roots, feeder roots and leaves (including the stem) was determined after which these components were dried at 60 °C for 48 hours to determine the physical parameters.

4.1.2.5.2. Disease assessments

Prior to drying, the root systems were assessed for necrosis and root infection. The percentage of the total root system of a given plant that was necrotic was estimated visually according to the key of Duvenhage *et al.*

(1992). Brittle and excessively discoloured roots were considered to be necrotic and white to light brown fleshy roots to be alive and healthy.

Necrosis was also determined with the aid of a Geotron root measurement apparatus (WLM1). The technique involved measuring the total root length of each plant, followed by a second measurement after removal of the necrotic roots. The percentage of necrotic roots was calculated from these two measurements.

Root infection was determined by plating 10 randomly selected 1-cm-long feeder root segments of each plant on a *Phytophthora*-selective medium (PARPH) (Kelham & Coffey, 1985). *Phytophthora* recovery was recorded after three days incubation at 23 °C in the dark. Root infection was expressed as the percentage of root segments yielding *P. cinnamomi*.

4.1.2.5.3. Physical parameters

The percentage increase in fresh mass for each treatment was calculated for the post –inoculation period. The dry mass of the total root component as well as the different root components (i.e. structural and feeder roots) were used to calculate the percentage of feeder roots present at the end of the trial. According to Zentmyer (1980), *P. cinnamomi* only invades the small feeder roots or absorbing roots, which have no root hairs. The fungus does not progress from the small feeder roots to the more mature secondary or structural roots and only rarely invades larger roots. The percentage feeder roots were therefore used as an indication of the plant's ability to cope with stress situations under various conditions.

The viable root to shoot ratio (VRSR) was calculated according to the formula of Ploetz & Schaffer (1989):

$$VRSR = [RT - (RT \times RN)] / ST$$

where RT is root dry mass, RN is necrosis as the portion of the total root system that was necrotic, and ST is shoot dry mass.

4.1.2.6. Re-isolation of *Phytophthora cinnamomi* and estimating root infection

Isolations from the media as well as the roots were done for confirmation of the presence or absence of *P. cinnamomi* .

Prior to the removal of the plants from the media, the medium in each container (except for the Schwalbach system) was baited with whole avocado fruit (Zentmyer *et al.,* 1960). In addition to this, a sample of each medium per container was collected and baited with citrus leaf discs (Grimm & Alexander, 1973). Five citrus leaf discs and five avocado discs (taken from the surface of avocado fruit) per container were transferred to a hymexazol selective medium for the isolation of *Phytophthora* spp. (Tsao & Guy, 1977). Fifty healthy looking root tips per treatment were collected to ensure that infection was still in an active stage. The root tips were incubated on the medium of Tsao & Guy (1977).

4.1.3. RESULTS AND DISCUSSION:

4.1.3.1. Root necrosis and recovery of *Phytophthora cinnamomi*

No *P. cinnamomi* could be isolated from roots of the noninoculated treatments, although some necrosis was evident. This indicates that the necrosis found in the inoculated treatments could partly have been due to factors other than *P. cinnamomi*. Nevertheless, recovery of *P. cinnamomi* from necrotic roots in the inoculated treatments was positively correlated with the extent of root necrosis, the only exception being the soil medium (Figure 4.6). Sand, vermiculite, water, and mist are therefore suitable as screening media to test avocado plants for tolerance to *P. cinnamomi*. Soil, on the other hand, displayed a lot of necrosis that could not be attributed to *P. cinnamomi* and should rather be avoided.

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Working in a peat-perlite potting medium and a Rockdale fine sandy loam soil, Ploetz & Schaffer (1989, 1992) also found a percentage of root necrosis in noninoculated, flooded and nonflooded treatments that could thus not be attributed to *P. cinnamomi*. They did, however, not explain the phenomenon.

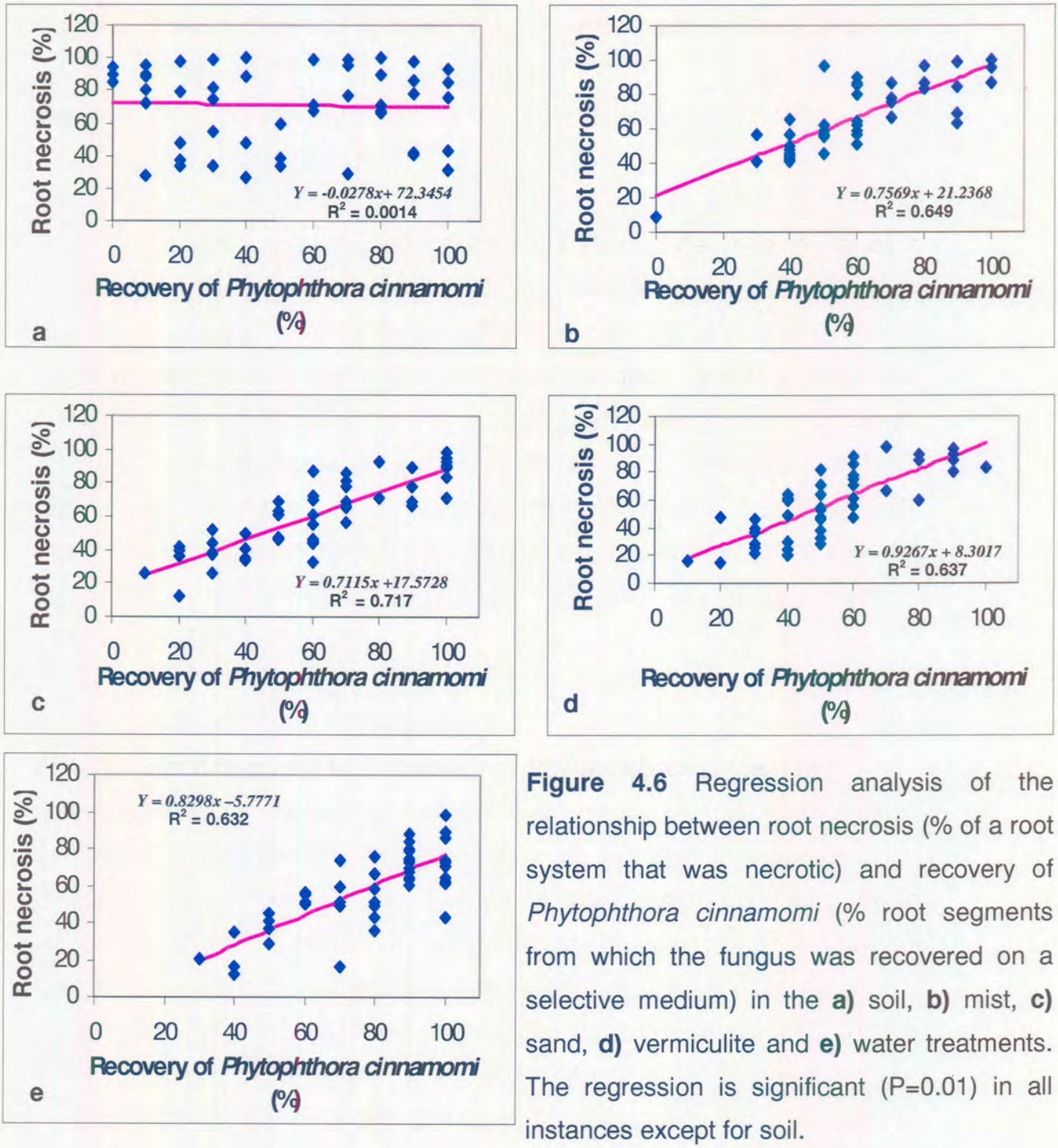

Figure 4.6 Regression analysis of the relationship between root necrosis (% of a root system that was necrotic) and recovery of *Phytophthora cinnamomi* (% root segments from which the fungus was recovered on a selective medium) in the **a)** soil, **b)** mist, **c)** sand, **d)** vermiculite and **e)** water treatments. The regression is significant (P=0.01) in all instances except for soil.

Since it is often difficult to recover *P. cinnamomi* from parasitised tissue (Ploetz & Schaffer, 1987), *P. cinnamomi* may have been responsible for even a larger portion of root necrosis in this study than data on the recovery of the pathogen might suggest.

4.1.3.2. Plant response

Growth measured as fresh mass during the stabilising period, prior to inoculation was significantly better in soil than in sand, vermiculite, water and mist (Schwalbach system) (Figure 4.7) . This was expected as soil is not an inert medium and contains minerals that are available to the plants. Growth of plants in the sand, vermiculite, water and mist did not differ significantly from each other. This indicates that moisture stress was not a factor in any of the media at this stage of the trial as irrigation was maintained at container capacity and no *P. cinnamomi* inoculum had been applied.

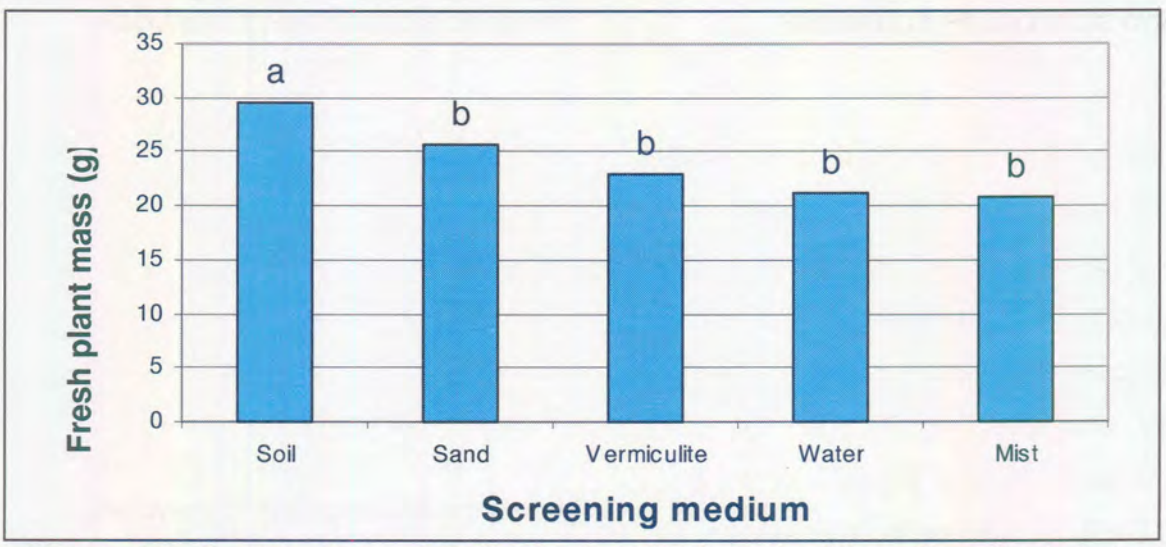

Figure 4.7 Growth of clonal Duke 7 avocado plants in different media during a four week stabilising period (pre-inoculation period) (Treatments followed by the same letter do not differ significantly at P=0.05).

After flooding and application of *P. cinnamomi* mycelium, the results of the control treatments (-P-F) indicated that overall growth of the plants had increased in the sand and vermiculite and that it had decreased in the soil, mist and water treatments (Table 4.3).

Table 4.3 Influence of *Phytophthora cinnamomi* root rot and flooding on the growth of clonal Duke 7 avocado plants in different media

Treatment		Increase in fresh mass (%)[w]		Feeder roots (%)[x]		Necrosis (%)[y]		Viable root to shoot ratio	
Vermiculite	-P-F[v]	19.19	c[z]	67.85	a	9.58	fg	0.31	abc
	-P+F	23.27	bc	62.38	ab	10.94	fg	0.30	abc
	+P+F	-4.76	d	46.55	cde	56.75	cd	0.21	bcd
Sand	-P-F	36.94	a	64.67	ab	9.37	fg	0.26	bcd
	-P+F	32.97	ab	57.27	abc	12.06	f	0.34	ab
	+P+F	18.21	c	35.43	e	60.26	bc	0.17	cd
Soil	-P-F	20.95	bc	58.81	abc	26.22	e	0.23	bcd
	-P+F	-17.36	de	48.89	cd	46.86	d	0.23	bcd
	+P+F	-23.41	e	38.11	de	70.93	a	0.12	d
Water	-P	-16.93	de	56.35	abc	17.58	f	0.45	a
	+P	-23.17	e	47.19	cde	58.12	c	0.17	cd
Mist	-P	-40.08	f	69.10	a	3.20	g	0.46	a
	+P	-27.47	ef	54.05	bc	67.41	ab	0.13	d

[v] -P and +P = noninoculated and inoculated with *P. cinnamomi* , and -F and +F = nonflooded and flooded, respectively.
[w] Percentage increase in fresh mass = fresh mass in the pre-inoculation period – fresh mass in the post-inoculation period
[x] %Feeder roots = feeder root dry mass divided by total root mass x 100
[y] Percent necrosis was measured for each root system at the end of the trial.
[z] Means within columns followed by the same letter are not significantly different at P=0.05

The decrease of growth in non-inoculated water and mist treatments indicates that these media could not sustain growth for the given period without additional nutrients. Soil was the only medium where flooding significantly reduced overall growth. This could be attributed to the low air-filled porosity and slow draining between flooding periods.

Fresh mass was significantly reduced by *P. cinnamomi* in vermiculite and sand.

The percentage increase in fresh mass could not be used to distinguish between the uninoculated (-P) and inoculated (+P) water and mist treatments but did differ significantly with regard to the soil, sand and vermiculite. Although increase in fresh mass was significantly influenced by *P. cinnamomi*

in the soil medium it did not differ significantly from the noninoculated but flooded (-P+F) treatment.

Contrary to the belief of Kellam & Coffey (1985) no detrimental effect was experienced by the removal of the plants from the media and the subsequent baring of the roots during the weighing process prior to flooding and inoculation. This was consistent with previous observations (not documented).

Flooding had no influence on the feeder root percentage in the sand, vermiculite and soil treatments. Percentage necrosis was not influenced by flooding except in the soil treatment.

Feeder root percentages were significantly reduced by *P. cinnamomi* in all the media except soil and water. A similar tendency was evident with root necrosis. Percentage necrosis was a good indication of *P. cinnamomi* infection except in the soil treatment. Viable root to shoot ratio (VRSR) significantly distinguished between the inoculated and uninoculated sand, water and mist treatments.

4.1.4. CONCLUSION

All the media tested except soil are suitable as screening media to test avocado plants for tolerance to *P. cinnamomi*. It is, however, evident from Table 4.3 that the evaluation criterion depends on the medium that is used. Feeder root percentage was a good criterion for clonal material in all the media except soil and water, whereas necrosis was a good criterion in all the media except soil where the high level of necrosis could have been due to factors other than *P. cinnamomi*.

Sand and vermiculite emerged as the best media for screening based on growth. Both these media had no adverse effect on growth of the plants after flooding. The matrix potentials of sand and vermiculite apparently were conducive to the development of *P. cinnamomi* zoospores and consequent infection of the feeder roots. The silica sand that was used in this trial is the

more expensive of the two media but can be steam-sterilised with ease whereas vermiculite tends to collapse during sterilising and would have to be replenished or replaced every screening season, making it more expensive in the long run.

Soil was an unreliable screening medium as a high incidence of necrosis, attributed to the slow drainage following flooding, was evident. Different soils would probably also give different results, indicating the risks involved in using soils.

As shoot growth in sand and vermiculite did not readily reflect the root situation, screening of seedlings would still depend on a subjective evaluation of the roots since determining feeder root percentages of 10 000 individual plants is not viable. Percentage necrosis could prove valuable as it can be done with a root measuring apparatus. Sand and vermiculite are, however, effective for the screening of genetic material that relies on root regeneration as a mechanism of resistance.

The Schwalbach system (mist) reflects stress in the root region readily in the shoots. Root regeneration is not relevant, as the effect of *P. cinnamomi* is intense and rapid in the absence of additional fertiliser or foliar feeds.

A further trial, incorporating vermiculite, sand and mist, to evaluate the effect of the medium and *P. cinnamomi* over time is recommended. This would entail assessing samples throughout the screening period for stress symptoms. Various methods with regard to stress evaluation have been disregarded due to financial constraints and it would be beneficial if these could also be included.

4.2. SCREENING OF CLONAL AVOCADO SELECTIONS FOR TOLERANCE TO *PHYTOPHTHORA CINNAMOMI*

4.2.1. INTRODUCTION

Rootstock material, consisting of foreign and local rootstock selections, is maintained in a gene source block at Nelspruit, where it is utilised in a breeding programme (Bijzet *et al.*, 1993; Koekemoer *et al.*, 1994; Breedt *et al.*, 1995; Bijzet, 1998.). The resulting seedlings undergo an initial screening with regard to *P. cinnamomi* to eliminate inferior genotypes. Material recovered from apparently resistant trees found in the field also undergoes an initial screening. Selections from the initial screening are multiplied and incorporated in a statistical screening to determine their performance relative to Duke 7, the major rootstock in South Africa and regarded as the standard or control. Selections with a performance better than Duke 7 are promoted to a *P. cinnamomi* field test and are incorporated into horticultural trials to determine their production potential.

Each of the selections from the initial screening comprises one seedling plant with a taproot. The degree of inherent resistance/tolerance compared to the standard clonal Duke 7 has thus not yet been determined. Horticultural field trials are costly and require space. A screening of clonal material was thus required to ensure that the best candidates are promoted to field-testing.

The 91 selections, remaining after the initial screening, have been tested with the detached root inoculation method (Van der Merwe, 1995) and selections that were not significantly better than the standard cultivar Duke 7, were discarded. None of the selections was, however, significantly better than Duke 7 and 46 selections equal to Duke 7 remained. This is still a high number of selections to be promoted to a field trial. The purpose of this study was therefore to determine if any of the selections had a better root regeneration ability than Duke 7 and hence a better chance of survival. Only 14 selections could be etiolated successfully and thus be multiplied for the trial.

4.2.2. MATERIALS AND METHODS

4.2.2.1. Plant material, inoculation and evaluation

Fourteen potential rootstock selections (Table 4.4), including Duke 7 and Edranol, were multiplied to have 40 clonal plants of each selection (Bijzet *et al.*, 1997).

Table 4.4 Short codes and descriptions of selections

Code	Selection	Description
A	92-4-3	Seedling of Duke seedling
B	2-64-1	Seedling from an unknown seedling tree in Paarl
C	92-2-2	Barr Duke seedling
D	94-2-4	Barr Duke Seedling
E	94-9-1-2	Teague seedling
F	2-62-1	Seedling from an unknown seedling tree in Paarl
G	92-1-2/5	Duke 7 seedling
H	92-1-1/1	Duke 7 seedling
I	94-1-12	Duke 7 seedling
J	Duke 7	Duke 7 clonal
K	SA-RS97/1	Escape tree (unknown seedling rootstock)
L	92-1-2/1	Duke 7 seedling
M	92-5-1	Seedling of a Duke seedling
N	92-1-2/2	Duke 7 seedling
O	94-9-1	Teague seedling
P	Edranol	Edranol clonal

Each plant was planted in a separate container in 100 % silica sand (used in swimming pool filters). The trial layout was a randomised block design in a greenhouse with natural light and a temperature regime of 18 to 30 °C. All the equipment and media were steam-sterilised (Baker & Roistacher, 1957) prior to commencement of the trial. Each treatment consisted of five replicates and four plants per replicate. As soon as the plants have acclimatised, 20 plants of each selection were inoculated with *P. cinnamomi* according to the method of Duvenhage & Maas (1990). The remaining 20 plants served as controls. The sand was flooded for 48 hours after inoculation and the water was drained to container capacity. Flooding and draining were repeated twice with a 48-hour interval, after which the sand was maintained at container capacity. Three months after inoculation, the root system of each plant was harvested

and divided into structural and feeder roots (Van Vuuren, 1997). The roots were dried and the feeder root mass calculated as a percentage of the total root mass.

4.2.2.2. Re-isolation of *Phytophthora cinnamomi*

After removal of the plants, the sand in each container was baited with whole avocado fruit (Zentmyer *et al.,* 1960). In addition a sample of sand was also taken from each container and baited with citrus leaf discs (Grimm & Alexander, 1973). Five citrus leaf discs and five avocado discs (taken from the surface of whole avocado fruit) per container were plated on a hymexazol medium selective for *Phytophthora* spp. (Tsao & Guy, 1977). Ten healthy looking root tips per plant were collected from all the treatments to ensure that infection was still in an active stage. The root tips were incubated on the medium of Tsao & Guy (1977).

4.2.2.3. Data analysis

The data were tested for normality with the Univariate procedure of SAS and analysis of variance was done with the GLM procedure of SAS. The means were also compared with a protected Fisher test at 95 % level of significance.

4.2.3. RESULTS AND DISCUSSION

4.2.3.1. Overall influence of *Phytophthora cinnamomi* on the root components

P. cinnamomi had a significant effect on root development (Table 4.5). The mean total and structural root component were significantly higher in the inoculated treatments than in the control treatments. Feeder roots, however, had a lower dry mass in the inoculated treatments than in the control treatments.

Table 4.5 Overall effect of *Phytophthora cinnamomi* on root development of clonal avocado selections.

	Mean dry mass (g)	
Root Component	Control	Inoculated
Structural	4.58b*	5.48a
Feeder	2.96a	2.53b
Total	8.00a	8.59b

* Values followed by the same letter within a row do not differ significantly at P=0.05

4.2.3.2. Variation amongst selections irrespective of *Phytophthora cinnamomi* inoculation

Feeder root percentages relative to actual dry mass of the total root component for the control treatments of the 14 selections are given in Figure 4.8. The influence of *P. cinnamomi* is thus excluded.

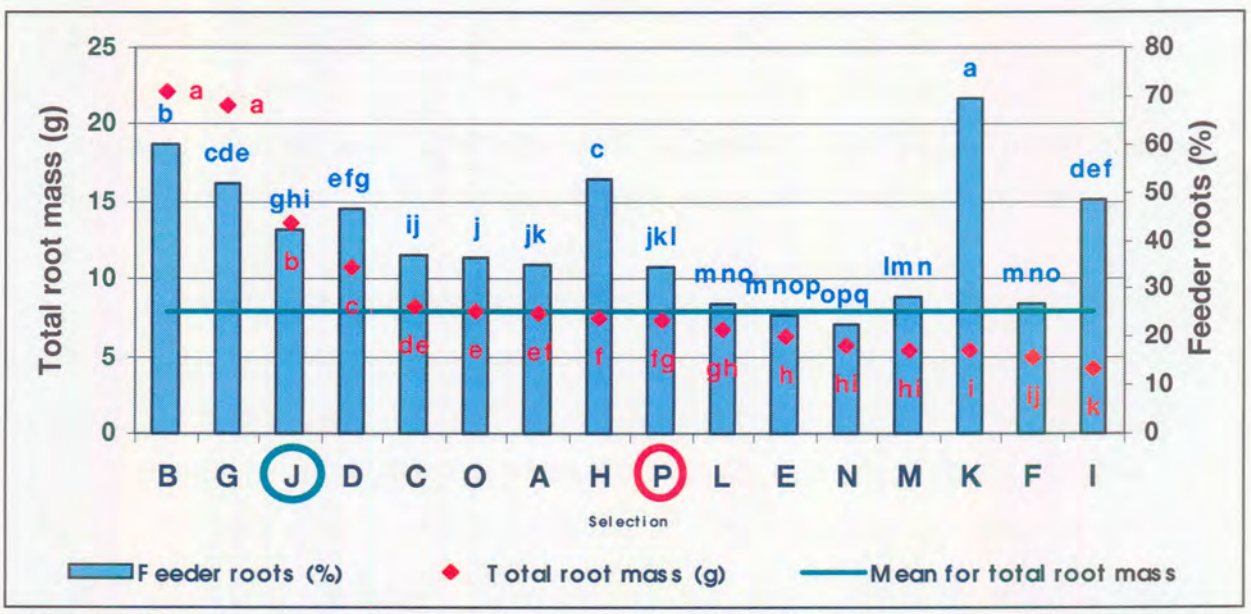

Figure 4.8 Feeder root percentages relative to actual dry mass of the total root component for the control treatments of 14 clonal avocado selections including the standard cultivar Duke 7 (J) and a susceptible control Edranol (P). Values within parameters followed by the same letter do not differ significantly at P=0.05.

Figure 4.8 indicates that the feeder root percentages of selections K and B were significantly higher than the feeder root percentages of the other

selections, selection K having the highest feeder root percentage overall. Selection B had the highest root mass (although not higher than G) as well as the second-highest feeder root percentage. By contrast, selection K, with the highest feeder root percentage, had a total root mass significantly lower than that of the standard rootstock Duke 7. The root mass of selection K was even lower than the mean for the 14 selections included in the trial. This indicates that, although feeder root percentage is a good criterion for clonal material, it is not a representative parameter for comparing tolerance to *P. cinnamomi* amongst genetically different selections.

4.2.3.3. Total root component before and after inoculation

Table 4.5 indicated that the total root mass was overall significantly higher in the inoculated treatment than in the control treatment. If this is viewed in detail, two groups can be distinguished (Figure 4.9). Group A represents the selections of which the total dry root mass differed significantly between the control and inoculated treatments, whereas group B includes the selections that did not display significant differences.

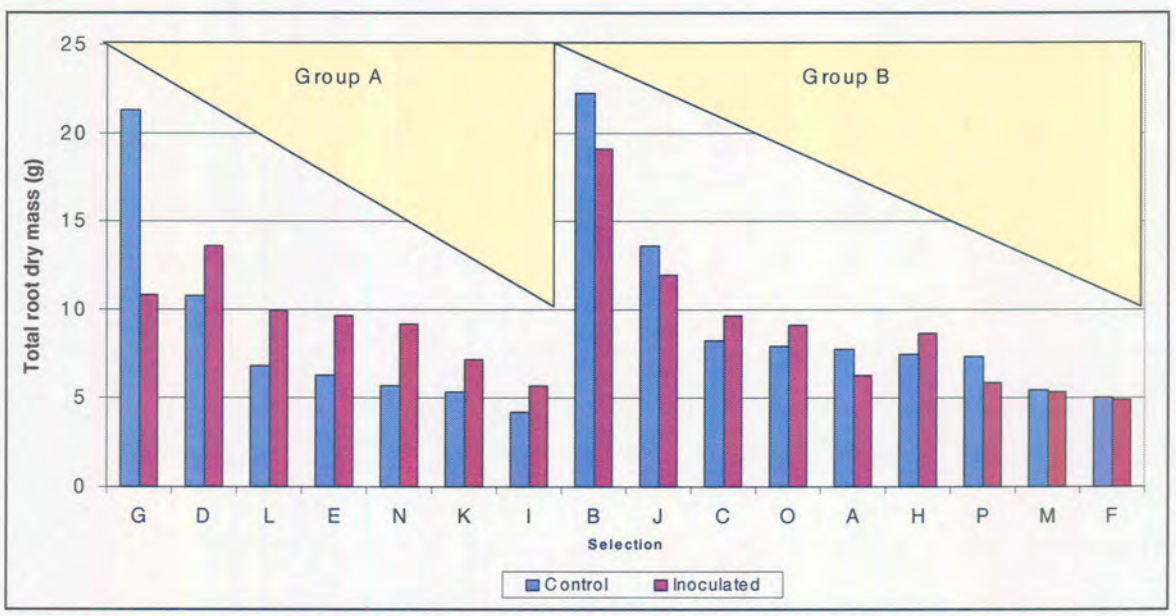

Figure 4.9 Comparison of the dry mass of the total root component in the control and the inoculated treatments with regard to significant (group A) and a non-significant (group B) differences.

In group A, only selection G showed a decrease in root mass whereas the other selections (D, L, E, N, K and I) had an increase in root mass following inoculation.

4.2.3.4. Effect of *Phytophthora cinnamomi* on the feeder root component

In order to identify the best selection with regard to tolerance or resistance the influence of *P. cinnamomi* on the feeder root component was studied (Figure 4.10).

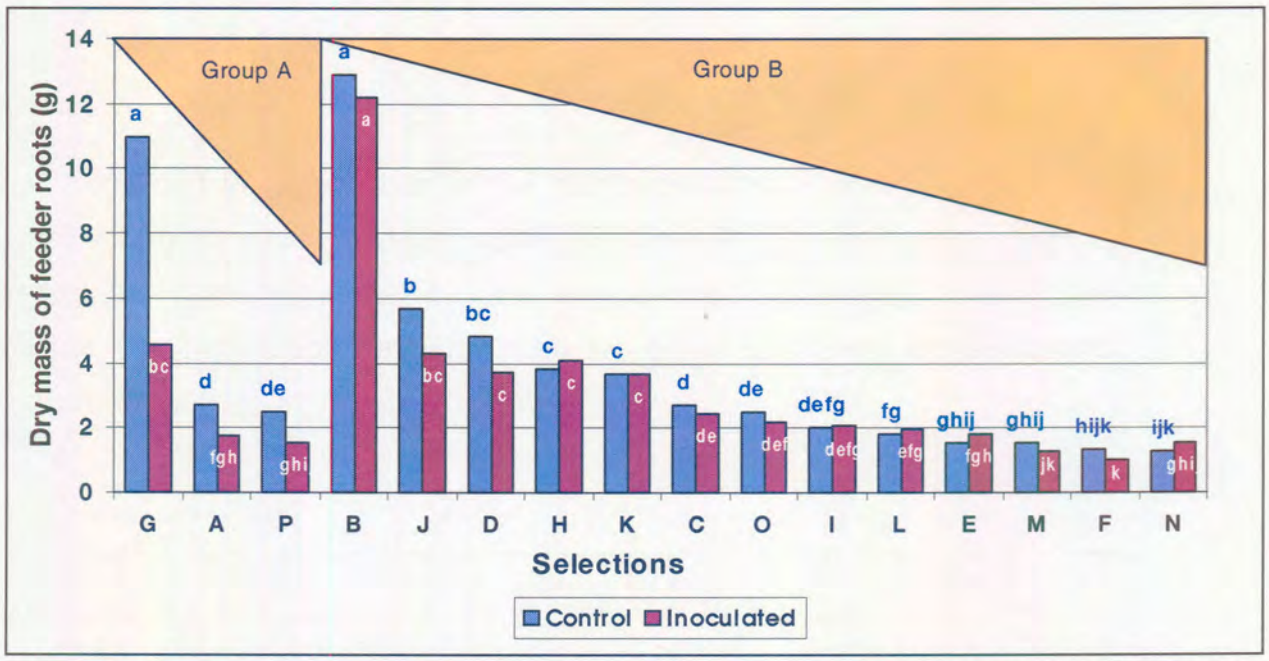

Figure 4.10 Comparison of the dry mass of the feeder root component in the control and the inoculated treatments with regard to significant (group A) and non-significant (group B) difference.

The data in Figure 4.10 indicate that, with the exception of selections G, A and P, none showed a significant reduction in feeder root mass and would thus seem to be equally tolerant to *P. cinnamomi*. Selections that could definitely be discarded at this stage would thus be G, A and P. This, however, still leaves twelve selections that are apparently equal to Duke 7 in feeder root regeneration. Although Figure 4.10 showed that the feeder root component of selection D, L, E, N, K and I (Group B) did not increase significantly, Figure 4.9 indicated an increase in dry mass of the total root component of these

selections, thus suggesting an increase in the structural root component. According to Schaffer *et al.* (1991) the formation of adventitious (fleshy, white) roots is a reaction to severe stress situations and is supposedly aimed at increasing the area for oxygen absorption.

In order to determine the most probable selections for further evaluation, a summary of the root characteristics of the fourteen selections and two control cultivars is given in Table 4.6.

Table 4.6 Comparison of clonal avocado selections with regard to their root components as affected by *Phytophthora cinnamomi*.

(Values = difference between control and inoculation values)

Total (g)		Stuctural (g)		Feeder (g)		Feeder %	
B	19.11	A	9.16	B	12.19	B	64.61
J	11.95	J	7.26	J	4.29	H	47.84
C	9.63	P	4.05	H	4.10	G	42.74
O	9.12	M	4.04	D	3.70	J	37.69
A	6.26	F	3.83	K	3.68	A	30.25
H	8.66	D	9.64	C	2.46	P	28.73
P	5.84	L	7.99	O	2.20	M	24.15
M	5.33	E	7.84	I	2.06	F	21.59
F	4.89	N	7.60	L	1.95	N	17.12
D	13.58	C	6.73	E	1.78	K	57.13
L	9.94	O	6.48	N	1.56	I	38.35
E	9.64	H	4.48	M	1.27	D	28.54
N	9.18	I	3.40	F	1.01	C	28.64
K	7.18	K	2.84	G	4.55	O	27.31
I	5.66	B	6.49	A	1.74	L	19.65
G	10.84	G	6.14	P	1.51	E	18.63

Difference between Control and Inoculation values

No significant difference

Significant Increase

Significant Decrease

Selection G in Table 4.6 indicates the danger of using feeder root percentage instead of mass when comparing different genotypes with each other. The feeder root percentage of G was the third highest of all the selections but the actual feeder root as well as the total root mass have been reduced drastically as is evident from Figures 4.9 and 4.10.

Avocado rootstocks need to be more than just tolerant to *P. cinnamomi*. Factors such as compatibility, precocity, yield and effect on quality should be taken into account as well (Bijzet & Sippel, 2001) and can only be determined

in a field trial. It can, however, be hypothesised that a larger root system would enhance the foraging ability of the avocado tree.

A large number of physiological processes in plants depend on leaf water potential and the avocado is no exception (Sterne *et al.*, 1978). A genetically larger root system would thus not be so easily prone to water stress as a smaller root system. The feeder root mass is also important, as the development of new increments of growth by continued elongation of existing roots and the initiation of new feeder roots are considered to be important features with regard to absorption by roots (Esau, 1977). This growth creates new absorbing surfaces, and it brings these surfaces in contact with new areas of soil.

The selections thus destined for further evaluation are B, H, D and K. Selections C, O, I, L, E, M, F and N are temporarily disregarded due to their total root mass (Figure 4.8) being significantly lower than that of Duke 7.

4.3. CONCLUSION

The detached root inoculation method (unpublished data) were only able to eliminate 45 of the original 91 selections. An alternative method was needed as the remaining number of selections was still to high to be promoted to the field. Comparison of feeder root percentage in non-inoculated and inoculated treatments were also not sufficient to facilitate the final selection of candidate rootstocks from a large number of potential selections with initial characteristics similar to that of Duke 7.

Four selections could be made based on the hypothesis that a larger root system will be a better forager and thus enhances the horticultural aspects of the rootstock scion combination. This hypothesis has, however, not yet been proven with regard to different selections and should be tested. It is also worthwhile to repeat the trial grafting the selections with a scion such as Hass, which is known to exert a further drain on the rootstock.

A revised method of Kelham & Coffey (1985) can be suggested for enhanced differentiation of the apparently similar selections, where the influence of *P. cinnamomi* is assessed over time. This method will identify the selections with an ability to maintain root regeneration, facing *P. cinnamomi* pressure, over time.

CHAPTER 5

Clonal propagation as a selection criterion of potential new avocado rootstocks

5.1. INTRODUCTION

A comparison of avocado (*Persea americana* Mill) rootstock selections amongst each other and with the standard Duke 7 necessitated clonal multiplication of the selections. This task was left to the ITSC nursery, but it did not tie in with the normal nursery activities and it was soon reported that some of the selections were difficult to multiply. Clonal multiplication is an important nursery practice and was included, in addition to *Phytophthora* tolerance, as selection criterion for avocado rootstocks. As each new selection comprises only one plant, clonal multiplication was required to provide at least 40 plants to be used for additional *Phytophthora* tests. This number excluded plants needed for horticultural evaluations.

The main methods of propagating avocado clonally were described by Frolich & Platt (1972), Ernst (1978) and Moll & Wood (1980). The method of Moll & Wood (1980) was preferred to multiply selected *Phytophthora* root rot tolerant seedlings since limited material was available for grafting and a large number of plants are required for additional *Phytophthora* tests as well as for horticultural evaluations that might follow at a later stage. This process represents only one of many methods available for multiplying avocado rootstocks. Thus, if a rootstock is found to be difficult to multiply by the method selected for the purpose of this project but has an excellent tolerance/resistance to *Phytophthora,* it could still be recommended for further investigation. This will, however, only be possible if sufficient material can be produced, by means of a different method, for these tests. If commercial release of such a rootstock is not viable due to the difficulty, and subsequent high costs involved in multiplication, the rootstock might still be useful as a breeding parent.

5.2. MATERIALS AND METHODS

The trial was performed with the fourteen selections listed in Chapter 4, Table 4.4.

Seeds of a sun-blotch-free Edranol avocado tree were planted, as nurse seeds, in small plastic bags (70 mm diameter, 150 mm high) containing a well-drained sterile medium. Care was taken to ensure that the nurse seeds were fairly uniform in size and mass. After germination, the nurse seedlings were grafted with the selections to be multiplied.

At bud burst the nurse plants were transferred to a dark-room for etiolation. Two methods were followed from this step, depending on the growth habit of the selection:

1. When leaves developed during the etiolation process, cuttings were taken while in the dark, when the shoot had stopped growing actively (approximately 300-400 mm). (Figure 5.1) Cuttings were taken at internode length and placed in a mist bed with bottom heating at 26 °C and a mist blow of two seconds every minute.

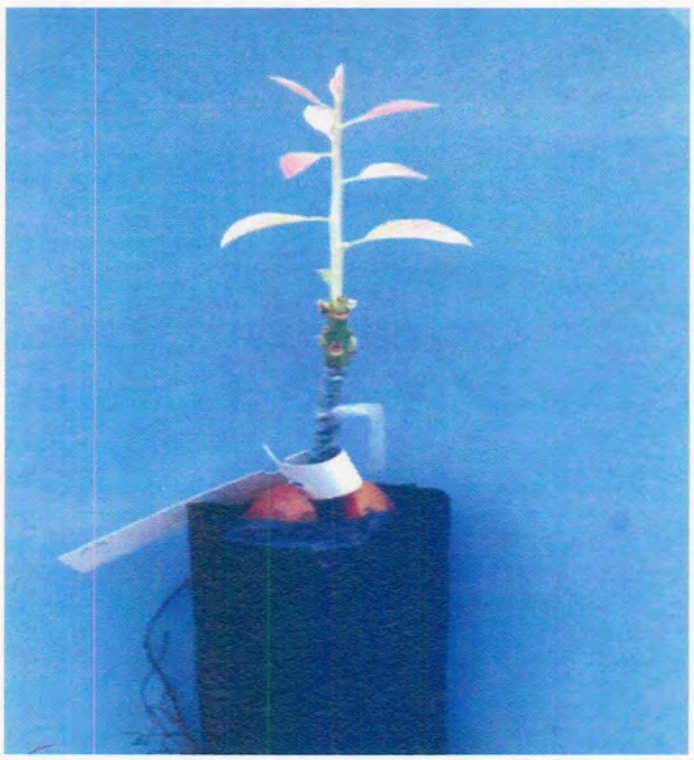

Figure 5.1 Etiolated avocado selection with leaves that had developed.

2. Selections that did not develop leaves in the dark were removed from the etiolation chamber when etiolated shoots were approximately 200-300 mm long. The shoots were painted black with a bitumen-based tree-sealing compound (Figure 5.2) and the plants were left in daylight to develop normal green leaves. When one or two leaves had developed, the shoot was cut and placed in a mist bed as described above.

Figure 5.2 Etiolated avocado selection without leaf development

Figure 5.3 Rooted internode-length cutting

Roots developed approximately 4-8 weeks later (Figure 5.3). When the roots had developed, the cuttings were transplanted to the same size bag with medium as the nurse seedling. The transplanted cuttings were left under the mist spray for a week whereafter they were hardened off.

Duke 7	F	H	**Duke 7**	L
N	O	O	N	D

Selections that consistently excelled are marked in green and ones that consistently failed are marked in red. The standard Duke 7 is marked in blue. K, a local escape tree, was the best overall performer. Some selection like Duke 7 and C were variable with regard to their performance. The overall performance of selection O was disappointing.

Scions from the selections were grafted on nurse seedlings during November and transferred to the dark as previously described. The material was inspected every fourth day. Cuttings were taken and placed in a mistbed as described above. The dates of each action were recorded. In each instance Duke 7 was regarded as the standard for the sake of comparison. Easy and effective clonal multiplication of avocado rootstocks was quantified by the following measures:

- Number of days from grafting to first cut
- Number of cuttings per nurse seed
- Percentage of cuttings rooted
- Number of days to rooting

Data were statistically analysed as a randomised block design with SAS. A selection refers to a single seedling plant that was selected during the initial screening. Limited material was thus available for multiplication purposes. Four bud sticks were taken from each selection and grafted on four different nurse seeds. These were considered as the replicates. The data were tested for normality with a UNIVARIATE procedure and an analysis of variance was done with the GLM procedure of SAS. The means were also compared with a protected Fisher test at 99 % level of significance.

5.3. RESULTS

The coefficient of variance ranged form 18 % to 28 % for the various measurements. The distribution of the data was fairly normal for each measurement except for the number of cuttings that were taken per graft, in which case a transformation was done. This resulted in the coefficient of variance for this specific analysis being lowered from 18 % to 7 %. The replicates did not differ significantly (P=0.01) for any of the analyses that were done.

5.3.1. NUMBER OF DAYS FROM GRAFTING TO FIRST CUT AND BETWEEN CUTS

The prolific nature of each selection regarding the production of new shoots was determined by noting the number of days from grafting the selection until the first cut could be made. Complementary to this the time from cut-to-cut, i.e the number of days that elapsed until another internode length cutting/cuttings was available, was also recorded.

Table 5.1 Time from grafting of clonal avocado selections on nurse seedlings until the first cutting could be taken.

Selection	Mean number of days*
N	80.0 a
Duke 7	72.5 a
E	70.5 ab
O	63.3 abc
A	45.0 bcd
B	45.0 bcd
D	44.8 bcd
I	44.8 bcd
K	43.0 cd
G	41.3 cd
C	39.0 cd
H	38.8 cd
M	38.3 cd
L	38.0 cd
F	34.3 d

* Values not followed by the same letter differ significantly according to a protected Fisher test (P=0.01)

The time from grafting to first cut varied between 34.3 and 80 days (Table 5.1). In this case the selections with the shortest time between grafting to first cutting made are the most efficient.

The time that elapsed until another internode length cutting or sometimes more than one cutting was available from each graft per selection is given in Table 5.2. There is a need for a prolific rootstock that produces new cuttings at shorter intervals in order to produce larger numbers of rooted cuttings in a shorter period of time.

Table 5.2 Time that elapsed until another internode cutting or cuttings per nurse seedling was available

Selection	Mean number of days between cuts*	
O	39.8	a
F	31.5	ab
B	31.3	ab
G	29.7	abc
E	29.6	abc
Duke 7	26.0	bcd
H	25.6	bcd
D	23.2	bcde
L	23.0	bcde
M	22.7	bcde
A	21.3	bcde
I	18.9	cde
N	16.8	de
K	13.9	e
C	13.3	e

* Values not followed by the same letter differ significantly according to a protected Fisher test (P=0.01)

The number of days between cuts varied from 13.3 to 39.8. Only two selections were significantly better than Duke 7 namely K (Sa-RS97/1) and C (92-2-2).

5.3.2. NUMBER OF CUTTINGS PER NURSE SEED

The data did not fit a normal distribution very well so a transformation was done. The data in Table 5.3 represent a transformation back to normal values. Ten of the selections were significantly more prolific than Duke 7. K, G and C, however, performed significantly better than any of the other selections, including Duke 7.

Table 5.3 Mean number of cuts that were produced per nurse seedling per selection.

Selection	*Mean number of cuttings per graft	
K	50.0	a
G	30.7	b
C	29.0	b
D	14.0	c
M	13.7	cd
L	12.6	cde
I	12.0	cde
N	11.1	cde
E	11.0	cde
A	11.0	cde
F	9.9	def
B	9.2	ef
Duke 7	7.2	fg
H	6.3	g
O	5.7	g

* Values not followed by the same letter differ significantly according to a protected Fisher test (P=0.01)

The number of cuts per nurse seed is also important as it enhances the final number of cuttings that will be available for rooting. It furthermore has implications with regard to space requirements for etiolation, as fewer bags with

grafted nurse seeds are needed for a selection that produces a large number of cuts per nurse seed.

5.3.3. PERCENTAGE OF CUTTINGS THAT ROOTED

The percentage of cuttings that can be successfully rooted is the most important selection criterion with regard to clonal ability. It is at this stage that many selections fail to produce results. The rooting ability of the selections that were evaluated is depicted in Table 5.4.

Table 5.4 Rooting ability of selections as determined by the percentage of cuttings that rooted.

Selection	Mean percentage of cuts that rooted*
K	82.5 a
E	77.3 ab
B	74.1 abc
F	69.4 abcd
G	65.9 abcd
L	64.5 abcd
H	62.5 bcd
I	55.8 Cde
C	53.2 De
A	52.0 De
D	39.7 ef
M	38.2 ef
O	26.3 fg
Duke 7	21.7 fg
N	9.4 g

* Values not followed by the same letter differ significantly according to a protected Fisher test (P=0.01).

The best selection was K but it did not differ significantly from E, B, F, G and L. It could be argued that a rooting percentage of less than 70 % is not economically feasible, which excludes most of the selections, including Duke 7.

5.3.4. NUMBER OF DAYS TO ROOTING

Another factor that has an influence on the clonal ability of a selection is the period of time that elapses from the day that the cutting is put into the mist bed until it has formed roots and can be transplanted. Saving time is always important but in this instance the primary need for a short period from cutting to rooting is the constant possibility of contamination with pathogens and the consequent loss of material. Table 5.5 shows the time that elapsed for each selection from entering the mistbed to being transplanted as a rooted cutting.

Table 5.5 Time from entering the mistbed to being rooted.

Selection	Mean number of days*	
D	114.5	a
L	92.4	b
O	83.0	bc
I	73.5	cd
B	71.8	cd
A	66.3	d
H	64.2	d
E	62.2	d
M	45.6	e
C	45.4	e
F	44.3	e
G	39.4	e
K	31.1	ef
N	23.3	f
Duke 7	19.0	f

* Values not followed by the same letter differ significantly according to a protected Fisher test (P=0.01).

Duke 7 performed very well with regard to this characteristic, but not significantly better than selections N and K. The variation amongst selections ranged from 19 days to 114.5 days.

In Table 5.6 a summary is presented of the data given in the graphs and the best selections are highlighted. Selections are arranged from the best performer to the worst performer per characteristic. Areas under each heading with the same colour represent the selections that did not differ significantly from each other. Selections under the same heading shaded in a specific colour differed significantly from those shaded differently.

Table 5.6 Summary of cloning ability of avocado selections

# days to 1st cut	#days : cut-cut	# of cuttings per nurse seed	% of cuts that rooted	# of days from cut to rooting
F	C	K	K	Duke 7
L	K	G	E	N
M	N	C	B	K
H	I	D	F	G
C	A	M	G	F
G	M	L	L	C
K	L	I	H	M
I	D	N	I	E
D	H	E	C	H
B	Duke 7	A	A	A
A	E	F	D	B
O	G	B	M	I
E	B	Duke 7	O	O
Duke 7	F	H	Duke 7	L
N	O	O	N	D

Selections that consistently excelled are marked in green and ones that consistently failed are marked in red. The standard Duke 7 is marked in blue. K, a local escape tree, was the best overall performer. Some selection like Duke 7 and C were variable with regard to their performance. The overall performance of selection O was disappointing.

5.4. CONCLUSION

The cloning ability of the different selections varied considerably even though they shared the same parent. One of the mechanisms with regard to tolerance in Duke 7 (one of the parents of these selections) is its considerable root growth potential (Coffey, 1991). It would thus be expected that the percentage cuttings that rooted as well as the number of days from cut to root should give an indication of the potential of the selections with regard to one of the mechanisms for tolerance. This will be taken into account during further experiments with these seedlingThe 91 selections, remaining after the initial screening, have been tested with the detached root inoculation method (Van der Merwe, 1995) and selections that were not significantly better than the standard cultivar Duke 7, were discarded.s.

Most of the selections performed better than the standard cultivar Duke 7 except for time from cut to root. This would be expected to some extent as the Duke 7 material was taken from a mature tree whilst the single plant selections were never older than two years, when the material for cloning was taken. Kadman (1976) concluded that getting material from mature trees to root was more difficulty than to root material still in a juvenile state. Any selection thus not performing to the same standard as Duke 7 is not acceptable.

The largest constraint experienced during this trial was the inability to etiolate some of the selections, which could thus not be rooted or undergo further testing. These selections might possess beneficial genes and will not be discarded but will be incorporated as mother trees in the breeding programme.

CHAPTER 6

General conclusion

6. GENERAL DISCUSSION

Phytophthora root rot caused *Phytophthora cinnamomi* Rands is a devastating disease of avocados (*Persea americana* Mill.), with immense financial implications to the producer. A review of the literature indicated the existence of complex host-pathogen interactions with regard to avocado and *P. cinnamomi*. It is evident that vertical resistance to *P. cinnamomi* does not exist in *P. americana*. Avocado rootstock germplasm that is currently available does, however, include individuals with different mechanisms of tolerance that can be utilised in a breeding programme. The incompatibility between the group of *Persea* spp. that exhibit resistance and the group that *P. americana* belongs to is a complicating factor in a breeding programme aimed at establishing *Phytophthora* root rot resistant rootstocks for avocado production. In order to find a beneficial phenotype to aid increased tolerance with regard to *P. cinnamomi*, an unknown number of multiple genes have to be recombined.

Detection of a beneficial genotype is the most crucial element in a breeding programme. Possible variation in host-pathogen interactions could thus be a further complication and these interactions must be taken into account when screening of newly created phenotypes is considered. Various physical and physiological factors as well as other pathogens can influence screening results. Ignorance with regard to these factors will induce high costs and render the breeding programme ineffective.

Various physical constraints such as flower morphology and behaviour as well as expensive and time-consuming field trials are further complicating factors. The literature should thus be utilised to optimise the screening process in order to ensure that beneficial genotypes are not overlooked or that unnecessary material does not make the breeding programme unwieldy and costly.

An overview of the breeding programme between 1991 and 1997 has shown that the methods previously used by the breeding team at the ARC-ITSC, were not scientifically based. The following problems were foremost:

- Inoculum was either derived from local isolates of the pathogen or from old storage cultures. Local isolates were not positively identified as *P. cinnamomi* by a mycologist whilst loss of virulence and various genetic degeneration or attenuation of strains maintained in culture could have occurred.
- Soil from an avocado orchard with *P. cinnamomi* symptoms was used without determining the pathogen complex or even determining whether *P. cinnamomi* was present or not.
- Methods were not consistent from season to season

The material that was initially selected between 1991 and 1997 nevertheless proved to be equal to the standard cultivar Duke 7. Only three of these selections were discarded, at the end of the trials done for this dissertation, due to their inability to match the characteristics of Duke 7.

With regard to choice of screening media it was indicated that soil is not an effective medium for the initial screening of breeding products. A high incidence of necrosis, attributed to the slow drainage following the flooding of the soil, was evident. Soil is not an inert standardised medium and repeatability from season to season will be difficult. The inherent properties, for instance the mineral status and clay content, could differ between seasons.

All the other media tested, namely sand, vermiculite, water and the Schwalbach system, were equal in performance. The medium that will be used in future will depend on the preference of the breeder as each medium has its own pro's and con's. The initial capital outlay of both the water and Schwalbach systems is high. These two systems also need intensive care, as temporary clogging of nozzles in the Schwalbach system and interrupted

delivery of oxygen to the water system, could cause the results to be inaccurate or even unattainable.

Vermiculite is prone to weathering and it is expected that continuous use would not be an option. Steam sterilising of vermiculite could contribute to a loss of volume of this medium. Personally, sand was preferred with the precept that only silica sand should be used due to its unique characteristics. Optimal germination of avocado seeds has previously been achieved in vermiculite. It is thus recommended that seeds be first germinated in vermiculite after which the seedlings can be transplanted to sterilised silica sand for the screening process as was described in this dissertation.

Sand and vermiculite are effective for the screening of genetic material that relies on root regeneration as a mechanism of resistance. Shoot growth in the sand and vermiculite does not reflect the root situation and screening of seedlings will still be dependent on a subjective evaluation of the roots as determining feeder root percentage of 10 000 individual plants is not viable. Percentage necrosis could prove valuable as this can be done mechanically. Although the Schwalbach system (mist) reflects stress in the root region readily in the shoot growth, as indicated in Table 4.3, root regeneration is not facilitated as the effect of *P. cinnamomi* is intense and rapid in the absence of additional fertiliser or leaf feeds.

It was also evident from Table 4.3 that the evaluation criterion to be used depends on the medium that is used. Feeder root percentage was a good criterion for clonal material in all the media except for soil and water. Necrosis was a good criterion for clonal material in all the media except for soil.

There was a definite difference with regard to the cloning ability of the different selections even where they shared the same parent. An inability to be etiolated was displayed by some of the selections and these could thus not be vegetatively propagated and were not tested further.

One of the mechanisms involved in the tolerance of Duke 7 (one of the parents of these selections) is its considerable growth potential. It would thus be expected that the percentage cuttings that rooted as well as the number of days from cut to root should give an indication of the potential of the selections with regard to this mechanism of tolerance. This, however, does not reflect clearly when comparing Tables 4.6 and 5.6.

Most of the selections performed better than the standard cultivar Duke 7. This would be expected to some extent as the Duke 7 material was taken from a mature tree whilst the single plant selections were never older than two years when the material for cloning was collected. Any selection not performing to the same standard as Duke 7 is not acceptable, as rooting cuttings taken from mature trees are more difficult than rooting material still in a juvenile state.

Valuable information was obtained with regard to various media and the criteria to be used during screening. This knowledge must be taken into account in the planning of future breeding projects. Knowledge of the clonal ability of a potential new rootstock is important for both the nursery and the producer from a financial point of view.