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Phenotypic variation in three *Phytophthora cinnamomi* populations from macadamia growing areas in Kenya

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Key words
Macadamia, management, Phytophthora, variation, root rots.

1 SUMMARY

In Kenya macadamia (*Macadamia integrifolia* Maiden and Betche and *Macadamia tetraphylla* L.A.S. Johnson) is grown by over 100,000 small scale rural farmers. However root rots and trunk cankers caused by *Phytophthora cinnamomi* Rands are major production constraints. Macadamia tree death due to the two diseases is currently estimated at 60%. No single effective method exists for management of the *Phytophthora* induced root rots. Knowledge on variability within species is a pre-requisite to development of strategies for effective disease management. In this study morphological and physiological characteristics of 76 *P. cinnamomi* isolates recovered from rhizospheres, stems and roots of symptomatic macadamia trees in different regions of Kenya were investigated. Phenotypic variations were demonstrated in radial growth rate, colony morphology and sporangial dimension. To determine pathogenicity and virulence, green apples were inoculated with each of the isolates. The isolates differed significantly (P=0.001) in growth rate on apples. There was a significant relationship (X^2_4 =94.1, P<0.001) between colony type and isolate sub-population. Colony morphology was influenced by temperature. Colonies were predominantly petaloid at 24 °C. Thirty five out of the 76 isolates were pathogenic. The homothallic isolates were the most virulent and killed macadamia seedlings 29 days after inoculation. *Phytophthora cinnamomi* was isolated 10 cm above the inoculation point from asymptomatic seedlings. These findings of large phenotypic variation among isolates have important taxonomic and disease management implications. This is the first such study undertaken in Kenya. The knowledge generated will be crucial in development of integrated management strategies for macadamia root rots and trunk canker in Kenya.

2 INTRODUCTION

Macadamia in Kenya is currently grown by over 100,000 small scale rural farmers in Central, Eastern and Coast provinces (Muthoka *et al.*, 2005). Kenya is the fourth largest world producer of macadamia nuts after Australia, Hawaii and South Africa (Wilkie, 2008). In 2009, 14,742 tons in-shell nuts were produced. The biggest importer of the in-shell nuts was china (3752 tons) while the United States of America imported the largest share of the processed nuts (1034 tons) (Onsongo, 2009). There are many players in the macadamia production value chain and the crop has potential to create employment,

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increase rural income and alleviate poverty and hunger (Gitonga *et al*., 2009). However, root rots and trunk cankers are major macadamia production constraints in Kenya (Mbaka *et al*., 2009) as in other countries such as South Africa (Manicomb, 2003), Hawaii (Hine, 1961) and Australia (Drenth, 2007).

The two major diseases in Kenya are caused by the soil borne pathogen *Phytophthora cinnamomi.* The pathogen causes a rot of the fine feeder roots leading to smaller, light green to yellow leaves, branch die back and substantial reduction in growth (Fig. 1); and stem cankers above the soil line (Fig. 2). The cankers girdle and kill the trees (Lòpez-Herrera & Pérez-Jiménez 1995; Zentmyer, 1984). Macadamia nut yield losses due to the two diseases are currently estimated at 60 % in Kenya with no recommended disease management strategies in place (Mbaka *et al*., 2009)

Figure 1: Macadamia root rot infected tree in a rural orchard

Figure 2: *Phytophthora cinnamomi* induced trunk cankers on a macadamia tree.

Phytophthora cinnamomi is one of the most easily identified *Phytophthora* species with distinguishing features including coralloid hyphae, prominent hyphal swellings and abundant chlamydospores. Host baits are used for recovery of *P. cinnamomi* from soil and the apple pathogenicity test is simple and accurate in separating pathogenic from non pathogenic *P. cinnamomi* and *Pythium* species (Serfontein *et al*., 2007). The pathogen has been the subject of several morphological studies, primarily as a basis for diagnostics, classification (Stamps *et al*., 1990) and to describe phenotypic variation (Hüberli *et al*., 2001). *Phytophthora cinnamomi* is heterothallic and requires crossing of A_1 and A_2 mating types for production of oospores. Production of oospores as a result of sexual reproduction increases the genetic variation in a population (Zentmyer, 1980). While designation of mating types as A_1 and A_2 for a particular isolate of *P. cinnamomi* was primarily based on behavior in pairing in pure culture, Shepherd *et al*. (1974) found differences in the morphology and behavior of Australian A_1 and A_2 that allowed recognition of the compatibility type with a degree of certainty. Isolates of *P. cinnamomi* vary widely in pathogenicity and the variation is not related to mating type (Dudzisnki *et al*., 1993). The occurrence of greater variation may enhance the potential for the pathogen to survive adverse conditions or overcome or resist control measures. Where the level of pathogen variation is high, there is the potential for it to evolve and adapt to its environment as selection pressure creates a situation where the pathogen is forced to change to survive. To

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develop successful management strategies for a disease, it is important to determine the extent of phenotypic variation within the causal pathogen population. When *P. cinnamomi* was first described, Rands (1922) showed that isolates varied in pathogenicity in stem inoculated *Cinnamomum barmanni* trees. Since then, there have been numerous reports on macro-morphological (colony type and growth rate) and micro-morphological (sporangial and gametangial morphology) variation among world wide collections of *P. cinnamomi* isolates (Dudzisnki *et al*., 1993). Sporangia can be ovoid, obpyriform or ellipsoid to elongate-ellipsoid in shape, with a petalloid, rosaceous or undefined pattern (Erwin & Ribeiro, 1996).

The current study examined the macro-morphological, micro-morphological and pathogenicity characters among three sub-populations of *P. cinnamomi* recovered from roots, stems and soil from rhizospheres of symptomatic macadamia trees from different parts of Kenya. It was expected that this study would improve knowledge of the diversity and structure of the pathogen population in macadamia growing areas. The knowledge is crucial in the development of strategies for effective management of *Phytophthora* induced root rots and trunk cankers of macadamia

3 MATERIALS AND METHODS

Root, stem and soil samples were collected from symptomatic macadamia trees during disease surveys carried out in Baringo, Bungoma, Embu, Kirinyaga, Machakos, Maragua, Meru Central, Murang'a, Nyeri, Taita-Taveta and Thika districts between December 2005 and April 2006. *Phytophthora cinnamomi* was recovered from soil by use of avocado fruit baits and from roots and stem pieces by direct baiting on Corn Meal Agar (CMA). Identification was based on morphological features such as presence of hyphal swellings and mode of zoospore release from sporangia (Mbaka *et al*., 2009). In total 76 *P. cinnamomi* isolates were identified. To determine the mating type, each isolate was paired with each of the two tester *P. cinnamomi* isolates, A_1 (VQ4856) and A_2 (VQ 4857) previously acquired from the Royal Botanical Garden, Australia. Isolates that formed oospores with the A_2 were designated A_1 and isolates that formed oospores with A_1 tester isolates were designated A_2. Isolates that formed oospores without being paired were thought to be homothallic (forming gametangia on one thallus). The 76 isolates were classified in the three populations (A1, A2, and H) and stored in labeled Bijou bottles containing 2 % Potato Carrot Agar (PCA) at 15 °C for further experimental work (Table 1). The isolates were later confirmed to be *P. cinnamomi* through DNA sequencing done at Stellenbosch University, South Africa in September 2009.

Table 1: Kenyan *P. cinnamomi* isolates used in this study, their source, type and year of isolation.

S/No	Isolate*	Farm code	District	Source	Year isolated	Mating type
1	06-070	Bar05-06	Baringo	soil	2006	H
2	06-071	Bar06-06	Baringo	soil	2006	A_1
3	06-072	Bgm3-06	Bungoma	soil	2006	A_1
4	06-073	Bgm4-06	Bungoma	roots	2006	A_1
5	06-034	Emb30-06	Embu	soil	2006	A_1
6	06-035	Emb30-06	Embu	soil	2006	A_2
7	06-036	Emb19-06	Embu	soil	2006	A_1
8	06-037	Emb19-07	Embu	soil	2006	A_2
9	06-038	Emb26-06	Embu	roots	2006	A_1
10	06-039	Emb26-06	Embu	roots	2006	A_2
11	06-040	Emb27-06	Embu	stem	2006	A_1
12	06-041	Krg3-06	Kirinyaga	soil	2006	A_1
13	06-042	Krg6-06	Kirinyaga	soil	2006	A_1
14	06-043	Krg8-06	Kirinyaga	soil	2006	A_1

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15	06-044	Krg8-06	Kirinyaga	roots	2006	A_2
16	06-045	Krg21-06	Kirinyaga	roots	2006	A_1
17	06-046	Krg21-06	Kirinyaga	soil	2006	A_2
18	06-047	Krg27-06	Kirinyaga	stem	2006	A_1
19	06-048	Krg27-06	Kirinyaga	stem	2006	A_2
20	06-049	Krg27-06	Kirinyaga	soil	2006	H
21	06-050	Mcks1-06	Machakos	soil	2006	A_1
22	06-051	Mcks1-06	Machakos	stem	2006	A_2
23	06-052	Mcks1-06	Machakos	soil	2006	H
24	06-053	Mcks25-06	Machakos	roots	2006	A_2
25	06-054	Mcks14-06	Machakos	soil	2006	A_2
26	06-055	Mcks14-06	Machakos	soil	2006	H
27	06-056	Mcks16-06	Machakos	stem	2006	A_1
28	06-057	Mcks16-06	Machakos	roots	2006	A_2
29	05-004	Mar2-05-	Maragua	stem	2005	A_1
30	05-005	Mar2-05	Maragua	soil	2005	A_2
31	05-006	Mar2-05	Maragua	stem	2005	H
32	05-007	Mar3-05	Maragua	soil	2005	A_2
33	05-008	Mar13-05	Maragua	soil	2005	A_1
34	05-009	Mar13-05	Maragua	stem	2005	A_2
35	05-010	Mar 30-05	Maragua	soil	2005	A_2
36	05-011	Mar28-05	Maragua	roots	2005	A_2
37	05-012	Mar28-05	Maragua	soil	2005	H
38	05-013	Mar26-05	Maragua	roots	2005	A_1
39	05-014	Mar26-05	Maragua	soil	2005	A_2
40	06-058	Mru-10-06	Meru Central	roots	2006	A_1
41	06-059	Mru-10-06	Meru Central	roots	2006	H
42	06-060	Mru-10-06	Meru Central	soil	2006	A_2
43	06-061	Mru-28-06	Meru Central	soil	2006	A_1
44	06-062	Mru-30-06	Meru Central	stem	2006	H
45	06-063	Mru-27-06	Meru Central	soil	2006	A_1
46	06-064	Mru-15-06	Meru Central	stem	2006	A_2
47	06-065	Mru22-06	Meru Central	soil	2006	A_2
48	06-066	Mru20-06	Meru Central	soil	2006	A_1
49	06-067	Mru22-06	Meru Central	soil	2006	H
50	06-068	Mru30-06	Meru Central	soil	2006	A_2
51	06-069	Mru24-06	Meru Central	soil	2006	A_2
52	05-015	Mur3-05	Murang'a	soil	2005	H
53	05-016	Mur9-05	Murang'a	roots	2005	A_1
54	05-017	Mur9-05	Murang'a	soil	2005	H
55	05-018	Mur18-05	Murang'a	soil	2005	H
56	05-019	Mur18-05	Murang'a	roots	2005	A_2
57	05-020	Mur20-05	Murang'a	stem	2005	A_2
58	05-021	Mur20-05	Murang'a	soil	2005	H
59	05-022	Mur25-05	Murang'a	stem	2005	A_2
60	05-023	Mur25-05	Murang'a	stem	2005	A_1
61	05-024	NY-25-05	Nyeri	soil	2005	A_1
62	05-025	NY-7-05	Nyeri	roots	2005	H
63	05-026	NY-13-05	Nyeri	soil	2005	A_1
64	05-027	NY-13-05	Nyeri	soil	2005	A_1

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65	05-028	NY-14-05	Nyeri	roots	2005	A_1
66	05-029	NY-18-05	Nyeri	stem	2005	A_1
67	05-030	NY-19-05	Nyeri	soil	2005	A_2
68	05-031	NY-20-05	Nyeri	roots	2005	A_1
69	05-032	NY-25-05	Nyeri	soil	2005	A_1
70	05-033	NY-26-05	Nyeri	stem	2005	A_1
71	06-074	Tt -10-06	Taita-Taveta	soil	2006	H
72	06-075	Tt -10-06	Taita-Taveta	stem	2006	A_1
73	06-076	Tt -8-06	Taita-Taveta	roots	2006	H
74	05-001	Tk-10-05	Thika	soil	2005	A_1
75	05-002	Tk-15-05	Thika	stem	2005	A_2
76	05-003	Tk-22-05	Thika	soil	2005	A_1

* The first two digits indicate the year of isolation; the other three digits are the accession number as per storage.

3.1 Radial growth rate and colony morphology: Hyphal tipped colonies of the 76 *P. cinnamomi* isolates were grown on corn meal agar (CMA) for 3 days. Agar discs (5 mm diameter) were cut from the edge of the actively growing colonies on CMA using a sterile cork borer. These were placed with the mycelia facing down at the centre of individual Petri plates (90 mm diameter) containing 10 ml of Potato Dextrose Agar (PDA) (Oxoid Ltd. Basingstoke, Hampshire, England). Plates were sealed with Parafilm® (American National Can. Chicago) and incubated at 20, 24 and 28 ˚C in the dark. The plates were arranged in a complete randomized design (CRD) in laboratory incubators with four replicate plates for each treatment. Radial growth was measured after 4 days along two lines intersecting at right angles at the centre of the inoculum disc. The radial growth rate in mm per day was calculated by taking the average of all radial measurements, subtracting the inoculum disc radius and dividing by four. The experiment was repeated 3 times. Colony morphology of each isolate at each temperature was recorded as rosaceous, petaloid, or non pattern (Erwin & Ribeiro, 1996).

3.2 Sporangia morphology: Each isolate was grown on clarified V8 juice agar at 25 ˚C in the dark for 3 days. Agar plugs of young actively growing mycelia were covered with non sterile soil extract solution (Jeffers & Aldwinckle, 1987) and incubated under continuous light from fluorescent lamps (20 W, cool white) suspended 22 cm above the cultures at room temperature (22 ± 2 ˚C) to induce production of sporangia. After 24 h, individual plugs of each isolate were mounted in distilled water and observed microscopically (200 - 400×). For each isolate, dimensions (length and breadth) of 20 randomly selected first generation sporangia were measured, and the length: breadth ratio was calculated (Duan *et al*., 2008). Sporangial shape was recorded using the descriptors of Erwin and Ribeiro (1996).

3.3 Pathogenicity to green apples: The 76 isolates were screened for their pathogenicity on green apples (*Malus domestica*). Green apples were purchased from a local supermarket. Care was taken to only select apples with no visible blemish. The apples were sterilized by dipping in 70 % ethanol and blotter dried on a laminar flow bench in the laboratory. Incisions (10× 10 mm) were cut in the apples using a sterile scalpel. Plugs (5 mm diameter) were cut from the edges of 4 day old *P. cinnamomi* cultures on V8 juice agar and inserted mycelia facing down on the incisions on the apples. The points of inoculation were sealed by wrapping with parafilm. Controls were inoculated with V8 juice agar discs and sealed in the same manner above. There were four replicate apples for each isolate and control treatments. The fruits were arranged in a complete randomized design (CRD) in disinfected plastic trays and incubated at 24 ± 2 °C in the dark for eight days. Presence of hard brown rots was indication of pathogenicity of the isolate. The length of each externally visible lesion extending from the point of inoculation was measured. The daily lesion extension (mm $^{d-1}$) was calculated by dividing the mean extension length by 8. Re-isolation of *P. cinnamomi* from the symptomatic and asymptomatic apples was done by direct plating of inoculated tissue on CMA.

3.4. Pathogenicity to macadamia seedlings: Isolates pathogenic to apples were further tested for

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their capacity to kill macadamia seedlings in a screen house experiment at KARI-Thika (1° 00' 10" South, 37° 04' 30" East). Sterile Miracloth (Calbiochem, Corporation, Canada) discs, 10 mm diameter were placed onto V8 agar (30 per plate). Each disc was inoculated with 5 mm agar disc of actively growing hyphal tipped *P. cinnamomi* isolates and incubated at 22 ± 2 °C for 10 days. Stems of two month old macadamia seedlings were bark wounded with a sterile scalpel blade mounted on a scalpel holder no. 3. The inoculated Miracloth discs were placed on the wounds and sealed with parafilm. Control plants were inoculated with sterile miracloth previously placed on V8 juice agar. There were four replicate plants per isolate and control treatments arranged in a completely randomized design (CRD) in the screen house. Watering using a can was done three times a week. Temperatures ranged between 21 to 24 °C through out the duration of the experiment. Data recording commenced three weeks after inoculation when the first symptoms (leaf necrosis) were observed. Plants were recorded as dead when all the leaves were necrotic and crisp. Dead plants were harvested and five one-centimeter sections above the point of inoculation were cut and plated on CMA and incubated at 24 ±2 °C in the dark for recovery of *P. cinnamomi*. Similar isolation was done from asymptomatic seedlings.

3.5 Data analysis: Data were examined for normality of distribution and variance homogeneity using the Levene option of the Hovtest. Transformations were performed where required in parametric tests (Dytham, 1999; Fowler and Cohen, 1990). The arcsine square root transformation was performed on proportion data. One way analysis of variances (ANOVAs) and the Least Significant Difference (LSD) test (P=0.05), using the procedure general linear model, GLM (SAS Institute, 2003) were used to test for significant treatment effects and means comparison, respectively. Pearson's test and the Spearman Rank Test were used to test for correlation between data. Colony data were tabulated into a three way contingency table using the variables of colony morphology (non- pattern, petaloid and rosaceous), temperature (20, 24 and 28 ˚C) and isolate sub-population (A_1, A_2 and H). Frequencies of sporangia shapes (eight shapes) were cross tabulated by isolate sub-population (A_1, A_2 and H) or non pattern. Non –pattern was classification was given to those isolates that did not display any characteristic pattern as described by Erwin &Ribeiro (1996). The log- linear model which best fitted the data indicated significant two way relationship between colony morphology and isolate sub-populations (X^2 =94.1, P<0.001). Colony morphology was influenced by temperature as indicated by a significant interaction (X^2=94.1, P<0.001). All the isolates had different colony morphology at all temperatures. At 24 and 28 ˚C all the isolates were generally petalloid (Table 3).

4 RESULTS

4.1 Radial growth rate: The mean radial growth rates differed significantly between the isolates (P<0.001) at different temperatures. The highest mean radial growth rates for all the isolate types were obtained at 24 °C and the lowest at 20 °C (Table 2). The H type isolates had higher radial growth rates at all the temperature conditions with an overall mean rate of 5.94 mm d^{-1} compared to the A_1 and A_2 which had mean radial growth rates of 4.2 and 4.43 mm d^{-1} , respectively.

4.2 Colony morphology: Colony morphology of the isolates was described as rosaceous, petaloid

Table 2: Mean radial growth rate of mating type A_1 andA_2 and homothallic (H) *P. cinnamomi* isolates from macadamia in Kenya, cultured on PDA at 20, 24 and 28 °C.

Temperature (˚C)	**Radial growth rate (mm^{d-1})**		
	A1 isolates n=34	**A2 isolates n=26**	**H isolates n=16**
20	3.45 ±0.01c*	3.64 ± 0.05c	5.60 ± 0.11c
24	4.76 ± 0.04a	5.05 ± 0.58 a	6.31 ± 0.05a
28	4.34 ± 0.03b	4.60 ± 0.04 b	5.80 ± 0.04 b
Mean	4.18	4.43	5.94
LSD	0.25		
P value	< 0.001		

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*Means in the same column followed by different letters are significantly different according to the LSD test (P=0.05).

Table 3: Colony morphology of A_1 andA_2, and homothallic (H) Kenyan *P. cinnamomi* isolates on PDA 4 days after incubation at 20, 24 and 28 °C*.

Colony type % at		**Isolate sub-population**		
20°C		A1	A2	H
§	Non-Pattern	5a	20b	40.7c
§	Rosaceous	18a	20a	0b
§	Petaloid	77a	60b	59.3b
24°C				
§	Non-Pattern	14a	25b	47c
§	Rosaceous	50a	50a	23b
§	Petaloid	36a	25c	30b
28°C				
§	Non-Pattern	0a	13b	35c
§	Rosaceous	33c	20b	15a
§	Petaloid	61b	67c	50a

* Row values followed by the same letter are not significantly different (P>0.05)

4.3 Sporangia morphology: All isolates formed non-papillate sporangia (Fig. 3).

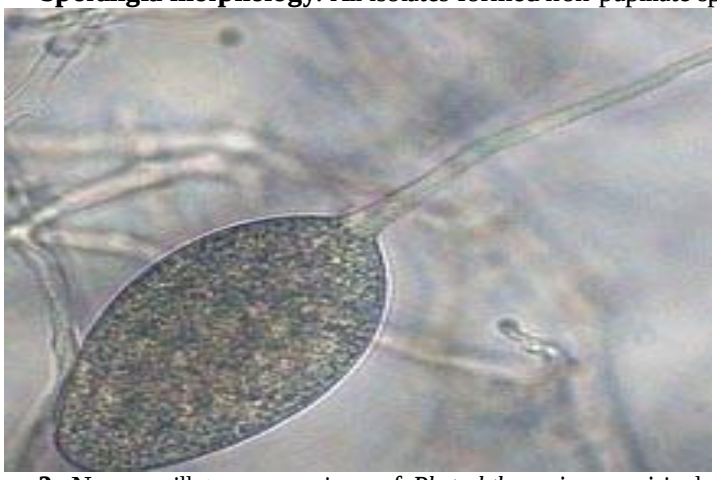

Figure 3: Non papillate sporangium of *Phytophthora cinnamomi* isolates from macadamia trees in Kenya (× 400).

Eight sporangial shapes (limoniform, ovoid, ellipsoid, conical, globose obpyriform, obvoid, and cylindrical) were recorded. The dominant sporangial type was limoniform (59.3 -74.2 %) followed by ovoid (13.1 -18.2 %) (Table 4). There was a strong correlation (r=0.67, P=0.001) between sporangia shapes and isolate sub-population. Homothallic isolates had the highest frequency (74.2 %) of limoniform sporangia. No cylindrical sporangia were recorded in A2 mating type isolates There was wide variation in sporangia shapes and size both within and among isolate populations. In general the shape of sporangia was pre-dominantly ovoid to ellipsoid. The mean lengths (L) of sporangia ranged from 43.4 to 77.8µm and the mean breadths (B) ranged from 25.3 to 48.6 µm. The L: B ratios of sporangia ranged from 1.50 to 1.93 (Table 4). For all isolates, sporangia averaged 57.9 × 34.8 µm (Length × breadth) with an L: B ratio of 1.66 (Table 4). There were no significant differences in sporangia lengths (P=0.066), breadth (P=0.058) or L/B ratios (P=0.064) (Table 5)

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Table 4: Sporangial shapes of mating type A1 and A2, and homothallic *P. cinnamomi* isolates associated with root rots and stem canker of macadamia in Kenya.

Sporangial shapes (%)	**Sub-populations**		
	A1	**A2**	**H**
Limoniform	59.3 ± 4.5	58.2 ± 5.1	74.2 ± 6.1
Ovoid	18.2 ± 3.2	17.1 ± 6.6	13.1 ± 2.1
Ellipsoid	7.2 ± 2.1	7.9 ± 2.2	4.3 ± 1.9
Conical	5.8 ± 1.8	7.6 ± 2.1	2.3 ± 0.8
Globose	4.2 ± 0.7	5.1 ± 1.8	2.6 ± 0.1
Obpyriform	3.1 ± 0.2	2.3 ± 0.1	1.8 ± 0.1
0bvoid	1.1 ± 0.1	1.8 ± 0.1	1.2 ± 0.2
Cylindrical	1.1 ± 0.1	0	1.1 ± 0.1

Table 5: Sporangial dimensions of mating types A_1 andA_2, and homothallic *P. cinnamomi* isolates associated with macadamia root rots and stem canker in Kenya.

Isolates	**Sporangia dimension**		
	Length (L) (µm)	**Breadth (B) (µm)**	**L / B ratio**
A1	58.4 ± 2.47	34.7 ± 2.48	1.68 ± 0.04
A2	56.5 ± 2.80	34.4 ± 2.08	1.64 ± 0.04
H	58.93 ± 4.80	34.7 ± 1.97	1.68 ± 0.59
P value	0.066	0.058	0.064

4.4. Pathogenicity to green apples: Of the 76 *P. cinnamomi* isolates examined in this study, 35 were pathogenic to green apples as indicated by development of hard brown necrosis on the fruits. *Phytophthora cinnamomi* was re-isolated from the lesions. Contingency table analysis revealed a strong positive correlation (r=48.7, P=0.001) between the source of the isolates and pathogenicity. Of the 35 pathogenic isolates, 10 were previously recovered from soil in macadamia rhizospheres, 17 from stems and 8 from roots representing 23 %, 100% and 50% pathogenicity of isolates recovered from soil, stems and roots respectively. There was no significant correlation (r=2.9, P=0.65) between the isolate sub-population and pathogenicity. However, there was significant difference (P=0.001) in virulence among the isolates sub-population. The highest lesion extension rates (5.79 ± 0.3 to 6.33 ± 0.2 mm $^{d-1}$) were recorded in H isolate inoculations regardless of the source (Table 6).

Table 6: Mean lesion extension rate (mm $^{d-1}$) on green apples after inoculation with mycelia of A1, A2 and Homothallic(H) Kenyan *P. cinnamomi* isolates recovered from macadamia rhizosphere soil, stems and roots.

Isolate type	**Mean lesion extension rate (mm $^{d-1}$)** **Isolate source**		
	Soil	**Stems**	**Roots**
A1	5.67 ± 0.2ab	6.22 ± 0.6ab	5.29 ± 0.4a
A2	4.95 ± 0.3a	5.39 ± 0.3a	5.99 ± 0.5b
H	5.79 ± 0.3bc	6.33 ± 0.2bc	6.32b ± 0.3c
P value	0.001	0.001	0.0012
LSD=	0.71	0.90	0.30

*Means in the same column followed by the same letter are not significantly different according to the LSD (P=0.05) test.

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4.5 Pathogenicity to macadamia seedlings: The first symptoms of infection were observed 21 days after inoculation. Infected seedlings had chlorotic and necrotic leaves without any sign of new growth. The first seedling death occurred 29 days after inoculation with isolate 05-075 (H). Twenty four of the isolates caused death on macadamia seedlings 29-53 days after inoculation. The remaining eight isolates did not cause any visible disease symptoms. There was no apparent relationship between the isolate sub-population and capacity to kill macadamia seedlings (Table 7)

Table 7: Days to death of macadamia seedlings after wound-bark inoculation with Kenyan *P. cinnamomi.* Isolates recovered from macadamia rhizosphere, stems and roots

Isolate	Mating type	Mean days ± SEM
06-071	A1	41.8 ± 8.3
06-072	A1	45.5 ± 10.7
05-001	A1	34.0 ± 6.5
06-074	H	29.5 ± 6.4
06-039	A2	37.0 ± 2.1
06-040	A1	38.3 ± 2.9
06-042	A1	37.3 ± 5.3
06-045	A1	52.0 ± 4.1
06-047	A1	33.5 ± 2.3
05-002	A2	40.75 ± 8.4
06-049	H	51.25 ± 3.8
06-051	A2	40 ± 4.7
06-056	A1	34.3 ± 4.0
06-057	A2	55.3 ± 1.4
05-004	A1	38.*0* ± 3.3
05-006	H	45.2 ± 8.3
05-013	A1	53.8 ± 3.3
06-061	A1	37.8 ± 5.8
06-065	A2	45.8 ± 4.0
06-067	H	55.*0* ±4.*0*
05-019	A2	29.5 ±3.*0*
05-020	A2	52 ± 6.8
05-022	A2	51.2 ± 5.*0*
05-023	A1	42.5 ± 6.3

Phytophthora cinnamomi was recovered from all the dead and symptomatic seedlings 2 cm above the point of inoculation. The pathogen was also recovered from same region in symptom less seedlings.

5 DISCUSSION

5.1 Radial growth rate: The effect of growth temperature on fungal isolates is affected by the composition of the agar medium and isolation method (Zentmyer *et al.*, 1976). Consequently, comparisons among data sets are virtually impossible unless consistent agar media are used and populations of similar genetic background are compared. In this study, it was established that 24 °C on PDA was the optimum growth temperature for all the 76 *P. cinnamomi* isolates. The fastest growing isolates had mean radial growth rates of 5 to 6.6 mm^{d-1}. This was lower than that reported by Zentmyer *et al.*, (1976), who used fresh potatoes in their agar medium and found *P. cinnamomi* to have radial growth rates between 6.2 and 7.5 mm^{d-1}. The growth rates were however similar to those of South African *P. cinnamomi* isolates (5.1 to 6.3 mm^{d-1}) growing at 25 °C (Linde *et al.*, 1997) and the Australian *P. cinnamomi* isolates growing at the same temperature (Hüberli *et al.*, 2001) on synthetic PDA. The H isolates had the highest radial growth rate at all temperatures on PDA (5.60 to 6.31 mm^{d-1}).

5.2 Colony morphology: Considering that all isolates were from similar localities and disease situations, in this study, we demonstrated that colony morphology is not a stable character and

should be used with caution when identifying *Phytophthora* species. Over all, the rosaceous and petalloid colonies were the predominant types. This agrees with previous studies on PDA using world wide collections (Zentmyer, 1980). Temperature altered colony types for all the isolates and should be considered when using colony morphology for identification. Serfontein *et al* (2007) in their study on trunk cankers of macadamia in South Africa observed morphological similarities between *P. cinnamomi* and *Pythium vexans* isolates from soil in macadamia orchards.

5.3 Sporangia morphology: Sporangia were all non-papillate and their dimensions were a stable character in this study which agrees with Waterhouse's grouping (Waterhouse, 1963). Sporangial shape and release are used with certainty for the distinction between *Phytophthora* and *Pythium* species where the two occur together in isolations (Erwin & Ribeiro, 1996; Hardham, 2005). Molecular techniques have shown a clear association of species according to Waterhouse's grouping by sporangial papillation form (Crawford *et al*., 1996). Sporangia arguably form the most important spore-bearing stage in the life cycle of *P. cinnamomi*, providing the potential for immense inoculum production and infection of hosts following release of zoospores. While Byrt &Grant (1979) suggest that sporangial numbers and size do not necessarily provide an accurate presentation of the number of zoospores produced, Shea *et al*. (1978) found sporangial size and number to coincide with high densities of the pathogen in the soil and an increase in infection of susceptible hosts. Thus the ability of an isolate to produce greater numbers of sporangia may provide it with the potential to release more zoospores making it more virulent.

5.4. Pathogenicity and virulence to green apples: One would challenge the use of green apples for determination of *P. cinnamomi* pathogenicity. Previous intensive pathogenicity studies supported lack of specificity in *P. cinnamomi* isolates of Australian (Dudzinski *et al*., 1993), French (Robin &Desperez-Loustou, 1998) and South African (Linde *et al*., 1997) origin. Serfontein *et al*. (2007) found that the apple pathogenicity test is accurate due to the distinctive brown hard rots caused by *P. cinnamomi*. The test has been used for separation of pathogenic from non pathogenic *P. cinnamomi* isolates and co-isolated *Pythium* species. In this study, only 35 out of the 76 identified *P. cinnamomi* isolates were pathogenic to green apples. The H types were apparently more virulent than the A_1 and A_2 mating type isolates as indicated by the lesion extension rates. The same isolate types had the highest growth rate on PDA. This gives an indication that mycelial growth has a direct relationship with virulence.

5.5. Pathogenicity to macadamia seedlings: Twenty four of the isolates pathogenic to apples caused disease or death in macadamia seedlings. The capacity to cause death of macadamia seedlings ranged from mortality occurring in 53 days to non pathogenic with most of the isolates being intermediate. This large variation in pathogenicity was reported in Australian, French and South African *P. cinnamomi* isolates as discussed earlier. Absence of visible symptoms does not indicate that *P. cinnamomi* is absent from macadamia plants. The pathogen was recovered from symptomless seedlings in this study. This implied that the pathogen may cause latent infections undetectable by visual examinations. Presence of latent infections in seedlings has an impact on disease management. The Kenyan situation is that macadamia propagation is done mainly in government research institutions. However, due to the high demand for planting material, propagation is also done commercially by untrained nursery operators with no certification. *Phytophthora cinnamomi* may be introduced to new areas through macadamia seedlings with latent infections or in potting media. This could explain the wide distribution of the pathogen in all the macadamia growing areas of the country (Mbaka *et al*., 2009).

6 CONCLUSION AND RECOMMENDATIONS

This study established that phenotypic variation among Kenyan *P. cinnamomi* isolates is independent and continuous. No single or small group of characteristics was identified that clearly separates the sub-populations into sub-species as they overlapped with one another. Having been collected from one host, the range of phenotypic variation among the isolates indicated that no distinction was due to the host source hence they cannot be classified as sub-species. These findings of large phenotypic variation among isolates derived asexually from one clonal lineage have important taxonomic, management and resistance screening implications. The study established that there are

stable and non stable characters for *P. cinnamomi* as earlier established by Hüberli *et al*. (2001). The non stable characters such as colony morphology on potato dextrose agar warrant an adjustment of the species description to include these phenotypes (Hüberli *et al*., 2001). The apple pathogenicity test used in this study was accurate in separating pathogenic from non pathogenic *P. cinnamomi* recovered from soil and plant parts. This can be used by researchers conducting surveys to determine levels of *P. cinnamomi* inoculum in soils. The same can be used to evaluate efficacy of soil treatment (drenching with chemicals, fumigation and solarization) for control of *P. cinnamomi.* The finding that most of the *P. cinnamomi* isolates pathogenic to green apples were pathogenic to macadamia has an implication on management of diseases. Green apples and other susceptible host fruits such as avocado can serve as natural baits to pass disease inoculums from infected soil to pathogen free areas. This would be more so in areas where propagation for macadamia and other susceptible host crops such as avocado is done in the same nursery environment. To avoid such dangers, nursery operators should be educated on the importance of hygiene and sterilization of potting media in their nurseries.

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