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Quantification of inoculum density of *Phytophthora palmivora* in soil and its relation to disease incidence in papaw in far northern Queensland

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Abstract. A dilution-plate technique using five media selective for *Phytophthora* was evaluated at 16 and 26°C to develop a direct quantitative isolation method for *Phytophthora palmivora* Butler from naturally infested soil. P_{10}ARP+H medium at 26°C was found to be the most effective. This method was used to examine the relationship between inoculum density of *P. palmivora* and disease in papaw seedlings in the glasshouse. Results showed 100% plant mortality at an initial inoculum level of 100.4 cfu g^{-1} and significant primary root damage ($P < 0.05$) at ≥ 2.9 cfu g^{-1} after 10 weeks in naturally infested soil. Low to medium initial inoculum levels increased during the experiment by four to six times and the highest initial inoculum level increased by two-fold. A survey of 35 papaw-growing sites showed populations of *P. palmivora* were highest where growers followed papaw with papaw. In most cases, lengthy rotations with other crops and fallows reduced both inoculum levels and the incidence of tree lodging due to root rot.

Additional keywords: Carica papaya, *Phytophthora*-selective media, root rot, rotation.

Introduction

Papaw (*Carica papaya* L.) production in the wet tropics region of far northern Queensland (latitudes 16.48 and 17.26˚S) has under-gone a major expansion in the last 10–15 years. The majority of papaw plantations in north Queensland are small with an average size of about 2 ha. In recent years, there has been approximately 300 ha under papaw at any one time. In 1980, the papaw industry was valued at $A100 000 and by 1996 this had increased to some $A10 million with potential for further expansion (Phil Ross, personal communication).

The soilborne pathogen *Phytophthora palmivora* Butler which affects papaw by causing damping-off of seedlings, root rot, stem rot and fruit rot is a major constraint to the expansion of the papaw industry in far northern Queensland. *P. palmivora* is a ubiquitous fungal pathogen widely distributed in tropical and subtropical latitudes of the world. It infects more than 150 plant species (Erwin and Ribeiro 1996) including ornamental, horticultural and agricultural crops. Although *P. palmivora* infects a broad range of hosts, there appears to be no reference in the literature to host specificity within the species. Waterhouse (1973) postulated differences among isolates of *P. palmivora* from hosts such as cacoa, coconut and rubber. However, in a study of 100 isolates of *P. palmivora* from diverse geographical and disease situations, Oudemans and Coffey (1991) were unable to detect differences using isozyme analysis. Due to the apparent uniformity within *P. palmivora*, this paper assumes that all *P. palmivora* isolates are pathogenic to papaw.

P. palmivora attacks papaw plants of all ages but seedlings are most susceptible (Ko 1971). Research conducted by Ko (1971) indicated that mature root tissues of papaw plants were resistant to infection, with mature plants succumbing only during exceptional circumstances such as water-logging associated with cyclones (Teakle 1957). Symptoms in young trees include yellowing and collapse of leaves resulting from a soft, wet rot of the tap root which often extends into the trunk. In trees of bearing age, the tap root and lateral roots become severely decayed causing plants to topple during windy weather (Teakle 1957). Extensive losses to this disease occurred following Cyclone Winifred in 1986, Cyclone Joy in 1990 and Cyclone Rona in 1999 (D. Walker, personal communication).

Before cultural control methods such as crop rotation and soil amendments can be evaluated, it is necessary to develop a reliable and practical quantitative isolation method for *P. palmivora* from naturally infested soil and determine the disease potential of such soils. Chlamydospores of *P. palmivora* are the main survival propagules in soil and constitute the primary inoculum that infects roots in soil (Ramirez and Mitchell 1975). Detection methods involving an agar medium amended with pimaricin and vancomycin and the quantitative manipulation of soil samples have been used by researchers (Ramirez and Mitchell 1975) to quantify *P. palmivora* propagules in artificially infested soil. However, no record can be found of the quantification of *P. palmivora* from naturally infested field soil (Erwin and Ribeiro 1996). In tropical regions, *Pythium* spp. are often troublesome on

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Table 1. Recovery of *Phytophthora palmivora*, *Pythium* spp. and *Mortierella* spp. by dilution plating (1:8 dilution) on *Phytophthora*-selective media after 72 h incubation at 26°C

Selective medium	Mean No. colonies per plate[A]		
	P. palmivora	*Pythium* spp.	*Mortierella* spp.
PCH	0.00 a	0.0 a	0.00 a
P10VPP+BH	0.00 a	7.2 b	0.00 a
P10VP+H	0.00 a	8.2 b	0.00 a
BNPRA+H	1.25 ab	25.0 d	0.80 a
P10ARP+H	2.65 b	12.0 c	0.95 a

[A]Means in the same column followed by the same letter are not significantly different ($P > 0.05$).

Phytophthora-selective media due to their luxuriant growth which can mask slower-growing *Phytophthora* species (Tsao 1983). Similarly, bacteria can inhibit the germination of *Phytophthora* propagules on *Phytophthora*-selective media (Tsao 1983). The use of relatively low temperatures (e.g. 18°C) was reported to enhance *Phytophthora* recovery due to the suppression of some undesired bacteria and *Pythium* spp. (Tsao 1983).

This paper reports on the evaluation of five *Phytophthora*-selective media in combination with soil dilution for their ability to quantify the inoculum density of *P. palmivora* in naturally infested soil. The best method was used to relate soil inoculum density to disease in a glasshouse study on papaw seedlings, and in a survey of papaw fields in the wet tropics region of far northern Queensland during 1999 and 2000.

Methods

Quantitative isolation of P. palmivora from soil

In an *in vitro* experiment, five media selective for *Phytophthora* were compared for their efficacy in detecting *P. palmivora* in a naturally infested red kraznozem soil. The selective media used were P_{10}VP+H (Tsao and Ocana 1969), BNPRA+H (Masago *et al*. 1977), P_{10}VPP+H (Papavizas *et al.* 1981), PCH (Shew and Benson 1982) and P_{10}ARP+H (Jeffers and Martin 1986). The treatments were assessed at 18 and 26°C (Papavizas *et al.* 1981) using a dilution plating technique (Johnson and Curl 1972). One mL aliquots of a 1:8 soil suspension (20 g of a naturally infested soil in 160 mL distilled water) were spread over the surface of ten replicate culture plates per treatment and then placed in an incubator in the dark and set at the appropriate temperature. The mean dry weight of soil dispensed to each plate was obtained by transferring three parallel samples of each soil suspension to pre-weighed porcelain cruets and drying on a hot plate. After 24 h, the soil suspension was gently washed from the surface of each culture plate with a stream of sterile distilled water. Culture plates were inverted and returned to the incubator. *Phytophthora* colony counts were made after a further 48 h. Colonies were confirmed as being those of *P. palmivora,* from typical sporangia produced following the transfer of mycelial plugs to sterile distilled water in Petri dishes placed on a laboratory bench for 24 h. *Pythium* and *Mortierella* colonies were identified macroscopically and the number of colonies that developed on each medium was recorded.

Estimating inoculum density and inoculum potential of soil

Sufficient kraznozem soil was collected from around the root zone of mature papaw plants infected with *P. palmivora* and from a nearby field of similar soil-type in which papaw had never previously been grown. Each of the soils was air-dried for 24 h before being passed through a 10-mm-diameter sieve. Five-fold dilutions of infested to uninfested soil, 1/0, 1/5, 1/25, 1/125 and 0/1 (w/w), were thoroughly mixed in a concrete mixer for 5 min before 1500 g of each dilution was potted up in 15-cm-diameter free-draining pots. Five replicate pots of each dilution were placed in a completely randomised design on a glasshouse bench (ambient temperature range of 19–29°C). Four soil samples were taken at random from each pot using a modified cork-borer (10 mm diameter) and bulked to give a 20 g composite sample which was used to assess the initial P(i) *Phytophthora* populations. The number of colony forming units (cfu) was determined by spreading ten 1 mL aliquots of 1:2, 1:3 or 1:5 (w/v) dilutions of soil on P_{10} ARP+H selective medium and counting after incubation for 72 h in the dark at 26°C.

A single 9-week-old papaw seedling cv. Hybrid 29 was transplanted to each pot and 10 days later pots were placed in plastic trays filled with water to a depth of 25 mm to saturate the soil (Duniway 1979). After 3 days, pots were removed from the trays and the soil allowed to drain for 4 days before hand watering as required for the next 8 weeks. All plants in this glasshouse experiment were observed daily and plant mortality recorded. Final *Phytophthora* densities P(f), root rot severity ratings and plant growth assessments (plant height, fresh weight of roots) were conducted as plants died or on surviving plants 10 weeks after transplanting. Root rot severity was assessed using the following scale: 1, no root rot; 2, rot of secondary roots only; 3, rot of primary root only; 4, rot of primary root and feeder roots; 5, complete root rot resulting in the death of the plant. Fresh weights of roots were recorded after roots were dissected from the stem, gently washed and blotted dry on paper. Sections of diseased roots and stems were surface sterilised in 70% methanol for 1 min, blotted dry with sterile paper then transferred to P_{10}ARP+H selective medium. The plates were observed for growth of *P. palmivora* from the roots and stems after incubation in the dark at 26°C.

Inoculum potential of field soil

Fields used for growing papaws in the wet tropics region were surveyed during 1999 and 2000 to determine the incidence and potential for Phytophthora root rot of papaw. Thirty five fields, all of a similar kraznozem soil-type, were sampled by taking an appropriate number of composite soil samples from each field. Each composite sample consisted of ten samples taken at random from an area of approximately 0.1 ha and bulked. If the field was under crop, random samples were taken from the root zone of the crop. Previous cropping history and disease incidence (percentage tree lodging due to root rot) were recorded. *Phytophthora* inoculum densities were assessed from a 20 g sub-sample taken from each composite sample using P_{10}ARP+H selective medium and dilution plating as described previously.

Statistical analyses

Data from the *in vitro* experiment, which examined the efficacy of various selective culture media at 18 and 26˚C, were analysed using

Table 2. Effect of two temperatures on the detection of *Phytophthora palmivora, Pythium* spp. and *Mortierella* spp. from a naturally infested kraznozem soil using five selective agar media incubated for 72 h

Fungal species	[A]Mean No. of colonies per plate at temp. of	
	18°C	26°C
P. palmivora	0.00 a	1.56 b
Pythium spp.	14.30 b	6.66 a
Mortierella spp.	0.00 a	0.66 a

[A]Means in the same row followed by the same letter are not significantly different ($P > 0.05$).

analysis of variance with a factorial treatment structure. Treatment means were compared by the protected LSD procedure at the 5% level of significance. In the glasshouse experiment examining inoculum–disease relationships, an ANOVAR was used to analyse data from initial and final inoculum densities–log(x+1) transformation, the disease severity assessment, plant height and fresh weight of roots.

Results

Quantitative isolation of P. palmivora from soil

P_{10}ARP+H medium was more effective ($P < 0.05$) than all other culture media except BNPRA+H in the detection of *P. palmivora* from naturally infested soil using soil dilution plating (Table 1). Significantly ($P < 0.05$) more colonies of *Pythium* spp. were recovered on BNPRA+H than on the other media whereas there was no difference in the number of *Mortierella* spp. detected. No colonies of *P. palmivora, Pythium* spp. or *Mortierella* spp. were detected on PCH.

None of the culture media treatments incubated at 18°C for 72 h produced colonies of *P. palmivora* or *Mortierella* spp., but significantly ($P < 0.05$) more *Pythium* spp. were recorded at this temperature than at 26°C (Table 2). Bacterial contamination did not appear to be a problem on any of the media examined.

Estimating inoculum density and inoculum potential of soil

Initial inoculum densities P(i) were 0, 0.6, 2.9, 31.4, and 100.4 cfu g^{-1} (Table 3). Final inoculum densities P(f) showed a four- to six-fold increase in inoculum arising from low to medium P(i) levels and a two-fold increase at the highest level (Table 3). Plant mortality of 100% occurred at the highest inoculum level and significant primary root damage ($P < 0.05$) was recorded where initial levels of infestation were at or above 2.9 cfu g^{-1}. Initial inoculum of ≥ 2.9 cfu g^{-1} caused a decrease of 19–70% in plant height whereas there was a decrease of 80–97% in fresh weight of roots where the initial inoculum level was ≥ 31.4 cfu g^{-1}.

Inoculum potential of field soil

P. palmivora was present in most papaw fields, many of which showed no lodging which is a symptom of root rot. In 13 of the 15 fields that had inoculum levels greater than 30 cfu g^{-1} of soil (sites 1–15), the incidence of papaw lodging ranged between 5 and 90% (Table 4). The highest inoculum levels and subsequent damage to trees occurred where papaws were grown on replant ground. In another 13 fields surveyed (sites 16–28), inoculum levels ranged between 4 and 30 cfu g^{-1} of soil (Table 4). Minor tree lodging (5%) was recorded in three out of seven fields planted to papaw. Where papaws were grown following pasture, weed fallow or rotated with other crops, *P. palmivora* populations were generally low with nil to minor tree lodging recorded. At the seven remaining sites (sites 29–35), less than 4 cfu g^{-1} of soil were detected and there was no tree lodging in the one field planted to papaw (Table 4).

Discussion

The isolation of *Phytophthora* from soil using a selective culture medium depends on the use of chemicals that have little or no effect on the target species to inhibit associated bacteria and fungi (Erwin and Ribeiro 1996). Results from the *in vitro* experiment showed that P_{10}ARP+H medium was the most effective culture medium for the direct isolation of *P. palmivora* from soil. P_{10}ARP+H was superior to other media, not only because it had the highest colony recovery, but also because colonies were distinct and easy to identify. The incubation of all selective media treatments at 18˚C failed to detect any colonies of *P. palmivora* but did cause an increase in the recovery of *Pythium* spp.

Table 3. The effect of inoculum density of *Phytophthora palmivora* on disease severity, plant height and fresh weight of roots of papaw plants grown in a glasshouse

Soil dilution (w/w)	Inoculum densities[A] (cfu g^{-1})		Disease severity ((1–5)	Plant height (cm)	Fresh weight of roots (g)
	P(i)	P(f)			
0/1	0.00 a (0.0)	0.00 a (0)	1.0 a	35.3 a	13.77 a
1/125	0.46 b (0.6)	1.18 b (2.3)	2.6 b	24.7 b	7.85 ab
1/25	1.36 c (2.9)	2.44 c (10.5)	3.2 b	28.7 ab	10.54 a
1/5	3.48 d (31.4)	5.26 d (191.6)	4.8 c	15.2 c	2.82 bc
1/0	4.62 e (100.4)	5.46 d (233.7)	5.0 cd	10.5 c	0.46 c

[A]P(i) = initial and P(f) = final inoculum density. Numbers in parentheses are means of the log(x + 1) transformed values. Means in columns followed by the same letter are not significantly different ($P > 0.05$).

Table 4. Effect of cropping history of papaw-growing sites in the wet tropics region of far northern Queensland on populations of *Phytophthora palmivora* and papaw lodging resulting from root rot

Site No.	Cfu g^{-1} soil	% lodging	Cropping history 2000	1999	1998	1997	1996
1	73.8	5		Papaw	Papaw	Papaw	Pasture (G)[A]
2	38.4	5		Papaw	Papaw	Papaw	Sugar cane
3	156.5	5		Papaw	Papaw	Papaw	Sugar cane
4	162.0	10		Papaw	Papaw	Papaw	Papaw
5	32.1	5		Papaw	Papaw	Papaw	Weed fallow
6	92.5	10	Papaw	Papaw	Pasture (G)	Pasture (G)	Pasture (G)
7	55.5	20		Papaw	Papaw	Pasture (G)	Pasture (G)
8	106.0	5		Papaw	Pasture (G)	Pasture (G)	Pasture (G)
9	80.0	20		Papaw	Papaw	Papaw	Weed fallow
10	262.2	80		Papaw	Papaw	Papaw	Papaw
11	240.0	90		Papaw	Papaw	Papaw	Papaw
12	37.1	n/a[B]		Weed fallow	Papaw	Papaw	Papaw
13	35.5	n/a		Weed fallow	Papaw	Papaw	Weed fallow
14	71.0	20		Papaw	Papaw	Papaw	Weed fallow
15	57.3	5		Papaw	Papaw	Papaw	Sugar-cane
16	8.0	n/a		Banana	Papaw	Papaw	Papaw
17	4.0	0		Papaw	Pasture (G)	Pasture (G)	Papaw
18	7.8	n/a		Banana	Papaw	Papaw	Pasture (G)
19	13.0	0		Papaw	Papaw	Sugar cane	Sugar cane
20	6.0	n/a		Weed fallow	Papaw	Papaw	Sugar cane
21	6.0	0		Papaw	Pasture (G)	Pasture (G)	Pasture (G)
22	5.6	0		Papaw	Pasture (G)	Pasture (G)	Pasture (G)
23	18.8	5		Papaw	Pasture (G)	Pasture (G)	Papaw
24	25.7	n/a		Banana	Papaw	Papaw	Papaw
25	10.5	n/a		Taro	Papaw	Papaw	Papaw
26	12.6	n/a		Weed fallow	Weed fallow	Papaw	Papaw
27	4.3	5	Papaw	Weed fallow	Weed fallow	Papaw	Papaw
28	21.0	5	Papaw	Papaw	Sugar cane	Sugar cane	Sugar cane
29	0	n/a		Pasture (G)	Pasture (G)	Pasture (G)	Pasture (G)
30	0	n/a		Weed fallow	Weed fallow	Papaw	Papaw
31	2.7	0	Papaw	Papaw	Pasture (G)	Pasture (G)	Pasture (G)
32	0	n/a	Weed fallow	Papaw	Papaw	Weed fallow	Weed fallow
33	0	n/a		Banana	Papaw	Pasture (G)	Pasture (G)
34	0	n/a		Weed fallow	Weed fallow	Papaw	Papaw
35	0	n/a	Sugar cane	Sugar cane	Sugar cane	Sugar cane	Sugar cane

[A](G) denotes a grass pasture.
[B]n/a denotes that the assessment for papaw lodging was not applicable as papaw was not present.

The ability to detect *P. palmivora* on P_{10}ARP+H and not on either P_{10}VP+H or P_{10}VPP+BH medium was surprising, as a medium amended with vancomycin was successfully used by Ramirez and Mitchell (1975) in the isolation of *P. palmivora* from artificially infested, autoclaved soil. Jeffers and Martin (1986) similarly reported being unable to recover *P. parasitica* var. *nicotianae* on P_{10}VP+H medium from naturally infested soil. Replacing vancomycin with ampicillin + rifampicin is likely to have contributed to the improved recovery although there appears to be no reference in the literature to vancomycin being inhibitory to the growth of chlamydospores of *P. palmivora* found in soil. Jeffers and Martin (1986) concluded that the growth of bacteria on dilution plates contributed to the reduced detection of *P. cinnamomi* and *P. parasitica* var. *nicotianae*. However in my experiment, the growth of bacteria on dilution plates amended with vancomycin was not a problem, suggesting that the inhibitory effect on *P. palmivora* in naturally infested soil was due to some other cause.

The BNPRA+H medium was the only medium other than P_{10}ARP+H on which *P. palmivora* was successfully detected in naturally infested field soil. A problem often associated

with using many selective media in the isolation of *Phytophthora* spp. from soil is the overgrowth of plates by *Pythium* spp. Amending the medium with hymexazol is known to improve the selectivity of many of these media and reduce contamination by *Pythium* spp. (Masago *et al.* 1977). However, the BNPRA+H medium, although amended with hymexazol, had significantly more *Pythium* spp. after 72 h than the other media. These colonies were a hindrance as they were fast-growing and tended to obscure the colonies of *P. palmivora*. This result probably occurred because 1% potato-dextrose agar (PDA) is used as the basal medium of BNPRA+H. PDA is higher in carbohydrate than the preferred basal medium cornmeal agar (Eckert and Tsao 1962) used in most other selective media.

A quantitative soil assay developed by Shew and Benson (1982) that utilised a wet-sieving technique combined with PCH selective medium was reported to be effective in the enumeration of *P. cinnamomi* from naturally and artificially infested soil. Results from my research, however, showed *P. palmivora, Pythium* and *Mortierella* spp. were not detected by this medium. Other researchers (Tsao 1983) have reported that chloramphenicol used in PCH medium is toxic to mycelial growth of some *Phytophthora* spp. and is not the optimal choice in a selective medium.

Any assessment of the quantitative relationship of inoculum density of *P. palmivora* to disease incidence and severity in papaw is likely to be complicated by the many factors important to host infection and disease development (Mitchell and Kannwischer-Mitchell 1983). Previous research into inoculum density/disease incidence relationships under controlled conditions has, in many cases, neglected to address the influence these factors have on disease development (Ramirez and Mitchell 1975; Mitchell 1978). Because of the complexity of factors influencing infection, most researchers tended to create optimum infection situations by using artificially produced inoculum in a sterile soil environment (Ramirez and Mitchell 1975; Mitchell 1978). In my research, a susceptible, commercially grown papaw cultivar was transplanted into a naturally infested soil to simulate field conditions as closely as possible. Nine-week-old papaw seedlings were used in this experiment as plants of this age are most commonly used by growers as transplants to the field. The watering regime of 3 days flooding and 4 days of drainage not only favoured *P. palmivora* infection (Duniway 1979) but also simulated the wet season conditions of the wet tropics of far northern Queensland. Higher inoculum concentrations were required in my study to give levels of disease comparable to those of other researchers (Mitchell 1978; Ramirez and Mitchell 1975). This was possibly due to the suppressive physical, chemical and biological components of naturally infested soil (Malajczuk 1983; Nesbitt *et al.* 1979) although differences in isolates, growing conditions and susceptibilities of host could also have contributed.

In the survey of 35 papaw-growing sites, *P. palmivora* was found in higher numbers where growers had followed papaw with papaw. Data obtained from the field survey indicate that the selective medium P_{10}ARP+H and dilution plating are acceptable for the detection of *P. palmivora* across a range of cropping situations. The soil assay proved sensitive enough to detect low populations of *P. palmivora* from weed fallows following papaw and from the root zone of crops such as banana grown in rotation with papaw. Future soil assays could draw on DNA technology to detect *P. palmivora* from soil. The success of Liew *et al*. (1998) in developing a DNA-based detection method for *Phytophthora medicaginis* in field-infected roots of lucerne provides the opportunity for similar detection methods for species such as *P. palmivora*. However, detection from field soil still remains a challenge for researchers.

Tree lodging was recorded in three fields (sites 7, 8 and 28) which had not been previously planted to papaw. Discussions with the growers concerned suggest this was most likely due to poor land preparation. Hardpans and failure to plant on mounds (Peterson *et al*. 1998) have been shown to increase the incidence and severity of root rot. *Phytophthora* inoculum was detected at sites 17, 19, 21, 22 and 31 but no tree lodging was recorded. Sites 17, 21 and 22 were newly planted to papaw which could explain the lack of disease. However, sites 19 and 31 had been planted for some 18 months suggesting the soil was suppressive to disease.

Long histories of papaw growing without rotation crops did not necessarily result in a high incidence of tree lodging. Rotation with non-host crops such as bananas or pastures did, however, reduce inoculum levels and the incidence of lodging compared with continuous papaw. Most species of *Phytophthora* are known to be poor saprophytic competitors (Turner 1965) and, therefore, are not expected to persist in high populations in the absence of a susceptible host. However, the use of crop rotation to control root rot should be treated with caution as results from the inoculum potential study conducted in the glasshouse showed initial inoculum levels as low as 3 cfu g^{-1} can cause significant plant damage in young seedlings. Research conducted by Turner (1965) also showed that at intermediate levels of soil moisture, spore viability with *P. palmivora* ranged from a minimum of 6 months to more than 2 years. As a result, crop rotation is unlikely to give satisfactory control of *Phytophthora* root rot.

The information and data generated in this study on the quantification of *P. palmivora* in naturally infested soil and the relationship of soil inoculum density to disease may be useful in the development of a disease forecasting system for growers. It must, however, be remembered that the usefulness of a predictive soil assay must take into account cultivar susceptibility and the effect of adverse seasonal conditions such as high rainfall which have a marked effect on disease incidence and severity.

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