Plant-parasitic nematodes associated with breadfruit, *Artocarpus altilis* (Parkinson) Fosberg

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Biography

Jin-Wah Lau is a Tropical Plant Pathology graduate student at the University of Hawai‘i at Mānoa. She loves to eat fruit and developed an early appreciation for growing, picking, and eating fruits from her grandmother. She has worked killing weeds, small animals, and plant pathogens for the Maui and Kaua‘i Invasive Species Committees and managed the Kaua‘i Community College Farm prior to entering graduate school. Her interests used to be hiking and botanizing, but nowadays she spends her days singing nursery songs, changing diapers, and rediscovering the world with her one-and-a-half-year-old son Huki. If she could feed breadfruit to baby every day, she would! Jin-Wah is interested in research that protects agricultural crops in Hawai‘i from pests and pathogens.

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Abstract

A survey of plant-parasitic nematodes associated with breadfruit in Hawai‘i was conducted on 25 sites with breadfruit trees. Soil and breadfruit roots yielded seven genera of plant-parasitic nematodes (*Helicotylenchus*, *Meloidogyne*, *Mesocriconema*, *Paratylenchus*, *Pratylenchus*, *Rotylenchulus*, *Tylenchorhynchus*) and one Heteroderid. Morphometric and molecular techniques identified a root-lesion nematode found parasitizing breadfruit as *Pratylenchus coffeae*. Host ranges and pathogenicity of *Meloidogyne javanica*, *Pratylenchus coffeae*, and *Rotylenchulus reniformis* were tested on breadfruit cultivar ‘Ma‘afala’ in two experiments. In experiment 1, *P. coffeae* reproduced 17-fold, while *M. javanica* and *R. reniformis* did not replace the inoculum concentration. At termination, no visible symptoms were present on host plants, but a greater root weight of breadfruit inoculated with *P. coffeae* was measured. Experiment 2 showed similar trends despite higher greenhouse temperatures and pot-bound breadfruit trees. Reproductive factor results indicate breadfruit is a good host to *P. coffeae* and a poor host to *M. javanica* and *R. reniformis*.

Key Words: Hawaii, *Helicotylenchus dihystera*, host range, identification, Kaua‘i, Ma‘afala, Maui, *Meloidogyne javanica*, lesion, Ma‘afala, molecular, morphometric, morphology, O‘ahu, *Paratylenchus*, pathogenicity test, *Pratylenchus coffeae*, *Rotylenchulus reniformis*, survey, ‘ulu.

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1.1 Introduction

> *"If a man should in the course of his lifetime plant ten such trees, he would as completely fulfill his duty to his own and future generations…"*
>
> - Remarks on breadfruit from explorer and naturalist Sir Joseph Banks, 1769

Breadfruit (*Artocarpus altilis* Parkinson (Fosberg)) is a traditional crop cultivated throughout Oceania for its starchy fruit (Zerega *et al.*, 2004) that is becoming increasingly popular as an orchard tree in Hawai‘i and throughout the world. Although reported as relatively free of disease (Ragone, 1997, 2006; Taylor and Tuia, 2007), limited research has been conducted in regard to the plant and plant-parasitic nematodes. Plant-parasitic nematodes on non-major crops are often overlooked when it comes to assessing plant health issues because the nematodes are microscopic and symptoms of nematode infection are often indistinct, making their presence difficult to diagnose without soil assays. To date, no information is available on the association of different nematode pathogens with breadfruit plants or their relationship with tree decline in Hawai‘i. Thus, the goal of this research was to investigate plant-parasitic nematodes associated with breadfruit in Hawai‘i.

Transported vegetatively as root shoots, breadfruit was one of the plants that Polynesian voyagers transported over long ocean distances when they first inhabited the Hawaiian Islands. The breadfruit tree, with an important cultural role in the history of the Pacific, is referenced in Polynesian, Micronesian, and Melanesian mythology. From a utilitarian perspective, every part of the tree yielded useful resources. Most importantly, fruits from the tree were likened to freshly baked bread by European sailors (Ragone, 1997), carbohydrate-rich and providing an important source of minerals and vitamins (Graham and Bravo, 1981; Jones *et al.*, 2011, 2013). Breadfruit

trees are best known for abundant yields of fruit with minimal input. Trees can be observed producing bountiful harvests by residences, commercial areas, schools, parks, and botanical gardens and are present on all of the main Hawaiian Islands (Miller, 1976; Ragone *et al.,* 2016).

With the most widespread cultivation and use of breadfruit in the Pacific, Ragone (1997) noted a decline of cultivation and use in many regions that traditionally utilized resources the tree offers. Changes in food preferences favoring ease of storage and convenience over heritage and tradition have led to a downturn in cultivation, proficiency of harvesting, and consumption of breadfruit in younger generations (Taylor *et al.,* 2008). Globally, however, knowledge of breadfruit is broadening. In Fiji, breadfruit's popularity as an export commodity resulted in 6 tonnes exported to Australia, New Zealand, and Canada in 2006 (Taylor and Tuia, 2007). Breadfruit is also gaining popularity as a cash crop in Pohnpei (Englberger *et al*., 2007), Trinidad and Tobago (Roberts-Nkrumah and Legall, 2013), Seychelles (Moustache and Moustache, 2007), and the Marshall Islands (Englberger *et al*., 2007). Food security, agricultural sustainability, and expanding local biodiversity are being examined as major components in combating global hunger with neglected and underutilized crops like breadfruit being brought to the forefront of solutions (Jones *et al.*, 2012).

Breadfruit is a tropical tree that grows to 15-21 m with a trunk up to 2 m in diameter (Ragone and Paull, 2008). Like other members of the family Moraceae, lactifers which produce milky sap are present in breadfruit trees. Trees grow best at a temperature range between 15° to 40°C, a yearly precipitation of 150 to 300 cm, and altitudes from 0-1,500 m (Ragone, 1997). A crop yield of 6 tonnes/ ha^{-1} has earned breadfruit the reputation as “the most productive orchard crop in the world” (Fownes and Raynor, 1993; Sauerborn, 2002). Average yield for a single breadfruit tree is 269 fruits per year, each with an average weight of 1.2 kg (Liu *et al.,* 2014), or

322.8 kg of fruit per tree per year. World hunger and food security are concerns in tropical regions, where problems of poverty, disease, and lower agricultural productivity are amplified. Breadfruit could be one solution to alleviate hunger and increase food security in these regions (Jones *et al.*, 2012).

Despite its multipurpose utility and huge potential towards agricultural sustainability, breadfruit is considered an underutilized crop and given little attention for the purposes of large scale commercial cultivation, in part due to limited knowledge on agronomic practices, processing and preservation, and planting materials (Roberts-Nkrumah, 2007). These challenges continue to be addressed by researchers from many different disciplines. Preliminary information on fertilization regimes, pruning, and planting density for modern breadfruit orchard production is now available (Lebegin *et al*., 2007; Goebel, 2007). Processing and preservation studies (Nochera and Caldwell, 1992; Nochera and Moore, 2001; Oduro *et al*., 2007; Akanbi *et al*., 2011) have allowed diverse products to be developed to please international and interspecies palates including: breadfruit biscuits (Olaoye *et al.*, 2007, Omobuwajo, 2003; Bakare *et al*., 2014), infant food (Mayaki *et al.*, 2003; Nelson-Quartey *et al.*, 2007), breadfruit energy bars (Nochera and Ragone, 2016), breadfruit noodles (Adebowale *et al*., 2017), and meal for broiler chickens (Oladunjoye *et al*., 2010) and weaned pigs (Ortiz, 2013). In addition, studies on arbuscular mycorrhizal fungi found an abundance of taxa on older breadfruit trees, mostly of the genus *Rhizophagus*, whereas younger trees were dominated by species of *Glomus* (Hart *et al.*, 2014). Since 2008, methods established by Shi *et al.* (2007) and Murch *et al*. (2008) improved *in vitro* propagation, enabling propagules to be commercially produced, making quantities and select breadfruit cultivars much easier to obtain than in the past (www.globalbreadfruit.com).

As a result of this continual stream of knowledge and innovation, breadfruit plantings have steadily increased and breadfruit orchards have been established internationally. Since 2009, Trees That Feed Foundation has disseminated 120,000 breadfruit trees to farmers and co-ops in Jamaica, Haiti, Costa Rica, Barbados, Ghana, and other countries (T. Candler, personal communication, 30 March 2017). In Hawai‘i, the National Tropical Botanical Garden Breadfruit Institute's "Plant a Tree of Life-Grow 'Ulu" project dispersed over 12,000 breadfruit trees since 2012 (Ragone *et al.*, 2016). As breadfruit orchards become more common, monocropping of agricultural crops has been cited to increase risk of total crop failure due to pest problems and plant pathogens (Bridge, 1996). Because many seedless breadfruit cultivars are genetically identical to one another although very distinct morphologically and from different geographic areas in the Pacific (Zerega *et al.*, 2004), most trees are likely genetically identical in response to pathogens. Accordingly, infestation by pathogens in a breadfruit monoculture could spread rapidly throughout an orchard, yielding potentially devastating effects.

Plant pathogens can decrease production, reduce tree vigor, and lead to early mortality in breadfruit trees. *Uredo artocarpi*, present on *Artocarpus* spp. in India and the Philippines and on islands of the South Pacific, was first reported from Hawai‘i in 1991 (Gardner, 1991). Recorded bacterial and fungal diseases of breadfruit include: anthracnose (*Colletotrichum gloeosporioides*) (Ragone, 2006; Stice *et al*., 2007), *Diplodia* collar rot (Sangchote *et al.*, 2003), fruit, stem, and root rot caused by *Phytophthora* spp. (Cerqueira *et al*., 2006;), pink disease (*Erthricium salmonicolor*) (Sangchote *et al.*, 2003), Rhizopus rot (*Rhizopus* spp.) (Sangchote *et al.*, 2003; Ragone and Paull, 2008), and Pingelap disease which caused an epidemic decline of trees in the Pacific (Zaiger and Zentmyer, 1966) but was later determined to be caused by environmental factors. Coates-Beckford and Pereira (1992) reported *Pseudomonas* sp. and *Fusarium* sp. to be

most common on declining and non-declining breadfruit trees in Jamaica. Brown set/root rot (*Phellinus noxius*) (Ragone and Paull, 2008; Stice *et al.*, 2007) and fruit rot (*Phytophthora palmivora*) (Redfern, 2010) can also be problems to breadfruit in tropical areas. Like other crops of Oceania, lack of genetic diversity often results in a build-up of pathogen populations responsible for deterioration of their agronomic performance (Lebot, 1992).

Plant-parasitic nematodes are obligate roundworms belonging to the phylum Nematoda that feed on plant tissues (Sasser and Freckman, 1987). Symptoms of host plant infection include: reduced foliage, chlorosis or leaf-yellowing, stunted growth, curling and twisting of leaves and stems, loss of leaves, premature or delayed maturity, poor fruit/seed production, wilt, and early senescence or death of plants (Williamson and Hussey, 1996). Often pathogenic nematodes do not directly kill plants, rather they reduce the vigor of the host and make it more likely to be harmed by other factors. Certain nematode taxa can transmit viruses between plants while feeding. As nematodes access nutrients within host plants, they increase the plant's susceptibility to bacterial pathogens (Powell, 1971; Williamson and Hussey, 1996), creating a secondary detrimental effect on the plant.

Starting from eggs, nematodes undergo four juvenile stages prior to reaching the adult stage. Many undergo their first molt in the egg and enter the plant as a second stage juvenile. Under ideal soil conditions, nematodes develop into egg-laying adults within a month, however some species take as little as 2 weeks, while others require a full year. All plant-parasitic nematodes have a protrusible, hollow stylet to penetrate host plants during feeding (Hussey, 1989; Davis *et al*., 2000) and most species are soil-dwelling and parasitize on roots (Sijmons *et al*., 1994; Hussey and Grundler, 1998). Their stylet combined with other morphological characteristics allows for their classification to genera. Commonly, parasitic nematodes are named for morphological

characteristics or for damage they induce on their hosts. Some plant-parasitic nematodes present in Hawai‘i include: cyst (*Heterodera* spp.), foliar (*Aphelenchoides* spp.), lesion (*Pratylenchus* spp.), pin (*Paratylenchus* spp.), reniform (*Rotylenchulus* spp.), ring (*Mesocriconema* spp.), root-knot (*Meloidogyne* spp.), spiral (*Helicotylenchus* spp.), and stunt (*Tylenchorhynchus* spp.) nematodes. Besides specific morphology, each nematode has its own characteristics including adaptation to temperature ranges, soil, host crops and egg laying quantities, and survival strategies. Globally, crop loss from plant-parasitic nematodes averages 8-15% with a cost of $78-118 billion USD/year (Sasser and Freckman, 1987).

Having subtle symptoms and infecting from beneath the soil line, nematode pathogens are difficult to detect even for the trained eye, but have potential to cause great damage. Although ubiquitous and pathogenic to a wide range of crops, research into these pests in the tropics has been concentrated on commodity crops. Examples of plant-parasitic nematodes on breadfruit have been the subject of little research compared with other major crop staples. A few examples can be found that cite parasitic nematodes associated with breadfruit globally, including in Jamaica (Coates-Beckford and Periera, 1992), Brazil (Sharma, 1976), Malaysia (Razak, 1978), and New Caledonia (Grandison *et al*., 2009). However, the majority of literature on pathogenic nematodes associated with breadfruit is in the form of regional reports (Kirby *et al*., 1980; Grandison, 1990, 1996) and conference proceedings (Hutton, 1976). Studies specific to nematode pathogens pertaining to breadfruit in Hawai‘i are not available. In Hawai‘i, small-scale farmers are often unaware of the nature and harmfulness of nematode infestations and do not seriously consider the destructive effect of nematode pests on their crops. Thus, increasing interest in the development of breadfruit as a crop for production in Hawai‘i requires that consideration be given to pest and pathogen systems.

With substantial crop yields, a potential for low input, suitability in sustainable production systems, a reputation as pest free, and capability to thrive in the wet tropics unsuitable for conventional staple grains, breadfruit orchards are being established across the Hawaiian Islands. In-depth research on plant-parasitic nematodes associated with breadfruit trees in Hawai‘i will add to the body of research on breadfruit and be helpful to local breadfruit growers, integrated pest managers, and researchers aiming to improve tree health and productivity.

1.2 Objectives

The objectives of this research were to determine which plant-parasitic nematodes are associated with breadfruit in Hawai‘i and which plant-parasitic nematodes are pathogenic to breadfruit trees in Hawai‘i. Specific objectives were to: (i) determine the incidence of plant-parasitic nematodes associated breadfruit on the islands of Kaua‘i, Maui, and O‘ahu, (ii) identify *Pratylenchus* found on breadfruit species level, and (iii) determine pathogenicity and virulence of *Meloidogyne javanica, Pratylenchus* sp., and *Rotylenchulus reniformis* on breadfruit tree growth under greenhouse conditions.

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Chapter 2: Survey of Plant-parasitic Nematodes Associated with Breadfruit, *Artocarpus altilis* Parkinson (Fosberg), in Hawai‘i

2.1 Abstract

Twenty-five breadfruit plantings on the islands of Kaua‘i, Maui, and O‘ahu were surveyed for incidence of plant-parasitic nematodes. Soil and breadfruit root samples were collected from plantings in residences, parks, schools, botanical gardens, and breadfruit orchards. Soil samples were processed by elutriation and centrifugation for nematode extraction. Roots were subjected to mist chamber extraction for seven days. Seven genera of plant-parasitic nematodes (*Helicotylenchus, Meloidogyne, Mesocriconema, Paratylenchus, Pratylenchus, Rotylenchulus,* and *Tylenchorhynchus*) and one unidentified taxon in Heteroderidae were found. *Helicotylenchus dihystera, R. reniformis, Paratylenchus* sp., *Pratylenchus coffeae, Meloidogyne* spp., and a Heteroderid were found on all islands. *Helicotylenchus dihystera* was the most frequent species encountered, occurring at 68% of sites and was the most widespread plant-parasitic nematode of breadfruit on Kaua‘i. On Maui, *H. dihystera, R. reniformis* and *P.coffeae* were found in 40% of the samples collected. *Paratylenchus* was the most common plant-parasitic nematode to the island of O‘ahu, detected in 86% of samples. *Meloidogyne* spp. were found in 43% of breadfruit sites on Oah‘u, and 23% and 20% of that on Kaua‘i and Maui, respectively, but in low abundance on all islands. Soil samples yielded *Mesocriconema* at sites on Kaua‘i and O‘ahu, and *Tylenchorhynchus* on O‘ahu. Plant-parasitic nematodes were absent from 12% of the locations surveyed. An unidentified genus in Heteroderidae was detected in samples from each island, all from botanical gardens.

2.2 Introduction

For early Hawaiians, breadfruit or ‘‘Ulu’ (*Artocarpus altilis* Parkinson (Fosberg)), was central to culture and sustenance. The breadfruit tree is a high yielding, low input starch crop for tropical and subtropical areas with hundreds of named varieties (Ragone, 1997). Transported in voyaging canoes when the first inhabitants arrived in the Hawaiian Islands, only one cultivar of breadfruit was known in the Hawaiian archipelago up until the early 20th century (Rock, 1974). Traditionally, seedless breadfruit was cultivated from vegetative shoots from roots. However, *in vitro* propagation methods established by Shi *et al.* (2007) and Murch *et al*. (2008) allowed for mass cloning, making commercial distribution of breadfruit propagules much more feasible than in the past. Global concerns over food security with a focus on traditional underutilized crops have increased cultivation of cloned breadfruit trees as an orchard crop. Breadfruit plantings have steadily increased over the past 8 years in Hawai‘i, featured in private residences, parks, community centers, botanical gardens, and most recently in orchards. Between 2010-2016, more than 12,000 ‘Ma‘afala’ cultivar trees were planted in Hawai‘i, including more than 10,000 trees distributed through the through the National Tropical Botanical Garden Breadfruit Institute’s “Plant a Tree of Life - Grow ‘Ulu” project (Ragone *et al*, 2016).

Breadfruit trees have enjoyed the status of being a win-win plant: productive, low-input, and mostly free of pests and disease (Ragone, 1997; Taylor and Tuia, 2007). While it may be true that breadfruit is often little affected by pests or pathogens in cases of single specimen trees, intensive cultivation of breadfruit crops may exacerbate pathogen problems and amplify pathogen damage. For greater efficiency of harvesting, marketing, and maintenance, typically only a single cultivar is planted in a given orchard making a grove that is not only a monocrop, but genetically identical throughout. The lack of genetic diversity of traditional crops of Oceania has allowed for

a build-up of pathogen populations responsible for deterioration of crop agronomic performance (Lebot, 1992). As stands of breadfruit orchards become more common in Hawai‘i, incorporating mixed plantings of breadfruit cultivars and diverse species may help mitigate disease problems.

While information is available on insect pests and plant pathogens that affect the breadfruit crown and fruit, below-ground pathogens are not as well understood. Information on plant-parasitic nematodes associated with breadfruit is lacking. Surveys of plant-parasitic nematodes associated with breadfruit are limited to Jamaica (Coates-Beckford and Periera, 1992), Brazil (Sharma, 1976), and New Caledonia (Grandison *et al.,* 2009), with regional reports of pathogenic nematodes in Malaysia (Razak, 1978) and select areas of the Pacific (Kirby *et al.,* 1980; Orton Williams, 1980; Grandison, 1990, 1996). *Aphelenchoides* sp., *Helicotylenchus dihystera, H. multicinctus, Hemicriconemoides cocophilus, Meloidogyne* sp., *Paratylenchus* sp. and *Xiphinema brevicolle* were present on the roots and rhizosphere of breadfruit trees found in Fiji, Kiribati, Niue, Western Samoa, Tonga, and the Cook Islands (Orton Williams, 1980; Grandison, 1990). Kirby *et al.* (1980), Grandison (1996), and Grandison *et al.* (2009) reported *Achlysiella williamsi, Aphelenchoides bicaudatus, Criconemella denoudeni, C. onoensis, Ditylenchus* sp., *Gracilacus* sp., *Helicotylenchus dihystera, H.erythrinae, H. indicus, H. microcephalus, H. pseudorobustus, Helicotylenchus* spp., *Hemicriconemoides mangiferae, Heterodera* sp., *Meloidogyne incognita, Pratylenchus coffeae, P. loosi, Pratylenchus* sp., *Sphaeronema* sp., and *Xiphinema* sp. on breadfruit from islands of the Pacific including New Caledonia.

In Brazil, *H. dihystera* and *Tylenchus leptosoma* were associated with the roots of the breadfruit tree (Sharma, 1976). In Malaysia, *Meloidogyne* spp. caused galling and inhibited root development of feeder roots and elongation, causing “retarded growth, reduced trunk diameter, sparse branching, and general yellowing of the leaves and in extreme cases, plant mortality”

(Razak, 1978). *Meloidogyne* spp. was also reported on breadfruit in a survey conducted in Jamaica in the late 1950s (Hutton, 1976). In later surveys in Jamaica, Coates-Beckford and Periera (1992) sampled declining and non-declining breadfruit trees and confirmed previous findings of plant-parasitic nematodes from other countries associated with breadfruit trees including: *P. coffeae*, *Pratylenchus* spp., *Helicotylencus erthrinae*, *H. multicinctus*, and *Meloidogyne* sp. The causal agent of slow decline disease in Jamaica, which causes decline and reduces production of mature breadfruit trees, was reported to be *P. coffeae* (Coates-Beckford and Periera, 1992). To date, research on plant-parasitic nematodes found associated with breadfruit trees in Hawai‘i is unavailable.

2.3 Objective

The objective of this survey was to determine the incidence and abundance of plant-parasitic nematode fauna associated with the roots and soils of breadfruit trees on the islands of Kaua‘i, Maui, and O‘ahu.

2.4 Materials and methods

Surveys of plant-parasitic nematodes associated with breadfruit were conducted from May 2013 to July 2015. A total of 25 soil and root samples were collected using a stratified random method from the islands of Kaua‘ i, Maui, and O‘ahu (Fig. 2.1). Three types of breadfruit production systems were targeted for sampling: commercial plantings, breadfruit growing near homesteads, and specimen trees in botanical gardens. Beneath the breadfruit canopy, soil and root samples were collected at four locations at a depth of 15 cm along the drip line.

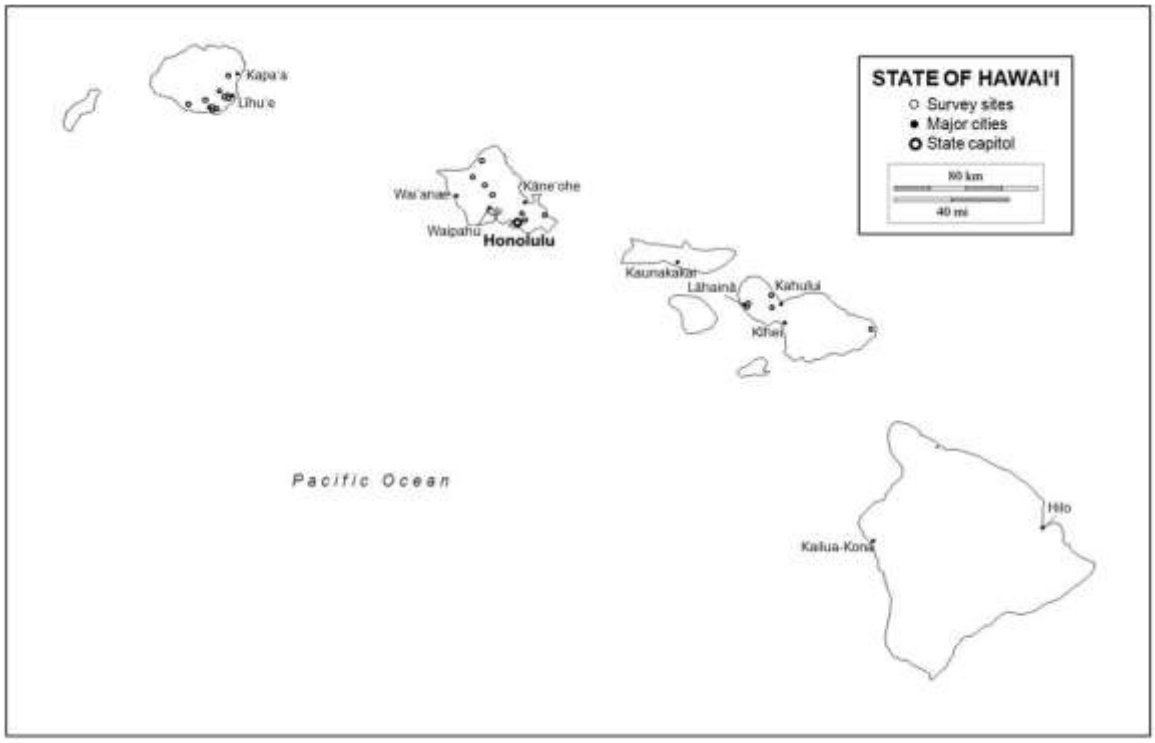

Figure 2.1: Breadfruit root and soil sampling sites in Hawai‘i, on Kaua‘i (13 sites), O‘ahu (7 sites), and Maui (5 sites).

With the exception of a few commercial plantings, most survey locations had single tree plantings. In commercial plantings, soil from multiple trees was composited into a single sample to represent the entire orchard. Roots and soils were collected in a zig-zag pattern from 10 trees encompassing no more than 1ha. Collected soil and root samples were gently mixed before 400 cm^3 of soil and 50 g of roots were subsampled and placed into plastic bags and placed in a temperature-controlled vessel for transport to the laboratory at the University of Hawai‘i at Mānoa. Breadfruit roots, identified by their red color, were separated from soil, cut into 2-cm long pieces and a 20g sample was used for nematode extraction in a mist chamber (Seinhorst, 1956). Nematodes were collected from the mist chamber after 7 days. Soils were screened through a 4-mm mesh screen. Nematodes were then extracted from a 200 cm^2 subsample using a semi-automatic elutriator (Byrd *et al.*, 1976). All plant-parasitic nematodes were identified to the genus level using a Leica DMIRB inverted microscope. Nematode counts were adjusted to 250 ml soil and 20 g root dry weight for comparisons. Mean, range, and "% frequency occurrence" of plant-parasitic nematodes by species were calculated (Table 2.1). To calculate "% frequency occurrence," positive sites were divided by the total number of sites surveyed.

2.5 Results and Discussion

The plant-parasitic nematodes associated with breadfruit in Hawai‘i are consistent with previous studies (Sharma, 1976; Hutton, 1976; Orton Williams, 1980; Grandison, 1990, 1996; Grandison *et al.*, 2009; Kirby *et al.*, 1980; Coates-Beckford and Periera, 1992). Plant-parasitic nematodes found in soils associated with breadfruit on Kaua‘i, Maui, and O‘ahu included *Mesocriconema, Helicotylenchus dihystera, Meloidogyne, Paratylenchus, Pratylenchus coffeae, Rotylenchulus reniformis, Tylenchorhynchus,* and an unidentified taxon of Heteroderidae.

Table 2.1: Mean number and range of plant-parasitic nematode species found on breadfruit from three Hawaiian Islands (20 g root samples and 250 ml soil samples) and frequency of occurrence across surveyed sites (%).

	Soil			Roots			% frequency of occurrence
Areas	K_x	O_y	M_z	K_x	O_y	M_z	
Number of samples	13	7	5	8	7	5	
Helicotylenchus	98 (0-570)	614 (20-1380)	72 (0-168)	41 (0-214)	140 (0-683)	39 (0-66)	68%
Rotylenchulus	367 (0-1032)	83 (0-290)	47 (0-96)	28 (0-290)	5 (0-20)	16 (0-32)	52%
Paratylenchus	24 (0-270)	54 (0-92)	58 (0-58)	32 (0-236)	31 (0-94)	45 (0-170)	44%
Pratylenchus	-	63 (0-154)	36 (0-120)	6 (0-20)	423 (0-2942)	1385 (42-2518)	36%
Meloidogyne	18 (0-156)	56 (0-177)	12 (0-62)	3 (0-10)	-	12 (0-82)	28%
Heteroderidae	42 (0-168)	27 (0-60)	207 (0-610)	-	-	-	12%
Mesocriconema	0 (0-6)	1 (0-10)	-	-	-	-	8%
Tylenchorhynchus	-	1(0-8)	-	-	-	-	4%

[x]K = Kaua'i, [y] O = O'ahu, [z] M = Maui, outside ( ) = mean, inside ( ) = range, and - = specified nematode not found.

Occurring at 68% of sites, *Helicotylenchus* was the most frequently encountered plant-parasitic nematode genus. *Paratylenchus* was the most frequently observed plant-parasitic nematode on breadfruit on O‘ahu, occurring at 86% of sites surveyed. On the other hand, *H. dihystera, R. reniformis,* and *P. coffeae* were the most prevalent nematode genera found on Maui, present in 40% of locations surveyed. *Meloidogyne* was detected less frequently on breadfruit compared to the other plant-parasitic nematodes among the three islands surveyed, occurring on O‘ahu at 43%, Kaua‘i at 23%, and Maui at 20% of the locations (Fig. 2.2). The most diverse occurrence of plant-parasitic nematodes genera was collected at Waimea Arboretum, O‘ahu, where six different nematode genera were detected and *Pratylenchus* were the dominant species.

Pratylenchus were mainly found in the roots of breadfruit trees surveyed. Root extraction from the mist chamber yielded as much as 2,942 *Pratylenchus* nematodes per 20g of roots on a site on O‘ahu, whereas elutriation of 200 ml of soil yielded only 36 *Pratylenchus* nematodes from the same site. Results of *Pratylenchus* from root and soil extraction from Maui were similar. Since Coates-Beckford and Periera (1992) cited *P. coffeae* as the cause of slow decline disease of breadfruit in Jamaica. The lesion nematodes found during our survey were found to bc *P. coffeae* also (Lau, Chapter 3). Whether *P. coffeae* is as devastating to breadfruit in Hawai‘i as it is in Jamaica remains unknown. Future studies should examine pathogenicity of *P. coffeae* on breadfruit cultivars commonly grown in Hawai‘i and interactions of *P. coffeae* with other pathogenic fungi and phytoparasitic bacteria to correlate with declining breadfruit (Coates-Beckford and Periera, 1992). Nonetheless, these findings suggest that breadfruit farmers should not accept breadfruit plants grown from root shoots or root cuttings as nematode contamination may be a potential risk.

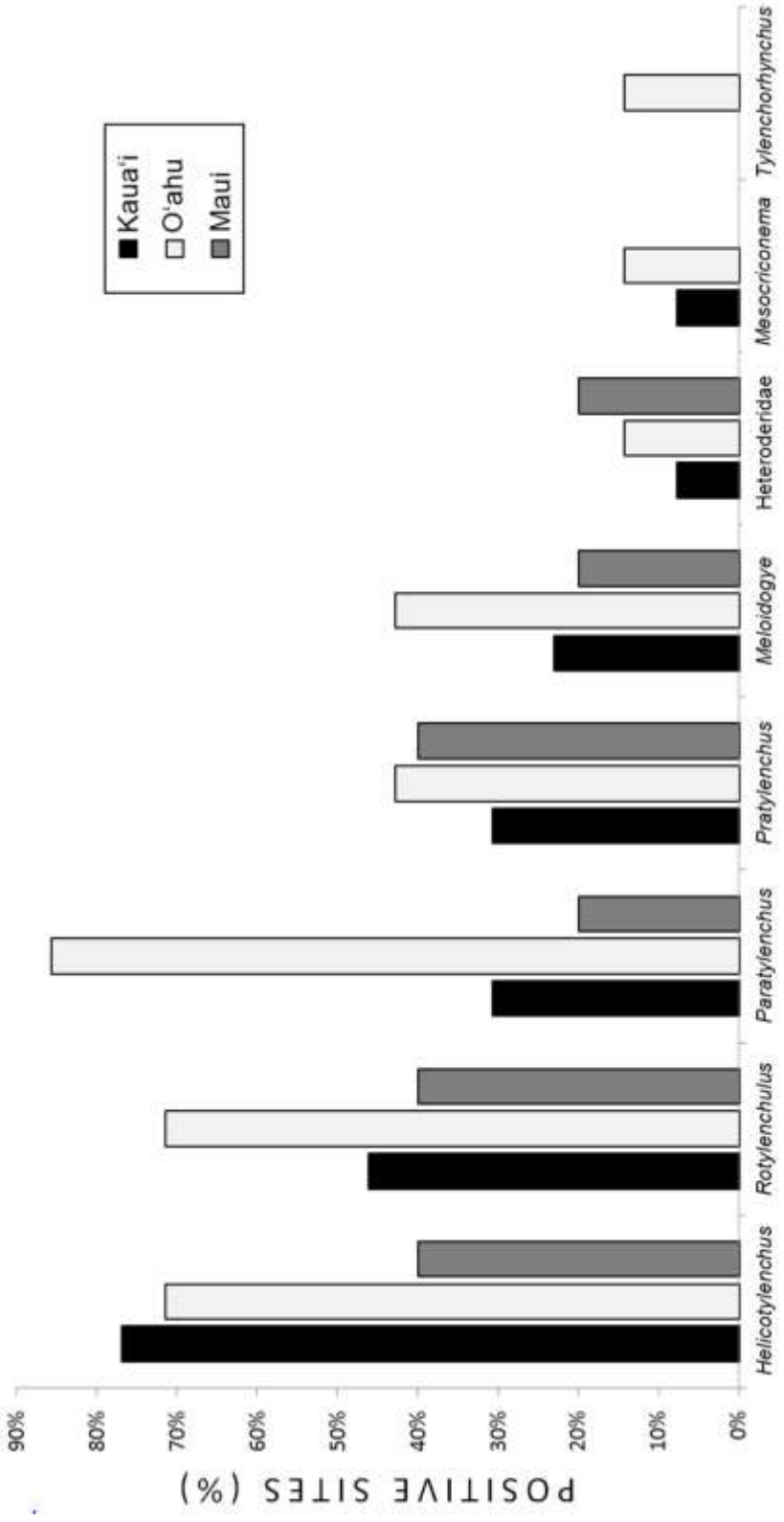

Figure 2.2: Plant-parasitic nematode occurrence on breadfruit sites in Hawai'i by island.

Detection of *Meloidogyne* was considerably less frequent in our survey, at just 28% of surveyed locations, compared to other surveys. Most of the breadfruit cultivars surveyed in this project were either ‘Ma‘afala’ or Hawaiian “Ulu’. Low occurrence and abundance of *Meloidogyne* may have been the result of these specific cultivars being poor hosts to *Meloidogyne*. A pathogenicity test for *Meloidogyne spp.* and other species of plant-parasitic nematodes on popular breadfruit cultivars could help determine breadfruit’s host range and potential damage to breadfruit trees by pathogenic nematodes.

Furthermore, reniform nematode was first described in Hawai‘i and has a wide host range (Linford and Oliveira, 1940; Linford and Yap, 1940). Thus it was no surprise that *Rotylenchulus* had a high incidence in the soils associated with breadfruit on all islands. As with *Meloidogyne*, the cultivars grown in Hawai‘i might be poor hosts to the reniform nematode.

An unidentified species of nematode in the Heteroderidae was detected at one site on each island, all of which were sampled from botanical gardens. Cyst nematodes are not common in Hawai‘i. Traditional propagation from root shoots combined with *Heterodera* presence specific only to botanical gardens could be indicative of the diversity of propagules of various plant species collected from different locales planted in these gardens and the sharing of root cuttings.

Several genera of plant-parasitic nematodes were commonly recovered by at low population densities. *Helicotylenchus* and *Paratylenchus* were found from many locations on all the islands. Since both *Paratylenchus* and*H. dihystera* were found primarily in soils, and are not major plant pathogens of tree crops, it is not likely that these nematodes are problematic to breadfruit. *Mesocriconema* was less prevalent in samples, only observed at one location each on Kaua‘i, Maui, and O‘ahu. *Tylenchorhynchus* was found at one location on O‘ahu. Plant-parasitic

nematodes were absent from 12% of locations surveyed, including one site on Kauaʻi and two on Maui.

Plant-parasitic nematodes were found to be prevalent in breadfruit roots and associated soil in some locations. However association is not proof of feeding nor an indicator of pathogen problems. Pathogenicity tests are needed to identify the level of virulence and damage of the specific nematodes commonly detected in this survey. Sourcing new plants from root shoots or root cuttings can spread endoparasitic nematodes to new planting areas, thus use of *in vitro* breadfruit propagation material free of nematodes should be recommended to farmers. The occurrence and abundance of specific nematode genera associated with breadfruit will aid in developing specific nematode management approaches appropriate to successfully growing breadfruit in Hawaiʻi.

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Chapter 3: Identification of *Pratylenchus* species Associated with Breadfruit

3.1 Abstract

An unknown *Pratylenchus* parasitizing breadfruit in Hawai‘i was identified as *P. coffeae* using morphometric and molecular techniques. A population was collected on breadfruit roots from the University of Hawai‘i at Mānoa Botany Garden. The population consisted of females, males and juveniles. The population was identified both morphometrically and molecularly. The molecular analysis was conducted with primers targeting the D2-D3 expansion region of the 28S rDNA. Nucleotide-nucleotide BLAST searches revealed a mean sequence identity of 93.9% and a mean query coverage of 99.1% to *Pratylenchus coffeae* in the GenBank sequence database. The next closest species match was to *Pratylenchus speijeri,* with a mean sequence identity of 93.4% and a 99.0% mean query cover. To resolve molecular identification similarities between *P. coffeae* and *P. speijeri*, measured morphometrics were revisited. *Pratylenchus coffeae* parameters shared body length (L), V%, and tail length with mean measurements from the breadfruit population of *Pratylenchus*, whereas *P. speijeri* had only T% and c-ratio in common with the unknown *Pratylenchus.*

3.2 Introduction

Pratylenchus were found in abundance during surveys of plant-parasitic nematodes parasitizing breadfruit roots in Hawai‘i on the islands of O‘ahu and Maui. *Pratylenchus* is linked to slow decline disease of breadfruit. In Jamaica, *P. coffeae* was cited as responsible for “premature fruit drop, leaf chlorosis and abscission, general unthriftiness, and branch dieback” and identified as the cause of slow decline disease of breadfruit, especially on trees older than 20 years and when paired with *Fusarium* spp. and *Pseudomonas* spp. (Coates-Beckford and Pereira, 1992). Similar

symptoms were noted in the 1950s to 1960s in the Pacific Basin without a causal agent identified (Zaiger and Zentmyer, 1966).

Nematodes of the genus *Pratylenchus* Flipjev, commonly called lesion nemaotdes, are economically important pests of agricultural, horticultural, and industrial crops (Oliviera *et al*. 1999; Seinhorst, 1998; Smiley *et al*. 2005). Lesion nematodes ranked third in the world for having the greatest economic impact on crops behind root knot and cyst nematodes. Lesion nematodes are ubiquitous in their distribution in almost every cool, temperate, and tropical environment, and have a wide host range of some 400 different crop plant species (Sasser and Freckman, 1987). While feeding, *Pratylenchus* spp. produce characteristic necrotic lesions on infected roots and deprive host plants of essential nutrients (Castillo and Volvas, 2007).

Pratylenchus are distinguished by a flat head, strong cephalic framework, and short, thick stylet with prominent basal knobs (Dropkin, 1980). Esophogeal glands usually overlap the intestine ventrally (Sher, 1973) and tails are cylindrical to conoid, two to three anal body widths (Dropkin, 1980). A long overlap, or small degree of overlap in the pharynx, is a distinguishing component when paired with other characteristics of *Pratylenchus* species (Al-Banna *et al*., 1997). In females, the vulva is located at 70-80% of the body length. In males, a distinct bursa reaches the tail tip (Evans *et al.*, 1993).

While it is straightforward to differentiate members from other genera, *Pratylenchus* species are very similar to each other making morphological identification within the genus difficult (De Luca *et al.*, 2012). Luc (1987) called *Pratylenchus* "a stenomorphic genus," meaning that limited morphological characters are present to diagnose a species. Because of intraspecific variabilities (Powers, 2004), like head and tail shape, high sample numbers are necessary to reduce likelihood of misidentification (Anderson and Townshend, 1980; Donald and Clark, 1983; Luc,

1987). Al-Banna *et al.* (2004) reported morphometric discrepancies amongst original descriptions of *Pratylenchus* spec even though these species are known to display a pronounced heteromorphism (Tarte and Mai, 1976; Tarjan and Frederick, 1978). Doucet *et al*. (2001) and Townshend (1991) found intraspecific variation a result of temperature differences and geographical locale, respectively. Because of these challenges in visual identification, advances in genetic identification and expansion of species-specific primers for root lesion nematode are replacing traditional morphological identification as primary characters for species identification of the genus *Pratylenchus*. In previous studies, little connection has been found between morphology and the evolution of rDNA, however analysis of genetic data have provided good phylogenetic resolution of *Pratylenchus* species (Nadler, 1992; Heise *et al*., 1995). To differentiate between two species of *Pratylenchus*, Uehara *et al*. (1998) used DNA amplification with species-specific primer sets to distinguish between *P. loosi* and *P. coffeae*. Al-Banna *et al*. (2004) found success with species-specific primers from the internal variable portion of the D3 expansion region of the 26S rDNA to distinguish between species of *P. neglectus, P. penetrans, P. scribneri, P. thornei,* and *P. vulnus*. Duncan *et al*. (2007) and Inserra *et al*. (2007) later found that addition of the D2 expansion segment to the D3 expansion region improves resolution, especially for diverse *P. coffeae* isolates when compared to closely related species (Duncan *et al*., 1999; Inserra *et al*., 2007).

Although sequence data to discriminate among *Pratylenchus* species has advantages including faster training and reduced sample numbers, DNA-based data should not be seen as a substitute for understanding and studying whole organisms when determining identities (Will and Rubinoff, 2004). In concurrence with Baldwin and Perry (2004), nematode morphology in the 21st

century should be an integration of diagnostic molecular approaches paired with traditional morphology.

3.3 Objective

The objective of this study was to identify the *Pratylenchus* species associated with breadfruit in Hawai‘i using morphometric and molecular techniques.

3.4 Materials and methods

Collection. Breadfruit roots were procured from Kaua‘i, O‘ahu, and Maui. Kaua‘i and O‘ahu survey sites where *Pratylenchus* was detected during breadfruit survey. Kaua‘i Community College, University of Hawai‘i at Mānoa Botany Garden, and Waimea Arboretum were revisited in July 2015 to collect additional specimen. Root samples collected from Kahanu Garden of the National Tropical Botanical Garden on Maui during September 2014 were stored and used for extraction. Breadfruit roots were surface sterilized in a 10% NaOCl solution, cut into 2-cm pieces, and set in the mist chamber to collect nematodes. Nematodes were collected after 7 days. Only the University of Hawai‘i at Mānoa Botany Garden yielded a sufficient quantity of *Pratylenchus* for species identification. Other sites where *Pratylenchus* had been found during breadfruit surveys had less than 10 specimen per sample and were not used in the morphological and molecular analysis.

Morphological analysis. During the morphological and morphometric analysis, living adult nematodes were used. Live specimens were immobilized with gentle heat and mounted on a slide for measurement and photographs (Esser, 1986). A Leica DMLB compound light microscope was used to record body length (L), body width, stylet length, tail length for females, position of vulva (V%) or spicule (T%) on body, body relative thickness (a) and tail relative length (c), presence/absence of males, and tail shape. Tails from relaxed specimens were described, then

photographed and compared to illustrated tail shapes of *Pratylenchus* spp. Illustrations were made transparent in Adobe Photoshop and then overlaid on photographs to compare to the unknown *Pratylenchus* to known species.

Measurements were made according to Hooper (1970), where specimens were fixed with heat then mounted for analysis. Specimen measurements were determined with an ocular micrometer. Ten females and 10 males were compared against original characterizations and diagnostic keys (Roman and Hirschmann, 1969; Frederick and Tarjan, 1989; Handoo and Golden, 1989, De Luca *et al*., 2012) in order to identify *Pratylenchus* to a species level.

Molecular analysis. Ten samples, five containing the DNA of one nematode and five with the DNA of two nematodes, were used for molecular identification. Nested PCR using species-specific primers was performed (Al-Banna *et al.*, 2004). Primers for *Pratylenchus* species differentiation targeted the D2-D3 expansion region of the 28S rDNA (Al-Banna *et al*., 1997). Forward primer sequences were selected for compatibility with the annealing temperature of the common reverse primer. The forward D2A (5’ -ACAAGTACCGTGAGGGAAAGTTG-3’) and reverse D3B (5'- TCGGAAGGAACCAGCTACTA-3') primers submitted by Subbotin *et al*. (2006) were used for amplification and sequencing of expansion regions of the 28S rRNA. Denaturation, amplification, and primer extension was completed on an Applied Biosystems 2720 Thermal Cycler at the University of Hawai‘i at Mānoa Nematology Laboratory in Honolulu, HI, USA. *Pratylenchus* male, female, and juvenile specimens were handpicked from mist chamber sample collections then placed singly in a 15 µl drop of sterile H_20 on a glass microscope slide.

Using a Roboz surgical micro 45° tool (model number RS-9421-06) under a Leica Wild MZ8 dissecting microscope, each nematode specimen was cut transversely, then pipetted into a 50 µl tube and stored over ice in preparation for PCR in 35 µl reactions (Cabos *et al.,* 2013). The

solution was kept in a bench chiller until ready for use. Per PCR tube, 24.5 µl of PCR reagent master mix was added. Readied tubes were placed into the PCR machine at 95°C for 2 minutes, 95°C for 45 seconds, 54°C for 30 seconds, then 72°C for 20 seconds. The cycle was repeated 39 times and then run at 72°C for 5 minutes before being reduced to 4°C for storage.

When loading the gel, 1xTAE buffer solution was added to the gel box to cover a 10% agarose gel. A 10 µl drop of marker ladder was added to the first and middle wells. PCR products were sequentially pipetted into each of the individual lanes. The gel was run at 90V for 54-60 minutes or until the dye marker was 5 cm from the end of the gel. The gel was photographed using a transilluminator UV box to indicate the presence of DNA. Selected samples that detected fluorescent DNA-binding dye with clear, bright bands were selected for submission to the University of Hawai‘i at Mānoa Sequencing Lab. The returned nucleotide-nucleotide BLAST search was compared against the GenBank sequence database for sequence similarities. The species analysis was considered alongside morphological characteristics for a final species determination.

3.5 Results

Morphological observations. Females: Photographs of the unknown female *Pratylenchus* species are presented in Fig. 3.1. Body vermiform, almost straight to open C-shaped, slender in young females and thicker in older ones, tapering towards both ends. Body width increased from lip region to the level of the median bulb and then decreased from the level of the vulva towards the tail tip. Cuticular annulations were visible. Lip region was slightly set off from the rest of the body. The stylet was strong with round basal knobs. The overlap was long and occurred

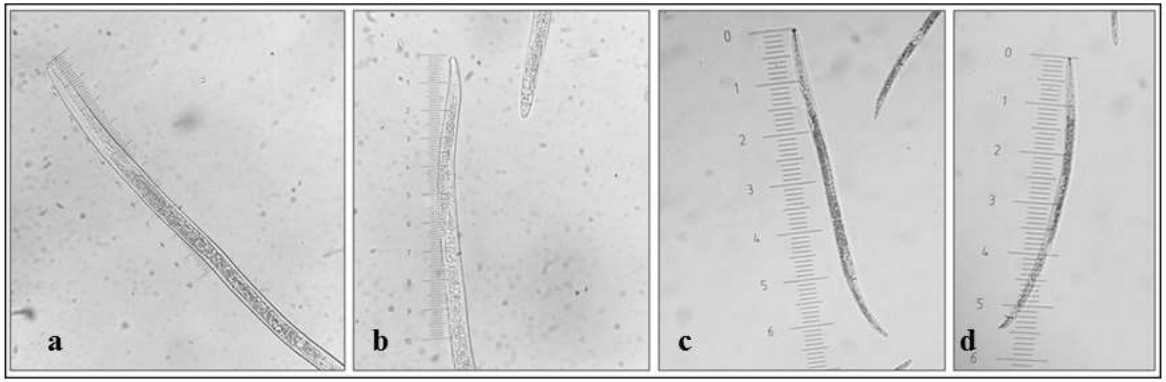

Figure 3.1: Photographs of unknown *Pratylenchus* spp. (a) female head, (b) female tail, (c) entire female, and (d) entire male as observed through Leica DMLB compound light microscope.

ventrally, slightly lateral. Vulva was distinct and occurred roughly at 75-80% of body length. Tails tapered ending in a broad flattened tail tip. The tail tip was smooth, blunt, and curved ventrally when relaxed.

Males: Photographs of unknown male *Pratylenchus* species are presented in Fig. 3.1. Males were common within the population and slightly less abundant than females, making up 38% of adult population. Males were morphologically similar to females, but smaller in body length and more slender. Spicules were ventrally curved. Bursa was conspicuous, extending before top of spicule to the tip of tail. Tail bent ventrally and ended in a pointed tip.

Morphometric observations. Females: Morphometrics of the females of the *Pratylenchus* spp. population collected off breadfruit roots from the University of Hawai‘i at Mānoa Botany Garden on the island of O‘ahu are presented in Table 3.1. The body length of females from the population ranged from 499.1 to 777.5 µm (mean: 654.2 µm). The average body width of the female population was 31.1 µm. The a-ratio of females ranged from 18.7 to 30.5 (mean: 21.2). The c-ratio ranged from 15.5 to 20.1 (mean: 18.09). Tail length of measured females ranged from 30.8 to 44.6 µm. The V-value of the populations ranged from 74.0 to 80.0% (mean: 76.8%). The stylet length of the females ranged from 14.5 to 17.6 µm (mean: 16.2 µm). Males: Morphometrics from males are presented in Table 3.2. The body length of males ranged from 413.1 to 557.2 µm (mean: 486.9 µm). The average body width of the male population was 22.2 µm. The a-ratio of the males ranged from 18.7 to 24.3 (mean: 22.0). The T-value ranged from 49.0 to 62.0% (mean: 55.6%). Stylet length of males ranged from 16.1 to 23.6 µm (mean: 19.8 µm).

Molecular analysis. The comparison of the sequences of the D2/D3 28S rDNA expansion segments of the unknown *Pratylenchus* yielded an amplicon ranging from 700 to 789 bp (mean:

Table 3.1: Morphometric measurements and ranges (in bold) of female *Pratylenchus* species from Hawai'i (in second column) followed by published values of *Pratylenchus coffeae* from Roman and Hirschmann, 1969; Frederick and Tarjan, 1989; Handoo and Golden, 1989; and *P. coffeae* and *P. speijeri* from De Luca *et al*., 2012.

		P. coffeae				*P. speijeri*
	Hawai'i population	Roman and Hirschmann, 1969	Frederick and Tarjan, 1989	Handoo and Golden, 1989	De Luca *et al.*, 2012	De Luca *et al.*, 2012
n	10	25	----	----	20	20
Body length (L) (µm)	654.2 **(499.1-777.5)**	592.67 **(516.4-721.2)**	580 **(460-630)**	**370-690**	602 **(529-799)**	533 **(473-563)**
Body width (µm)	31.1 **(25.6-34.6)**	24.92 **(19.8-28.8)**	----	----	21.2 **(17.5-25.5)**	19.5 **(16.0-23.5)**
Tail length (µm)	36.1 **(30.8-44.6)**	32.45 **(27.0-38.4)**	----	----	29 **(21-36)**	28 **(23-32)**
Stylet length (µm)	16.2 **(14.5-17.6)**	15.47 **(14.4-16.8)**	16 **(15-17)**	**15-18**	16.9 **(16.5-17.0)**	17.0 **(16.5-18.0)**
V (%)	76.8 **(74.0-80.0)**	78.60 **(74.0-79.0)**	79 **(76-82)**	**75.8-84.2**	80.5 **(76.0-82.5)**	80.0 **(77.6-82.0)**
a	21.2 **(18.7-30.49)**	26.31 **(20.1-33.7)**	25 **(21-30)**	**17.7-30.5**	28.7 **(23.4-34.0)**	27.7 **(23.4-32.7)**
c	18.1 **(15.52-20.14)**	18.19 **(14.9-20.8)**	21 **(17-27)**	**13.7-23.9**	20.9 **(17.0-31.0)**	18.8 **(17.1-21.6)**

V =position of vulva relative to body length from head
T = position of spicule relative to body length from head
a = body length/body width
c = tail length/body length

Table 3.2: Morphometric measurements and ranges (in bold) of male *Pratylenchus* species from Hawai‘i (in second column) followed by published values of *P. coffeae* males from Roman and Hirschmann, 1969 and *P. speijeri* males from De Luca *et al*., 2012.

		P. coffeae	*P. speijeri*
	Hawai‘i population	Roman and Hirschmann, 1969	De Luca *et al.*, 2012
n	10	----	10
Sex	M	M	M
Body length (L) (µm)	486.9 **(413.1-557.2)**	516.81 **(430.7-600.0)**	489 **(446-550)**
Body width (µm)	22.2 **(18.6-25.6)**	18.64 **(16.0-22.8)**	16.5 **(15.5-18.5)**
Stylet length (µm)	16.3 **(14.0-19.1)**	14.35 **(13.8-15.6)**	15.2 **(14.5-16.5)**
T (%)	55.6 **(49.0-62.0)**	51.36 **(36.0-68.0)**	35.0 **(28-45)**
a	22.0 **(18.7-24.3)**	27.82 **(23.5-32.2)**	29.7 **(27.1-33.0)**

752 bp) in length depending on the sample studied. Of ten samples, nine provided sufficient DNA for analysis in NCBI Blast. Matches with mean sequence identity of 93.9% (range: 93-95%) and a mean query coverage of 99.1% (range: 98-100%) were found with *Pratylenchus coffeae*. The next closest species match was to *Pratylenchus speijeri*, with a mean sequence identity of 93.4% (range: 92-95%) and a 99.0% (range: 98-100%) mean query cover.

3.6 Discussion

Many authors have suggested that identification of *Pratylenchus* requires multiple adult female specimens for reliable diagnosis (Al-Banna *et al*., 1997). According to Fortuner (1984) at least 20 specimens must be morphometrically analyzed before naming *Pratylenchus* to species. Loof (1991) called the family "not very homogeneous" and argued the number of specimens examined to be least 25 adult females per sample, while Roman and Hirschmann (1969) found it necessary to examine 20 aspects from each 25 females and 25 males before characterization. More nematodes do provide better resolution, however no sampling process has unlimited resources, thus 10 females and 10 males underwent morphometric examination during our study for comparison and were supplemented with a molecular analysis of 15 nematodes.

Morphometric observations were in concurrence with previously published descriptions overlapping at *P. coffeae* measurements. Mean measurements of the unknown species, including body length (L), stylet length, V%, and a- and c ratios were compared with published parameters from Roman and Hirschmann (1969), Frederick and Tarjan (1989), Handoo and Golden (1989), and De Luca *et al*. (2012) (Table 3.1). Body width, T%, tail length, and absence/presence of males were also compared when information was available. According to observations of 64 *Pratylenchus* spp. taken by Handoo and Golden (1989), *P. coffeae* and *P. penetrans* were the only species whose ranges corresponded with the means of the measured nematode for all available

criteria, including: L, stylet length, V%, a- and c ratios, and where males were “present, common.” The next closest species, *P. gibbicaudatus*, *P. kasari,* and *P. morettoi,* matched the stylet length, V%, and a- and c ratios of the unknown species, but our measured L mean did not fall within the range of that published, and males were cited as “present” rather than “present, common” by Handoo and Golden (1989).

Comparing mean observations to published ranges for L, stylet length, V%, and a-, and c ratios characterized by Frederick and Tarjan (1989) for 49 *Pratylenchus* species, no species met all five criteria. Five nematodes met four of the five measurement criteria compared by Frederick and Tarjan including: *P. coffeae, P. delattrei, P. flakkensi, P. kasari*, and *P. morettoi. Pratylenchus coffeae, P. delattrei* and *P. flakkensi* had a shorter range for L, and *P. kasari* and *P. morettoi* had a larger a-ratio than the unknown *Pratylenchus.* The other 44 *Pratylenchus* species matched three criteria or less. Descriptions by Roman and Hirschmann (1969) affirmed *P. coffeae* as a match for the unknown species from breadfruit. Mean measurements of L, width, tail length, stylet length, V%, a-, and c ratios were considered, with *P. coffeae* matching six of the seven measured means of the unknown nematode from our collection. The remaining *Pratylenchus* species matched three or less of the seven criteria compared.

De Luca *et al.* (2012) outlined parameters for *Pratylenchus speijeri,* a new species of nematode with its closest related species being *P. coffeae*. According to De Luca *et al*. (2012), “morphological characterization did not result in an unambiguous separation” from *P. coffeae* based on range overlaps for *P. coffeae* and *P. speijeri. Pratylenchus speijeri* had been tentatively identified during a survey of corn in Hawai‘i (Khaitong and Sipes, unpublished).However, comparing measured means of the *Pratylenchus* spp. from Hawai‘i breadfruit to ranges of *P. coffeae* and *P. speijeri*, *P. coffeae* matched more criteria than *P. speijeri*. In all, seven criteria were

available to compare to the female nematodes including: body length (L), body width, tail length, stylet length, V%, and a- and c ratios. Based on comparative measurements provided by De Luca *et al*. (2012) for females and De Luca *et al.* (2012) and Roman and Hirschmann (1969) for males, *P. coffeae* encompassed the range of the unknown nematode for L, V%, and tail length for females and body length, body width, and T% for males, while *P. speijeri* only overlapped at c-ratio for females, and body length and stylet length for males.

Several *Pratylenchus* species shared our female tail description of bluntly flattened with a smooth terminus. Of Roman and Hirschmann's (1969) illustrated tail shapes and descriptions, the broadly rounded tip was shared by *P. coffeae*, variations of *P. penetrans*, and *P. scribneri* (Fig. 3.2). Only the tail tips of *P. coffeae* and *P. scribneri* bend ventrally when relaxed, whereas the tail of *P. penetrans* bends dorsally above the vulva then straightens towards the tip of the tail, not matching the curvature of the unknown species. De Luca *et al.* (2012) illustrated closely related *Pratylenchus* species including *P. auracensis, P. coffeae, P. floridensis, P. jaehni, P. loosi, P. parafloridensis,* and 10 tail variations from *P. speijeri.* All six of these tail shapes visually matched a tail variation of our unknown nematode (Fig. 3.3). Included in the De Luca *et al.* (2012) examination of *P. speijeri* were 10 variations of tail shape (Fig 3.4) with special emphasis given to the tail terminus indentation, a common feature of *P. speijeri*. No such indentation was found in the unknown *Pratylenchus.* Frederick and Tarjan (1989), in describing 49 *Pratylenchus* spp., included a written tail type descriptor during species characterization. However, only a general key outlining fifteen different variables but without illustration were included to represent each species. Accordingly, *P. coffeae, P. penetrans,* and *P. scribneri* shared the same tail describers: blunty pointed and smooth tipped, but *P.coffeae* and *P. penetrans* are described as hemispherical

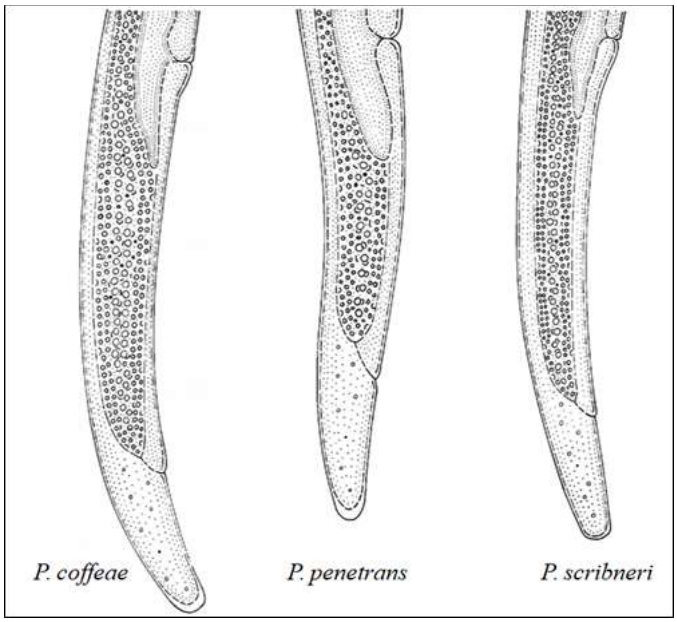

Figure 3.2: Tail tip shapes of *P. coffeae* (*left*), *P. penetrans* (*middle*), and *P. scribneri* (*right*) illustrated by Roman and Hirschmann (1969) match the unknown *Pratylenchus* spp. in Hawai‘i.

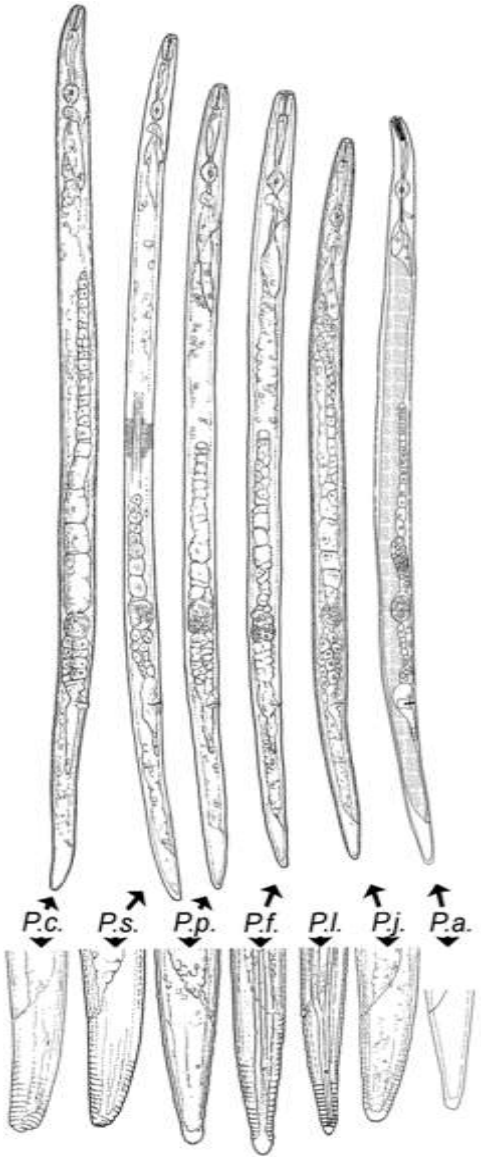

Figure 3.3: Tail shapes of nematodes closely related to *Pratylenchus coffeae* (P.c.) including *P. speijeri* (P.s.), *P. parafloridensis* (P.p.), *P. floridensis* (P.f.), *P. loosi* (P.l.), *P. jaehni* (P.j.), and *P. auracensis* (P.a.) (De Luca *et al*., 2012) with variations in tail shape matching a population from Hawai‘i.

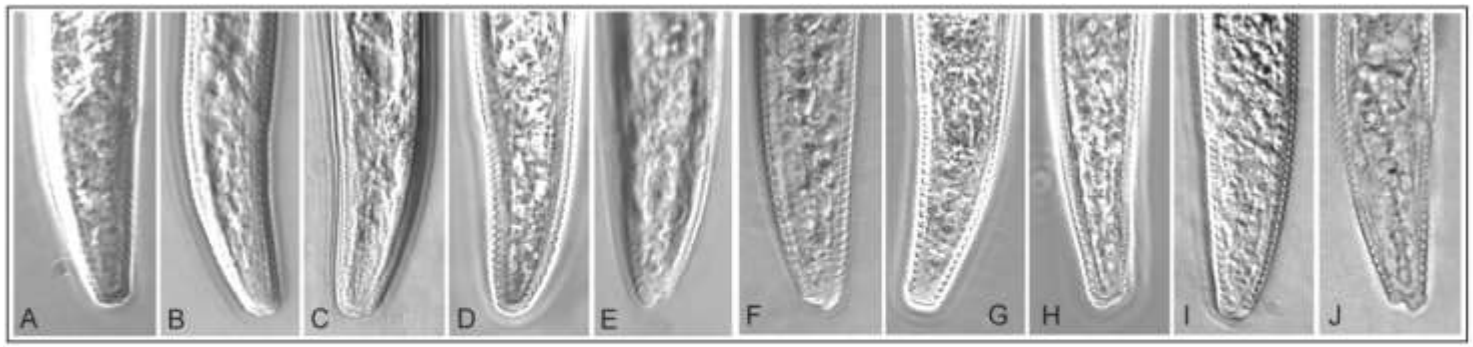

Figure 3.4 (A-J): Common tail shapes from *Pratylenchus speijeri* n. sp. (De Luca et al., 2012). Tail indentation (F, J) not observed on tail of unknown measured nematode.

while *P. scribneri* is described as semi-hemispherical. Also matching Frederick and Tarjan's (1989) tail classification "blunty pointed and smooth tipped" included: *P. bolivianus, P. crassi, P. delattrei, P. fallax, P. flakkensis, P. impar, P. kralli, P. microstylus, P. neglectus, P. pratensis, P. pratensisobrinus, P. typicus, P. mulchandi,* and *P. zeae.* Based on tail shape alone, a species conclusion could not be made with available material.

Molecular analysis confirmed the morphometric species identification of *P. coffeae,* but put into question the possibility of a species identification of *P. speijeri*. *Pratylenchus coffeae* and *P. speijeri* populations obtained from GenBank shared a range of 98.0-100% for query coverage, while the range for sequence identity encompassed a 1% difference (Table 3.3). The average length of our amplicon of 752 bp was closer to the published length of *P. coffeae* at 758 bp for the D2/D3 28s rRNA region, than the same region of *P. speijeri* which ranged from 780 to 795 bp in length. A comparison of morphometric data to compare to molecular analysis found that *P. coffeae* matched more criteria than *P. speijeri.* Combining molecular and classical methods to diagnose *Pratylenchus* to species demonstrates the utility of both to greatly improve identification capabilities or improve certainty of identification within the complex of lesion nematodes.

Table 3.3: Nucleotide sequence similarities from the GenBank database reveal likeness in the D2-D3 expansion region of the 28s rRNA between both *P. coffeae* and *P. speijeri* to theunknown *Pratylenchus* spp. extracted from the roots of a breadfruit tree in the Botany Garden at the University of Hawai'i at Mānoa.

Isolate no.	# of nematodes	Amplicon length (bp)	Description	Query cover	Sequence Identity	Accession numbers	Reference
1	2	789	*Pratylenchus coffeae*	99	95	KC490925.1	Shi and Zheng, 2013
			Pratylenchus speijeri	99	95	KF974703.1	Zhou *et al*., 2013
2	1	764	*Pratylenchus coffeae*	99	94	EU130845.1	Subbotin *et al.*, 2008
			Pratylenchus speijeri	99	93	KF974703.1	Zhou *et al.*, 2013
3	1	---	---	---	---	---	---
4	1	757	*Pratylenchus coffeae*	99	95	EU130845.1	Subbotin *et al.*,2008
			Pratylenchus speijeri	99	94	KF974703.1	Zhou *et al.*, 2013
5	1	709	*Pratylenchus coffeae*	100	93	EU130845.1	Subbotin *et al.*, 2008
			Pratylenchus speijeri	100	92	JN809845.1	De Luca *et al.*, 2012
6	2	789	*Pratylenchus coffeae*	100	92	KC490925.1	Shi and Zheng, 2013
			Pratylenchus speijeri	100	92	KF974703.1	Zhou *et al.*, 2013
7	2	742	*Pratylenchus coffeae*	99	94	EU130844.1	Subbotin *et al.*, 2008
			Pratylenchus speijeri	99	94	KF974703.1	Zhou *et al.*, 2013
8	2	788	*Pratylenchus coffeae*	99	94	KC490925.1	Shi and Zheng, 2013
			Pratylenchus speijeri	98	94	KF974703.1	Zhou *et al.*, 2013
9	2	700	*Pratylenchus coffeae*	99	94	KC490925.1	Shi and Zheng, 2013
			Pratylenchus speijeri	99	93	KF974703.1	Zhou *et al.*, 2013
10	1	730	*Pratylenchus coffeae*	98	94	KC490925.1	Shi and Zheng, 2013
			Pratylenchus speijeri	98	94	KF974703.1	Zhou *et al.*, 2013

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Chapter 4: Nematode pathogenicity on breadfruit

4.1 Abstract

Host status of *Meloidogyne javanica, Pratylenchus coffeae,* and *Rotylenchulus reniformis* on breadfruit, *Artocarpus altilis* cultivar 'Ma'afala' were determined. Two experiments were conducted with four replicates of *M. javanica, P. coffeae,* and *R. reniformis* on breadfruit. After 180 days or 360 days post-transplanting breadfruit plugs in 4-L paper-pulp pots, 1000 eggs of *M. javanica* or *R. reniformis* or 900 mixed vermiform stages of *P. coffeae,* or the water equivalent was administered to each plant. Nematode reproduction was recorded 180 days post-inoculation from soil and roots of the breadfruit.Plant measurements were recorded for trunk diameter, plant height, total plant weight, dry root weight, dry stem weight, and leaf chlorophyll content. Only *P. coffeae* reproduced and increased in population density. In experiment 2, *P. coffeae* was the only vermiform plant-parasitic nematode recovered. Reproductive factor of *P. coffeae* on breadfruit in experiment 1 showed a 17-fold growth and suggested that breadfruit is a good host to *P. coffeae.* In experiment 2, a trend of vermiform nematodes across two treatments indicated more successful reproduction of *P. coffeae* than *M. javanica* or *R. reniformis*. *Meloidogyne javanica* and *R. reniformis* were able to reproduce but population growth was not great enough to replace inocula concentrations indicating that breadfruit is a poor host to *M. javanica* and *R. reniformis*. Nematode infection had little impact on breadfruit growth with insignificant differences between inoculated and uninoculated plants, except for root weight in experiment 1. Breadfruit infected with *P. coffeae* had the greatest root weight ($p=0.0323$). Differences in nematode viability between experiment 1 and 2 may be accounted for by a combination of seasonal differences, pot environment, inoculation concentration, and tree age.

4.2 Introduction

One consideration that growers evaluate when selecting a crop is susceptibility to plant disease. Ragone (2006) and Taylor and Tuia (2007) reported that breadfruit trees are reputed to be relatively free of pests and disease. This may be true in cases of single specimen trees, but intensive cultivation of breadfruit crops may exacerbate pathogen problems and amplify pathogen damage. To meet a demand for breadfruit trees, a horticultural industry for mass propagation and distribution was established (www.globalbreadfruit.com). In 2011, over 5,000 breadfruit trees were distributed in the state of Hawai‘i for planting (Elevitch and Ragone, 2014), and 12,000 breadfruit trees, primarily the ‘Ma’afala’ cultivar were distributed through the National Tropical Botanical Garden Breadfruit Institute’s “Plant a Tree of Life - Grow ‘Ulu” project by 2016 (Ragone *et al*., 2016). A 4-ha breadfruit orchard incubator at Mililani Ag Park on Oah‘u planted in 2012, and a 1-ha ‘Ulutopia breadfruit orchard planted in 2014 on Kaua‘i are some of the most recent large scale plantings. This is not only the trend on Kaua‘i and O‘ahu; breadfruit trees are becoming increasingly popular statewide. Current breadfruit projects include: Ho‘oulu ka ‘Ulu, Breadfruit Harvest for Hunger, Breadfruit Phenology Project, Talking Trees: Learning from breadfruit in Hawai‘i, Hawai'i ‘Ulu Producers Cooperative, and Breadfruit vs. Potato. Annual Breadfruit Festivals held on Kaua‘i, Maui, O‘ahu, and the island of Hawai‘i continue to strengthen knowledge, cultivation, utilization, and consumption of breadfruit in Hawai‘i (Ragone *et al*. 2016). Much needed is better understanding of pathogens and diseases of breadfruit.

While information is available on fungal and bacterial diseases that affect the breadfruit tree crown and fruit, below-ground pathogens are not as well understood. Information on pathogenic nematodes associated with breadfruit is limited, but can be found in the form of nematological surveys of breadfruit and regional reports. Surveys are an accepted starting point to

study pathogens that cause damage to plants and are important in evaluating which nematode species could be pathogenic and virulent to breadfruit. In Hawai‘i, a survey of plant-parasitic nematodes associated with breadfruit found *Pratylenchus coffeae, Rotylenchulus reniformi*s, and *Meloidogyne* sp. Nematode presence in soil and roots of a breadfruit tree can serve as an indicator for potential damage from pathogens but does not define pathogenicity or virulence to the tree, nor does it prove that associated pathogenic nematodes are definitively damaging. Thus, as breadfruit production systems are expanding, it is essential that potential pathogenicity caused by nematode fauna on breadfruit trees be formally assessed. As breadfruit production systems expand throughout the state, an infectivity test to determine which nematode fauna are able to reproduce and affect breadfruit trees is lacking.

4.3 Objectives

The objectives of this study were to determine if *Meloidogyne javanica, Pratylenchus coffeae,* and *Rotylenchulus reniformis* could successfully reproduce on breadfruit trees and if an observable impact on tree health was measurable 6 months after inoculation.

4.4 Materials and methods

Nematode inoculum. The nematode inocula consisted of populations of *Meloidogyne javanica, Pratylenchus coffeae,* and *Rotylenchulus reniformis*. Pure cultures of *R. reniformis* and *M. javanica* were established from the eggs and juveniles of infected plants at University of Hawai‘i at Mānoa Magoon Research Station on O‘ahu. *Meloidogyne javanica* cultures were maintained on *Solanum lycopersicum* ‘Orange Pixie’ bush tomato. The nematode inoculum was separated from infected plants by shaking the *S. lycopersicum* roots in a 10% NaOCl solution in a sealed Erlenmeyer flask for 2 minutes, then pouring the solution over nested 250, 149, 44, and 20 µm-mesh sieves. Eggs and juveniles were collected on the bottom and rinsed with tap water. The

inoculum was adjusted to suspend 250 egg/ml and plants were inoculated 1000 eggs of *M. javanica*. *Rotylenchulus reniformis* stock cultures were maintained on cowpea (*Vigna unguiculata)* plants at the University of Hawai‘i at Mānoa Magoon Research Station. To remove *R. reniformis* from plants, *V. unguiculata* roots were shaken for 2 minutes in a 10% NaOCl solution contained within a sealed Erlenmeyer flask. Nematodes and eggs were rinsed in a 20-µm-pore sieve with tap water before being counted and adjusted to a suspension of 200 eggs/ml. Each plant was inoculated with 1000 eggs of *R. reniformis* per breadfruit plant.

Efforts to culture *P. coffeae* from specimens collected during the survey failed on a modified carrot disk method were unsuccessful (Moody, 1973). Therefore, vermiform *P. coffeae* were collected from breadfruit roots found in University of Hawai‘i at Mānoa Botany Garden and extracted via the Baermann funnel method. In order to obtain pure inocula, collected roots were surface sterilized in a 10% NaOCl and cut into 2-cm pieces and placed on a Baermann funnel filled with sterile water (Baermann, 1917; Walker and Wilson, 1960). After 24 hours at room temperature, 10 ml of solution was collected each day for 7 days and screened for *P. coffeae*. Using a Leica Wild MZ8 dissecting microscope, *P. coffeae* were consolidated on the counting dish and other nematode species were removed with a pick. The nematodes were adjusted to a concentration of 150 nematodes/ml and 900 vermiform nematodes were inoculated onto each breadfruit plant.

Plant material. Starter plugs of breadfruit cultivar‘Ma‘afala’ were obtained from Global Breadfruit, San Diego, CA. Soil free planting materials were certified nematode-free by the United States Department of Agriculture and Florida Department of Agriculture and Consumer Services. Upon arrival, breadfruit starters were transplanted into 4-L pressed paper-pulp pots filled with a water steam-sterilized silty clay soil and sand substrate mixture (1:1 soil:sand). Breadfruit plants

were placed on greenhouse benches and acclimatized for 180 (Experiment 1) or 360 (Experiment 2) days prior to inoculation.

Inoculation. Six or 12 months after transplanting, breadfruit plants were inoculated in the late evening by pipetting the nematode inoculume suspension into four 2-cm deep holes located 8 cm from the base of the plant in each pot. Similarly, control plants received distilled water. Plants were grouped by replication spaced at 60 cm between replicates and randomly within replications with100 cm between treatments to avoid cross contamination.

Plant measurements. Breadfruit measurements were recorded 2-5 days prior to infecting plants and 180 days after inoculation. Plant growth parameters taken prior to inoculation included: (i) stem diameter, (ii) plant height, (iii) plant weight, (iv) leaf size, and (v) chlorophyll levels. Pots were hand-watered twice daily and fertilized with an aqueous solution of Miracle-Gro Water Soluble All Purpose Plant Food (24-8-16) twice monthly. At the end of 180 days, the experiment was terminated, and stem diameter, plant height, plant weight, root weight, leaf weight, stem weight, and leaf chlorophyll levels were recorded. Fresh and dry weights were logged for roots, leaves, and stem.

Nematode reproduction. M. javanica, P. coffeae, and *R. reniformis* population density in the soil (P_f) was assessed at 180 days post inoculation. Nematodes were extracted using three methods: a root shake technique to disperse egg masses and nematodes from the host root surface, a modified Baermann funnel method to account for nematodes within roots, and an automated elutriator for nematodes present in the soil. Extraction of eggs and nematodes from infected breadfruit roots was completed from a modified protocol developed by Hussey and Barker (1973). Within a sealed Erlenmeyer flask, 30 g of chopped roots were submersed in a 10% NaOCl and shaken for 2 minutes. The liquid was then rinsed over nested 250, 149, 20-µm pore sieves.

Collected eggs and vermiform nematodes were subjected to sugar flotation and centrifugation (Jenkins, 1964) to clarify samples for counting. A Leica Wild MZ8 dissecting scope was used to count nematodes present. Motile nematodes were extracted using a modified Baermann funnel technique (Seinhorst, 1956). A fine mist was sprayed in 2-minute intervals over 10 g of chopped breadfruit roots placed in Kimwipe tissues and set in an unlined basket atop the funnel for 7 days. This time period allowed for hatching and exiting of nematodes from root pieces. Nematodes were collected daily and identified to genus on a Leica DMLB compound light microscope. Roots after mist chamber extraction or NaOCl extraction were then oven dried and weighed. Vermiform nematodes from the mist chamber were counted. Subsamples of 250 cm^3 soil from each experimental pot was utilized to extract nematodes using a semiautomatic elutriator (Barker *et al*., 1975) and centrifugation (Jenkins, 1964). The nematodes were counted and recorded. Total population of nematodes in a pot was calculated by adding the nematodes recovered from three extractions. The total population was then divided by the inoculation level to determine a Reproductive Factor (Rf).

Experimental design. Two experiments consisting of 4 treatments (nematodes and uninoculated) with 4 replicates per treatment were established in summer 2016 and winter 2016. Plants were maintained in the Magoon Research Station.

Data Analysis. Nematode and plant growth data were subjected to one way analysis of variance (ANOVA) using the general linear method (GLM) procedure in Statistical Analysis System (SAS Institute, Cary, NC). Changes in mean nematode population density and reproduction were separated using Tukey's test at $P < 0.05$ [SAS version 7 (TSP1)]. The differences between breadfruit plant growth parameters of inoculated and uninoculated plants were

determined using Tukey's test at $P < 0.05$. All data were tested for normality prior to ANOVA. Nematode numbers were log (x+1) transformed for ANOVA.

4.5 Results

Experiment 1

Only *P. coffeae* reproduced on the plants in the greenhouse. More *P. coffeae* were recovered from the roots than any of other nematodes (Fig. 4.1). *Pratylenchus coffeae* also had the highest population in the soil by *R. reniformis* and *M. javanica,* respectively. Nematode eggs were recovered from the roots of all treatments in experiment 1, with more eggs removed in those plants inoculated with *R. reniformis* than *M. javanica* or *P. coffeae* (Fig 4.2). The uninoculated plants also had a few eggs but these were likely from free-living nematodes. The Rf was significantly higher for *P. coffeae* in experiment 1, with inocula increasing 17-fold after 180 days (Table 4.1). *Meloidogyne javanica* and *R. reniformis* were able to reproduce, but not in numbers great enough to replace initial populations.

No visible symptoms were present at the termination of the experiment. Root galls and lesions, characteristic of infection by nematode pathogens were not present on breadfruit roots. Plant measurements from experiment 1 were not different between inoculated and uninoculated plants with the exception of root weight, but some trends could be seen (Figs. 4.3 and 4.4). *Pratylenchus coffeae* infected plants had the greatest root weight ($p=0.0323$) (Fig. 4.5). Breadfruit infected with *P. coffeae* had the first and second greatest dry leaf weight, dry stem weight, chlorophyll, height, and the third largest trunk diameter. Plants infected with *R. reniformis* plants had the second and third greatest values. Breadfruit infected with *M. javanica* had the lowest values except for height, for which it scored the highest value. The uninoculated plants had the greatest

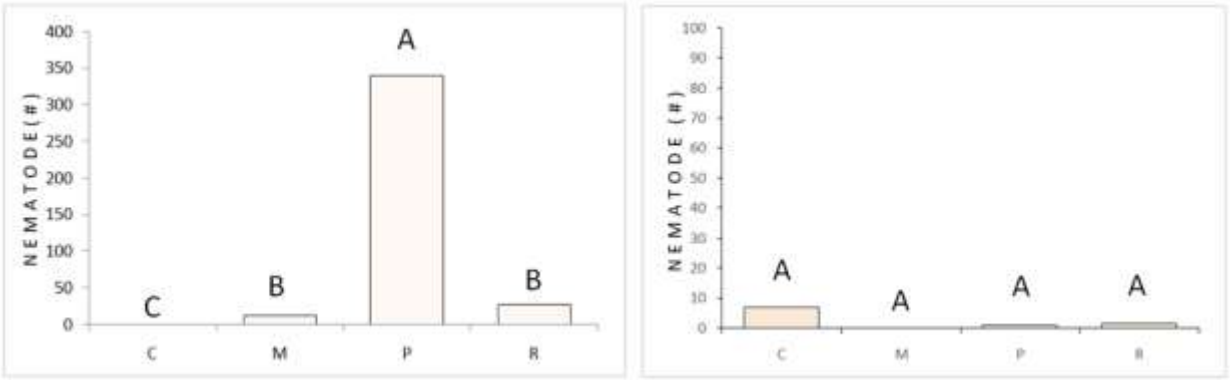

Figure 4.1: Egg and vermiform nematodes extracted from 30g of breadfruit roots inoculated with water (C), 1000 eggs of *Meloidogyne javanica* (M), 900 *Pratylenchus coffeae* (P), or 1000 eggs of *Rotylenchulus reniformis* (R) from experiment 1 (left) and experiment 2 (right) at 180 days post-inoculation. Analysis of variance was used to determine treatment significance, and treatment means were separated by the Waller-Duncan k-ratio *t*-test.

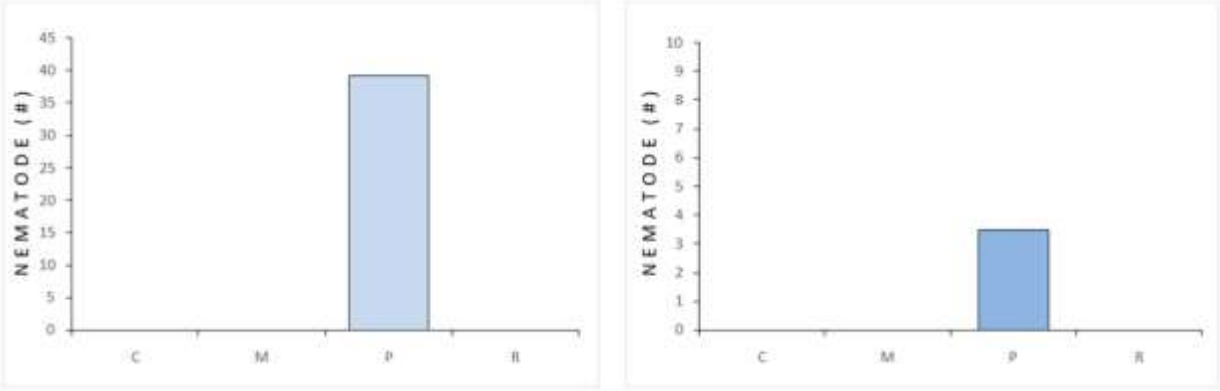

Figure 4.2: Vermiform nematodes extracted in mist chamber from 10 g of breadfruit roots inoculated with water (C), 1000 eggs of *Meloidogyne javanica* (M), 900 *Pratylenchus coffeae* (P), or 1000 eggs of *Rotylenchulus reniformis* (R) from experiment 1 (*left*) and experiment 2 (*right*) at 180 days post-inoculation. Analysis of variance was used to determine treatment significance, and treatment means were separated by the Waller-Duncan k-ratio t-test.

Table 4.1: Nematode population growth (P_f/P_i) on breadfruit 180 days after inoculation. Data are means of four replications. Means within a column followed by same letter(s) are not different according to Least Significant Difference (LSD) ($P < 0.05$).

	Experiment 1	Experiment 2
Meloidogyne javanica	0.40a	0.01a
Pratylenchus coffeae	17.83b	0.28a
Rotylenchus reniformis	0.34a	0.02a

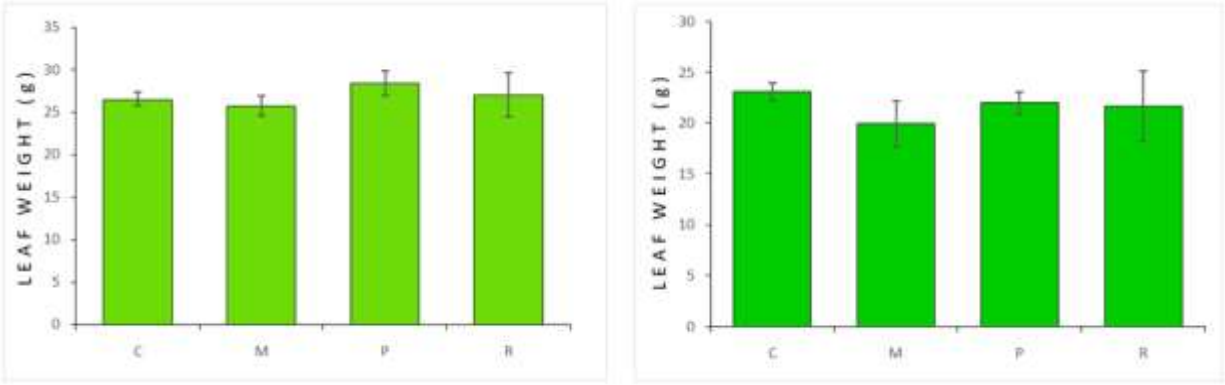

Figure 4.3: Dry leaf weights of breadfruit plants inoculated with water (C), *Meloidogyne javanica* (M), *Pratylenchus coffeae* (P), or *Rotylenchulus reniformis* (R) from experiment 1 (*left*) and experiment 2 (*right*) at 180 days post-inoculation. Means were separated using Tukey's test ($P<$ 0.05).

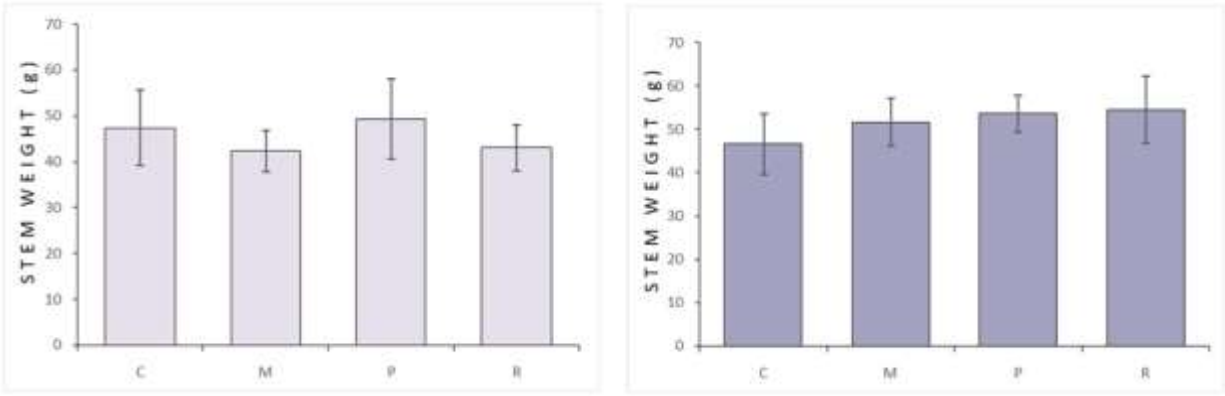

Figure 4.4: Dry stem weights of breadfruit plants inoculated with water (C), *Meloidogyne javanica* (M), *Pratylenchus coffeae* (P), or *Rotylenchulus reniformis* (R) from experiment 1 (*left*) and experiment 2 (*right*) at 180 days post-inoculation. Means were separated using Tukey's test (P <0.05).

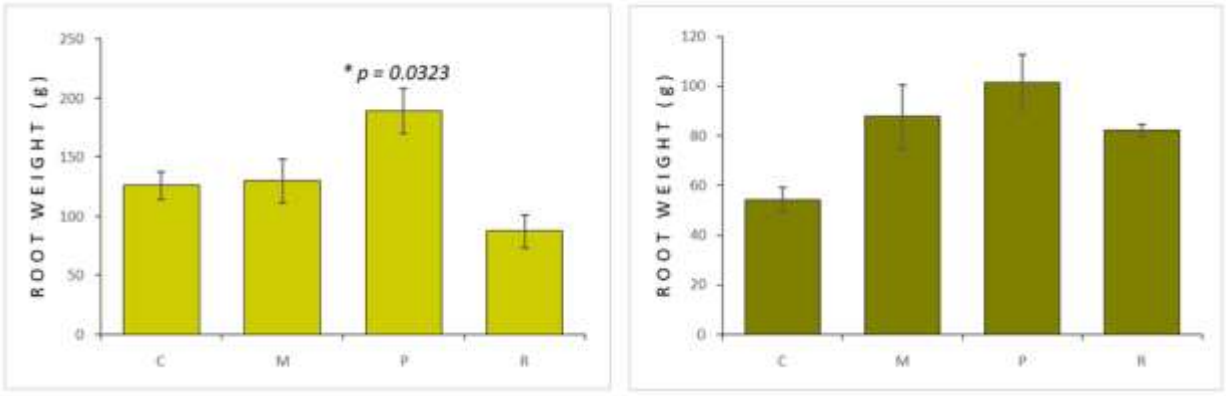

Figure 4.5: Dry root weights of breadfruit plants inoculated with water (C), *Meloidogyne javanica* (M), *Pratylenchus coffeae* (P), or *Rotylenchulus reniformis* (R) from experiment 1 (*left*) and experiment 2 (*right*) at 180 days post-inoculation. Means were separated using Tukey's test ($P < 0.05$).

range of values with the shortest heights, the largest trunk diameters and greatest dry leaf weights. The stem weight and chlorophyll content were in the middle compared to inoculated plants.

Experiment 2

Nematode reproduction differed between experiment 1 and experiment 2. Very few plant-parasitic nematodes were recovered in the second experiment (Fig 4.2). Contamination by free-living nematodes was more pronounced in experiment 2. None of the nematodes had a Rf greater than one (Table 4.1). Despite low Rf values, the trend indicating *P. coffeae* was the most capable of reproducing on breadfruit when compared to *R. reniformis* and *M. javanica* was consistent.

No root galls or root lesions were observed on breadfruit roots when the experiment was terminated. Plant measurements from experiment 2 lacked significant differences, but like experiment 1, a trend towards a higher root mass was visible (Fig. 4.5). Measurements including dry leaf weight (Fig. 4.3), dry stem weight (Fig. 4.4), chlorophyll, height, and trunk diameter had differing values for all of the treatments, however the control had the lowest measurements for height, total weight, dry root weight, and dry stem weight and *R. reniformis* had the highest values for height, dry stem weight, and chlorophyll.

4.6 Discussion

Despite low frequency and modest abundance of *Meloidogyne* on surveys and lack of symptoms in Hawai‘i, we found it warranted to test if *Meloidogyne javanica* could reproduce and damage breadfruit based on reports from Jamaica (Coates-Beckford and Pereira, 1992), Samoa (Grandison, 1996) and galls produced on breadfruit in Venezuela (Crozzoli, 2009). Heavy galling by *Meloidogyne* along the tap, lateral, and feeder roots averaging 15mm in size was cited by Razak (1978) on breadfruit, but was not apparent on breadfruit roots sampled in Hawai‘i. Based on

pathogenity results, *M. javanica* is not virulent to breadfruit. Breadfruit is a poor host to *M. javanica*.

Rotylenchulus reniformis has been associated with breadfruit in American Samoa (Grandison, 1996) and Jamaica (Coates-Beckford and Periera, 1992). With its wide distribution and broad host range (Robinson *et al*., 1997), it is not surprising that it was also found associated but breadfruit in Hawai‘i in low abundance (Chapter 2). Low levels of infection by *R. reniformis* was consistent in experiment 1. *Rotylenchulus reniformis* appears capable of reproducing on breadfruit at low levels, but breadfruit cultivar Ma‘afala is a poor host to *R. reniformis.*

The results confirm previous reports by Coates-Beckford and Periera (1992) indicating breadfruit was a good host to *P. coffeae*. However, the lack of significant plant data, with the exception of root weight where *P. coffeae* had a higher root weight, did not fall in line with symptoms reported from trees infested with *P. coffeae* (Coates-Beckford and Periera, 1992). In reviewing the work of Coates-Beckford and Periera (1992), tree age greater than 20 years was a significant factor in slow decline disease. Interestingly, the two sites with the highest populations of *Pratylenchus* on O‘ahu and Maui were from established trees in botanical gardens. Trees were likely greater than 20 years old. However, because collection methods aggregated multiple trees on a given site together, it is uncertain specifically from which of the trees that the nematodes were extracted. Nonetheless, any evidence of pests or pathogens present on breadfruit trees were duly noted including chlorosis, mid-day wilting, spider mites, scale, or mealy bugs, and no pests or symptoms were noted for these two *Pratylenchus*-concentrated locations.

Although pathogenic nematodes generally cause reduced plant growth, several researchers found low populations of pathogenic nematodes can enhance host growth (Lownsbery and Peters, 1955; Olthof and Potter, 1973) and were implicated in enhanced growth of root systems parasitized

(Chitwood and Buhrer, 1946). This stimulatory effect was often short-lived, but may have accounted for the higher dry root weights of *P. coffeae* inoculated breadfruit trees. Also likely was that a 180-day incubation period was not adequate to exhibit symptoms of slow decline disease. Lack of other significant differences in plant measurements could have been because nematodes did not occur in populations high enough to cause damage or that host plants were tolerant to nematodes tested.

Suppression of nematode growth in experiment 2 was likely limited by the greenhouse temperature, pot environment, and tree age. These elements may have characterized lower nematode reproduction between experiment 1 and 2. The greenhouse temperature may have been a factor in reduced nematode reproduction. Radewald *et al*. (1971) and Yokoo and Kudoka (1966) observed differences in the reproduction of various nematode species feeding in different crops when exposed to variations in temperature. Other studies found the duration of the lifecycles of nematodes to be temperature dependent and that heat up to 30ºC allowed tropical species to more quickly complete their lifecycle (Moens *et al*., 2005; Tuyet *et al*., 2013), while temperatures greater than 35ºC inhibited reproduction (Slack and Hamblen, 1961; Rebois *et al*., 1970). An upper temperature limit for life cycle completion was between 31 and 34.7°C on *Meloidogyne incognita* and *M. javanica* (Trudgill, 1995; Ploeg and Maris, 1999). Additionally, *Pratylenchus penetrans* hatched more at 20°C than at 25°C (Pudasaini *et al*., 2008). The day/night temperature at Magoon Research was 28/22°C measured at experiment 1 completion in December, and 31/24°C at experiment 2 completion in May.

Breadfruit growth may have varied between experiments with height differences between pre- and post inoculation more amplified in experiment 1 as a result of pot size. In experiment 2, root bound breadfruit trees had less differences in growth over the course of the experiment.

Despite a continuous supply of nutrients, it has been noted that pot-bound plants experience substantially restricted growth which affects shoot and root growth (Krizek *et al*., 1985; Poorter, 2012). An additional 180 days of growth in the original pots (360 days after planting) in experiment 2 may have accounted for growth differences between experiment 1 and 2. Impeded root growth, a result of being rootbound, disfavors nematodes that rely on root tips by reducing availability of feeding sites. Biological constraints of more roots and less soil per pot may have further contributed to the lack of nematode activity observed in experiment 2.

Breadfruit growth variations between experiments may have been partly attributable to differences in greenhouse temperatures at different times of the year in addition to duration after planting breadfruit tree plugs in soil (180d, 360d). Last, it should be mentioned that contamination by free-living nematodes was evident in experiment 2, as vermiform free-living nematodes were present in soil and roots from the control. Eggs of free-living and plant-parasitic nematodes were not differentiated. Thus, uninoculated host plants from experiment 2 yielded the highest number of eggs from the root shake, even though only vermiform *P. coffeae* were recovered from *P. coffeae* inoculated plants across all treatments.

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Chapter 5: The Future of Breadfruit

5.1 Concluding remarks

Plant-parasitic nematodes associated with breadfruit in Hawai‘i were consistent with previous in other breadfruit growing areas (Sharma, 1976; Hutton, 1976; Orton Williams, 1980; Grandison, 1990, 1996; Grandison *et al.*, 2009; Kirby *et al.*, 1980; Coates-Beckford and Periera, 1992). Commonly occurring plant-parasitic nematodes include *Helicotylenchus, Meloidogyne, Pratylenchus,* and *Rotylenchulus* in Hawai‘i. Identifying which nematode genera and species are associated with breadfruit through surveys is a first step towards establishing pathogenicity of specific nematodes to breadfruit.

An efficient host supports a higher population of parasitic nematodes than the population of parasitic nematodes supported by a poor host. Conversely, a non-host plant is a very poor host where nematodes fail to live, multiply, or face early death (Seinhorst, 1967). Nusbaum and Barker (2012) characterized host plants by “their relative suitability as a substrate for the parasite and relative vulnerability to damage,” a definition which seems subject to interpretation rather than having a hard and fast definition. According to Khan (2005), the concept of host status is greatly variable. During pathogenicity studies, we considered nematode pathogens that had a reproductive factor (P_f/P_i) greater than one as hosts, while nematodes reproductive factor values less than one but not zero were poor hosts.

Breadfruit was a host to *Pratylenchus coffeae* in greenhouse studies. Anecdotally, because *Pratylenchus coffeae* was the only nematode recovered from the roots at University of Hawai‘i at Mānoa Botany Garden after surface sterilization during root collections over a period of ten months for inoculum preparation for pathogenicity tests, a 17-fold reproduction replacement rate was not unexpected from a seemingly unfailing supplier of *P. coffeae*. Surveys of nematodes on

breadfruit in Jamaica (Coates-Beckford and Periera, 1992) and Brazil (Sharma, 1976) and regional reports from Samoa (Grandison, 1996) and Venezuela (Crozzoli, 2009) that identified *Pratylenchus* spp. associated with breadfruit reinforce the importance of surveys as a first step in determining nematode pathogenicity. Despite their importance in determining which plant-parasitic nematodes can infect breadfruit, surveys are no substitute for pathogenicity tests.

In the case of *M. javanica* and *R. reniformis* on breadfruit, reproductive factors for each species was just over a third, despite being discovered on breadfruit surveys in Samoa (Grandison, 1996) and Jamaica (Coates-Beckford and Periera, 1992), and breadfruit called "very susceptible to *Meloidogyne* attack" (Razak, 1978). In spite of a low reproductive factor, nematode recovery was uniform across all treatments in experiment 1 allowing a conclusion to be made that breadfruit is a poor host to *M. javanica* and *R. reniformis.*

The appearance of plant roots was normal at the end of the experiments. Symptoms of fewer and smaller leaves, leaf chlorosis, reduced fruit production, premature fruit drop, dieback of branches, and tree death were observed in Jamaica (Coates-Beckford and Periera, 1992). Coates-Beckford and Periera (1992) cited tree maturity as a significant factor of infection by *P. coffeae*, with breadfruit trees older than 20 years as most heavily infected. Although not symptomatic, the two locations with the highest concentration of *Pratylenchus* spp. were of the oldest trees surveyed, both at botanical gardens. In hindsight, a 180-day inoculation period may not be enough time to exhibit host symptoms of slow decline.

Damage by nematodes invariably remains hidden by many other factors especially the presence of multiple biotic and abiotic stress factors operating simultaneously on the crop. Plant-parasitic nematodes are easily and often misdiagnosed, thus it is not unexpected that nematodes are rarely considered as major limiting factors to crop production and frequently neglected (Bridge,

1996). When we are lucky enough to find information on pathogens in non-major crops such as breadfruit, plant nematodes are rarely mentioned (Raabe, 1981). That breadfruit is a confirmed host of an endoparasite should raise quarantine concerns, based on a history of movement of tropical and temperate plant-parasitic nematode species into new uninfested areas. Dissemination of burrowing nematode (*Radopholus similis*) and root lesion nematodes (*Pratylenchus* spp.) to nearly all areas where banana crops are grown is the result of lack of quarantine guidelines that address sharing of pathogens globally (Gowen, *et al*., 2005). Fortunately, the advent of micropropagation using mature axillary shoot buds (Murch *et al*, 2008) solves nematode quarantine issues on breadfruit, as most parasitic nematodes reside in or on roots.

However, practicality often outweighs prudence in Hawai‘i, and all too often breadfruit growers continue to opt for root shoots or root cuttings to avoid mass orders, shipping costs, and agricultural regulations for plant importation connected with the purchase of breadfruit propagules from out-of-state. Sourcing new plants from root shoots or root cuttings can transmit plant-parasitic nematodes to new planting areas, thus it is emphasized that *in vitro* breadfruit propagation material free of nematodes be transplanted when feasible. Despite results during these studies that might suggest that specific pathogenic nematodes do not cause damage to breadfruit, it is recommended that growers exploit different breadfruit cultivars and practice intercropping with non-host plants in breadfruit orchards. Solarization prior to planting and after planting has also proved successful in breadfruit orchard settings (Coates-Beckford and Periera, 1994).

Last, it should be mentioned that a small amount of white latex sap was often visible during root collection of woody breadfruit roots larger than 1 cm in diameter. Although milky sap was not visible in smaller roots, those that were woody released an imperceptible amount of sap which left a distinct gluey film on pruning shears and fingers during root processing. Sap and associated

stickiness was absent from fleshy feeder roots. In studying lactifers on breadfruit, Harvey (1999) noted that younger tissue from breadfruit had a higher liquid:congealant ratio in latex, and the rather clear liquid component was tacky. Laticifers are a type of elongated secretory cell that produce latex and rubber as secondary metabolites (Mahlberg and Sabharwal, 1968) and are present in all plants belonging to the family Moraceae including breadfruit. Milky sap is present in all parenchymatous tissues of the tree, including plant roots. According to Mahlberg (1993) laticifers are harder to see in the roots than other parts of plants that contain them. One of the evolutionary functions of specialized cells in laticifers is for the defense of plants against pests. Numerous studies have linked plant latex and associated metabolites of laticifers with defense against insect herbivores (Konno *et al*., 2004; Ramos *et al*., 2007; Helmus and Dussourd, 2005; Hagel *et al.*, 2008). Studies by Langenheim (2003) and Pickard (2008) postulate that glue-like exudates produced by lactifers are a plant defense mechanism that works by coating the mouthparts of foraging herbivores and sealing offplant wounds from pathogens. Studies are not available on interactions or effects of laticiferous roots with below-ground pathogens.

It would be of interest to determine if plant latex and associated metabolites of laticiferous breadfruit roots secreted during root damage reduce host desirability to pathogenic nematodes. Increased research on interactions of *P. coffeae* with pathogenic fungi and phytoparasitic bacteria correlated with declining breadfruit as outlined by Coates-Beckford and Periera (1992) might also provide a more holistic approach to the disease complexes that affect breadfruit. Furthermore, an investigation into how breadfruit-specific arbuscular mycorrhizae mentioned by Hart *et al.* (2014) affect nematode populations could broaden our understanding of how underground communities affect breadfruit trees above. Human migration based on DNA fingerprinting of plant-parasitic nematodes is yet another unexplored area. Since breadfruit dispersal throughout the Pacific was

human-mediated though root cuttings, divergence of nematode isolates that occur in roots and rhizosphere of breadfruit trees could prove useful in supporting evidence about human migration paths through Oceania as phytonematodes on breadfruit trees were relocated during colonization. Future studies are also recommended to test pathogenicity of *Meloidogyne* spp., *Pratylenchus* spp. and *Rotylenchulus reniformis* to Ma'afala cultivars in different time intervals and examine the host ranges of popular cultivars like the Hawaiian "Ulu', 'Otea' and 'Ulu Fiti.'

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