Journal of Experimental Biology and Agricultural Sciences

<http://www.jebas.org>

ISSN No. 2320 – 8694

PLANT PARASITIC NEMATODES ASSOCIATED WITH *Annona squamosa* L.

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Received – September 01, 2020; Revision – November 22, 2020; Accepted – December 15, 2020

Available Online – December 30, 2020

DOI: [http://dx.doi.org/10.18006/2020.8\(6\).818.827](http://dx.doi.org/10.18006/2020.8(6).818.827)

KEYWORDS

Annona squamosa

Plant Parasitic Nematodes

Rotylenchus

Criconemoides

Meloidogyne

ABSTRACT

The current study was conducted from December 2018 to January 2019 to identify plant-parasitic nematodes (PPNs) associated with *A. squamosa* in Rizal Province, Philippines. A total of 10 soil and root samples were collected from the surveyed plants. Nematodes from the collected soil samples were extracted using the modified Baermann tray method while root nematodes were extracted using H₂O₂ incubation. Results of the study revealed the presence of *Meloidogyne*, *Rotylenchulus*, and *Criconemoides* PPNS genera from the roots and soil samples. The study revealed that the number of nematodes associated with *A. squamosa* is not enough to cause infestation and damage. Further, various environmental factors such as moisture and potassium content of the soil, temperature, and pH make the conditions unfavorable for the proliferation of plant-parasitic nematodes in the study area.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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1 Introduction

Annona squamosa L. locally known as Atis is a fruit tree, belongs to the family *Annonaceae*. It is an evergreen plant mainly located in tropical and subtropical regions, including the Philippines (Narwade & Aher, 2019). It has a long history of ethnomedicinal uses such as tonic, apophlegmatisant, abortient, heart sedative, anticancer, antioxidant, antidiabetic, antihypertensive, hepatoprotective, antiparasitic, antimalarial, insecticidal, microbicidal, and molluscicidal activities (Sharma & Goray, 2009; Saha, 2011; Narwade & Aher, 2019). The fruits are also extensively used to prepare desserts and beverages.

Different crops have different parasites and some of which are the plant parasitic nematodes (PPNs). Nematodes are microscopic organisms that are considered as one of the pests of different crops. These are considered to be very diverse since many occupy all parts of the earth. They are also very hard to identify since they are very minute. In a global context, there is increasing demand for nematode taxonomists to assess community structure concerning soil function, to develop new tools for agronomic management, and to address quarantine regulations.

Plant parasitic nematodes are among the most important agricultural pests and cause serious crop losses worldwide. Nematodes mostly inhabit the soil and attack the underground parts of the plants that's why the management of nematodes is more difficult than that of other pests. Chemical nematicides are effective, easy to apply, and show rapid effects, but they have begun to be removed from the market in some developed countries because of public health and environmental safety concerns (Tian et al., 2007).

The plant parasitic nematodes associated with *A. squamosa* are not yet identified. Hence, this study was formulated with the aim of Identify plant parasitic nematodes genera associated with the roots and soil of *A. squamosa*. This identification of PPN was completely based on their morphological characteristics (size, shape, and body structure). Further, the effect of soil texture and its nutritive value concerning pH, nitrogen, phosphorus, and potassium on plant parasitic nematode occurrence was also studied in the present study. The morphological identification of the plant parasitic nematodes associated with *A. squamosa* can significantly contribute to the field of agriculture research because these plant parasitic nematodes could destroy the crops and affect the yield will be identified. Thus, this study could lead to proper management practices in controlling plant parasitic nematodes associated with *A. squamosa*.

2 Materials and Methods

2.1 Description of the Area of Study

The roots and soil sample collection of *A. squamosa* was carried out from the Sarian Farm, located at Teresa, Rizal, Philippines.

The farm specializes in propagating superior varieties of fruit trees and some selected crops. In the area of collection, some trees of *A. squamosa* exhibited signs of infestation such as wilting and chlorosis of leaves.

2.2 Sample Size and Sampling Technique

Five *A. squamosa* plants that are showing nematode infestation symptoms such as chlorosis and wilting of leaves were selected for the collection of the roots and soil subsamples (Xavier-Mis, 2016). Among these, three were mature and the other two plants were at the seedlings stage. The collection of soil samples was carried out around the drip line of each tree by removing 2 cm of the above soil using a trowel and probing the soil with a depth of 15 – 30 cm using a soil auger. Ten soil subsamples were collected from each tree, mixed, and put in zip lock bags. The fibrous roots were collected within the depth of 15-30 cm using a trowel around the drip line of each tree. These subsamples were stored together in a plastic bag with a seal, together with a little amount of soil to maintain its freshness (Abawi & Gugino, 2007; Xavier-Mis, 2016). After the collection, the plastic bags with samples were labeled clearly and systematically with the sample number or reference using a permanent marker and placed in a cool box. It was put in a cool shaded area since nematodes are very sensitive and perishable, thus, appropriate care must be taken to keep them in good condition (Nambiar & Wainer, 2011).

2.3 Nematode Extraction from Soil Samples using the Baermann Tray Method

For each soil sample, five replicates were prepared. One hundred cc soil samples were put in a wire mesh with 2ply tissue papers. After that, it was placed in a bowl with water. The bowl was covered with plastic to reduce the evaporation of water and was left for 3 days. After incubation, the suspension was collected and poured in stacks of sieves which are the 80, 325, and 400 mesh from top to bottom. Afterward, the soil nematode suspension was collected from the terminal sieve by concentrating the particles on one side and carefully poured into the glass vial. Then, nematodes were checked using the dissecting microscope (Ferris et al., 2004)

2.4 Nematode Extraction from Root Samples using Incubation in H₂O₂

The root samples were cut into pieces and were placed in a bottle with 100 ml of 1% of H₂O₂ and covered tightly. Then, it was incubated for 48 hours at room temperature. Afterward, all the solutions were poured on 225 mesh over 400 mesh sieves and was rinsed thoroughly. Then, the nematode suspension from the terminal sieve was collected and used for identification (Ferris et al., 2004; Coyne et al., 2007)

2.5 Preparation of Fixative, Mounting and Fixation

For fixing isolated nematode species, a fixative medium was prepared by mixing 100 mL of 40 percent formalin, 10 mL of glycerol, and 450 mL distilled water. This setup was done twice because the other one was used in producing the mounting medium. Once mixing is done, one of the finished solutions was poured into an amber bottle for storage. The remaining setup was continued by adding 560 mL distilled water to produce a 1X strength formalin-glycerol which was later used as a mounting medium. After mixing, it was also poured in a 1 L amber bottle for storage to avoid any reactions (Abawi & Gugino, 2007; Coyne et al., 2007).

For nematode fixation, nematode suspension was collected in a vial and these vials were immersed in a water bath for 40 seconds. The vials were removed and an equal amount of 2x fixative solution was added to the nematode suspensions. The vials were covered and left for 24 hours (Abawi & Gugino, 2007; Coyne et al., 2007).

Before mounting, the glass slides were placed in a ringer to make an even thickness ring to avoid smashing the nematodes to be mounted using nail polish. The nail polish ring was air-dried for it to harden before adding the mounting medium. When the nail polish ring hardens, a drop of mounting medium was placed in the center of the nail polish ring. On the other hand, the fixed nematode suspension was poured into an agar plate and it was examined under a compound microscope to find the target nematode using a low magnification and verify it at high magnification. The magnification was shifted back to the low to pick a nematode (Abawi & Gugino, 2007; Coyne et al., 2007).

Nematodes were picked with the help of a nematode picker, for this the medium surrounding the nematode was agitated so that the nematode can be picked easily. The picked nematodes were placed at the center of the drop of mounting medium of the glass slide. After ensuring the mounted nematode, it was covered using a coverslip and sealed with the nail polish. After sealing, it was labeled on both ends using slide labels with its identity, and the number of mounted specimen. On the other end, it was labeled indicating the name of the collector, plant, and the place of its collection (Abawi & Gugino, 2007; Coyne et al., 2007).

2.6 Morphological Identification

The morphological identification of plant parasitic nematodes associated with *A. squamosa* was done by using the scanner, LPO, HPO, and the oil immersion objective of the *Euromex* light microscope. The observations were recorded and photographs 100x were also taken. The “Identification Key for Agriculturally Important Plant-parasitic Nematodes Prepared for the International

Nematode Diagnosis and Identification Course”, Eskisehir, Turkey (2012), A Manual for Nematology (Mengistu et al., (2013) and the “Plant Parasitic Nematodes in Subtropical and Tropical Agriculture”(Luc et al.,2005) was used for the identification of these plant parasitic nematodes. Expert advice was also done by Mr. Romnick A. Latina, an expert in nematology and a professor at the University of the Philippines, Los Baños.

2.7 Plant Parasitic Nematode Population Count

A clear plastic dish with grids imprinted on the bottom was used as the counting dish. The plant parasitic nematodes solution was transferred into the counting dish carefully, avoiding splashing. The nematodes present were counted in the total suspension volume in the counting dish in a systematic way following the gridlines on the dish. The counted PPN's suspension was returned on the vial. The procedure was repeated for all the suspension samples (Coyne et al., 2007).

2.8 Soil Analysis

The soil sample was brought to the Bureau of Soils and Water Management in Quezon City for the analysis of its pH, nitrogen, phosphorus, and potassium content of the soil. Soil samples were analyzed using the colorimetric method for N, P, and K determination while pH was analyzed using a pH meter (Regalado & Dela Cruz, 2016).

3 Results and Discussion

3.1 Morphological characteristics of plant-parasitic nematodes

Three genera of the nematodes were identified from the soil and root samples of *A. squamosa*. The major identified genera are *Criconemoides* (ring nematode), *Meloidogyne* (root-knot nematode), and *Rotylenchulus* (reniform nematode). Further, nematodes species of the family *Criconematidae* feed ectoparasitically on common plant hosts such as grasses and woody perennial plant roots, wherein some are at root tips, others in more mature regions of the root, including areas where the root surface is disrupted by lateral root emergence

In this study, only female species were found due to the rarity of the male species, these results are in agreement with previous findings (Jaffee et al., 1989; Ferris et al., 2004). The entire female species that belong to *Criconematidae* is stout, sausage-like shape to cylindrical, with a length that ranges from 104.57 µm to 107.86 µm long and a width of 25.86 µm and is ventrally arcuate after heat relaxation, which can be seen in Figure 1A and 1D. This characteristic makes it distinguishable from any other plant parasitic nematodes. *Criconemoides* female species has a retrorse annulus with finely crenate edges, scales, or spines which is seen in all the figures 1A-D. The stylet is robust with an anchor-shaped

Figure 1 *Cricnemoides* observed under OIO. (A and D) entire female; (B) female labial region; (C) posterior end of the female; (D) entire female showing the vulva (blue arrow) and stylet (black arrow)

knob which is greatly seen in Figure 1D. It has also a long stylet which allows penetration and feeding on cells, several layers deep at the root tip or in the root cortex. Their labial area is about 12.84 μm and is not well separated from the rest of the body which is marked by one or two thinner annules. It is also variously shaped and its sclerotization is strong, as seen in Figure 1B. Their esophagus is not visible due to thick annulations. The vulva is located posterior with one genital tract, extending anteriorly (Hoffman, 1973; Ferris et al., 2004; Olson et al., 2017).

The *Heteroderidae* family is a family of plant-parasitic nematodes that are composed of sedentary obligate parasites of roots which leads to the formation of galls in roots in some cases. Among the genera that belong in this family, only juvenile *Meloidogyne* was found which was reported as the most common and destructive plant-parasitic nematode that greatly influence both the quality of the crop and crop yield (Bellafiore et al., 2015)

The juvenile *Meloidogyne*, as seen in Figure 2A is vermiform in shape and is about 388.95 μm in length and is 15.907 μm in width, as seen in Figure 2D. The anterior region, as seen in figure 3D, clearly shows the metacarpus, which is pointed by the black arrow, is one of the distinguishing characteristics of plant-parasitic nematodes from free-living nematodes, and the nerve ring which is pointed by the yellow arrow. The stylet of these juveniles is short and moderately sclerotized which is about 13.161 μm , and their labial region skeleton is weakly sclerotized (Figure 2B). The tail of the juvenile *Meloidogyne* species is long, conical-shaped with an irregular tip in outline, as seen in Figure 3C, is about 55.29 μm and is internally clear. However, mature females are not observed in the study because the only passive method was used to extract these plant parasitic nematodes which are only capable of extracting motile plant-parasitic nematodes to avoid the risk of sample destruction (Hunt et al., 2005).

Figure 2. *Meloidogyne* species observed under HPO (A) and OIO (B). (A) Entire juvenile; (B) head region showing the stylet (black arrow)

Figure 3 *Meloidogyne* species observed under HPO (C, D). (C) posterior part of the juvenile; (D) Anterior part of the juvenile showing metacarpus (black arrow) and nerve ring (yellow arrow)

Figure 4. *Rotylenchulus* observed under HPO (A,B,D) and OIO (C). (A-B) entire immature female; (C) head region showing the stylet (black arrow); (D) entire male

The family *Hoplolaimidae* is a family of plant-parasitic nematodes that are considered as obligate higher plant parasites with a migratory ecto-endoparasitic habit, and sometimes semi-endoparasites. Members of the family are vermiform-shaped nematodes which turn into kidney-shaped upon adulthood (Ferris et al., 2004; Subbotin et al., 2015). In this study, only male species and immature vermiform females were found.

The immature female of *Rotylenchulus spp.*, as seen in the Figures 4A and 4B, has a vermiform and small body with a length that is about 242.59 µm to 675.23 µm, and a width of about 23.75 µm to 11.51 µm, respectively, which are ventrally arcuate. The labial region is continuous with body contour which is rounded, striated and has a rounded outline and their labial sclerotization is medium (Figure 4C). This figure also shows the stylet, which has a length of 23 µm and is classified as medium strength, with rounded basal knobs. The esophagus is not visible in all the *Rotylenchulus sp.* that has been observed. Figure 5G shows the tail of an immature female with a length of 109.46 µm, that is conoid with rounded terminus and the vulva (red arrow) which is situated posteriorly with not

protuberant vulval lips and has two genital tracts, each with a double flexure. Figure 5.2E and 5F show the entire juvenile which resembles an immature female, but shorter and lacking vulva and genital tracts. It has a length of 207.44 µm and a width of 11.55 µm. The tail has a length of 34.22 µm. Figure 4D shows the entire male with a length of 465.36 µm and a width of 30.42 µm, which also has a vermiform body. The labial sclerotization is also medium developed. The stylet and esophagus of the male are not visible. Figure 5H shows their spicule that is located ventrally in the tail and is curved. The tail of the male is pointed (Eisenback, 2002; Nakasono, 2004; Hunt et al., 2005).

3.2 Total count of plant-parasitic nematodes of *A.squamosa*

Table 1 shows the population count of plant parasitic nematodes found in both soils and root samples. In soil samples, *Criconemoides* had the highest number (51), followed by *Meloidogyne* (12) and *Rotylenchulus* (2). Further, in roots samples, *Criconemoides* also had the highest number (42), followed by *Rotylenchulus* (13) and *Meloidogyne* (4).

Figure 5 *Rotylenchulus* observed under OIO (E,F) and HPO (G,H). (E) entire juvenile; (F) entire juvenile showing the tail; (G) posterior part of the immature female showing the vulva (red arrow) and anus (blue arrow); (H) posterior end of male showing the spicule (black arrow).

Table 1 Total count of the genus of plant-parasitic nematodes found associated with the soil and roots of *A. squamosa*

NEMATODE GENERA	COUNT IN SOIL SAMPLES (6.9 pH)	COUNT IN ROOT SAMPLES	TOTAL COUNT
Criconemoides	51	42	93
<i>Meloidogyne spp.</i>	12	4	16
<i>Rotylenchulus spp.</i>	2	13	15

Plant-parasitic nematodes are counted to identify the groupings and number of PPNs with the crop growing in the area and the soil characteristics. Also, this is an attempt to evaluate the occurrence and economic importance of these plant-parasitic nematodes associated with *A. squamosa* (Subbotin et al., 2015). As reported by Zehr et al. (1986), there are around 250 Criconemellas/100 cc of soil on its initial count and around PPN's in 100 cc of soil (Miller, 1980) which create damages on crops. These previous results are contradictory to the findings of the current study, where PPN's were found less than 100/100 cc.

3.3 Texture, pH, Nitrogen, Phosphorus, Potassium Content of Soil in the Sampling Site

The collected samples from the sampling site in Teresa, Rizal were reddish-brown and classified as silty clay due to its creamy and

soft texture. These soils are considered marginal for agriculture because this type of soil is acidic, which has low cation exchange capacity, and low water holding capacity (Needelman, 2013). In the current study, three soil samples were sent for analysis to the Bureau of Soils and Water Management, among these, the first sample was collected from this study site while samples 2 and 3 were from the study and used as a reference for the comparison of soil sample characteristics (Table 2). All three samples were acidic soils, characteristics for all the soils in the whole province of Rizal (Pouyat et al., 2007; Carating et al., 2014; Carpio et al., 2017;).

Nutrients in soil play a very important role not just in plants but also in other living organisms. As seen in Table 2 sample 1 has moderately low nitrogen content, sample 2 has low nitrogen content while sample 3 has high nitrogen content. The low to a

Table 2 Soil analysis of the soil samples from Sarian Farm, Teresa, Rizal, Philippines. December 2018.

Soil Sample	pH	Nitrogen	Test Results Phosphorus	Potassium	Texture Feel
1	6.9	Moderately low	High	Sufficient	Heavy
2	5.0	Low	Moderately high	Sufficient	Light
3	6.5	High	Medium	Sufficient	Heavy

Table 3 Standard soil data in Teresa, Rizal, Philippines

Soil Sample	pH	Nitrogen	Test Results Phosphorus	Potassium	Texture Feel
Standard	7	High	Moderately High	Sufficient	Light

moderately low level of nitrogen in soil indicates that the soil is low in organic content and acidic because the change in nitrogen indicates changes in soil pH (Lamb et al., 2014). The high nitrogen level in acidic soil, on the other hand, might indicate that it was recently added with fertilizers and there is a high amount of other nutrients such as phosphorus and potassium. In terms of phosphorus contents, the medium to high levels of phosphorus is because of the animal manures which were reported from the sampling site. According to Liu et al. (2013), animal manures are a rich source of phosphorus which might greatly affect the content of the acidic soil from the sampling site. In terms of sufficient levels of potassium in all three soil samples, it can be explained by the presence of clay particles in the samples which traps the small molecules of potassium. It is also sufficient in soil because small clay and humus particles which can be seen in the sampling site and the samples themselves develop negative electrical charge particles attract positively charged ions, or cations, which include potassium (Stack, 2016). In terms of the texture of the soil samples, it just depends on the location inside the sampling site, where some have wet silt clay and some have dry silt clay which is a characteristic of a soil that can be found in the Teresa series in Rizal province (Carating et al., 2014; Carpio et al., 2017;).

As compared to the sampling site soil (Table 3), the standard soil has a neutral pH of 7, high nitrogen, moderately high phosphorus, sufficient potassium content and a light texture in the feeling method (Carating et al., 2014; Carpio et al., 2017).

3.4 Association between the soil characteristics and plant-parasitic nematodes occurrence

Results of the study suggested that for the genus *Criconeimoides*, moisture fluctuations are said to be a major limiting factor. The reduction in the numbers of the genus under this family has been observed due to the rapid changes in moisture levels due to the higher temperatures. The optimum temperature for reproduction may vary with the host plant, as well as the genus under this family, evidence indicates that the range of 22 to 26°C is the best.

The pH of the soil sample is 6.9. It has been reported that pH 7.0 was significantly better for population increase. It has also been reported that lighter sandy soils are better than heavier clay soils in supporting nematode populations. *Criconeimoides* had the highest count among the other plant parasitic nematodes in the soil samples. However, comparing with the number of *Criconeimoides* reported in other studies, the count is still low since they are not in their optimum environment which should be in neutral pH with lower temperature soils (Chalanska & Labanowski, 2014).

The potassium is required for turgor build up in plants and long distance transport of water. Root-knot nematodes like *Meloidogyne spp.* disrupt the xylem and phloem of their hosts which compromised the transport of water and nutrients. In that case, the root-knot nematodes will be lower in potassium deficit plants. Sandy type of soil could help increase *Meloidogyne* population since there are macropores that allow the nematodes to penetrate easily. The soil sample in this study has sufficient potassium which gives rise to the occurrence of nematodes, but because of the type of soil which was heavy in texture, *Meloidogyne spp.*, has relatively low frequency in terms of their population (Warner, 2009).

The environmental factors, particularly the temperature, influence the juvenile *Rotylenchulus spp.* development (Nakasano, 2004). Plant parasitic nematodes are generally sensitive to heat. The juvenile *Rotylenchulus spp.* severely suffers from high temperature (33 °C) so its juveniles could not develop to young females or males in water medium and soil. The temperature at the sampling site was 31 °C and only one juvenile *Rotylenchulus spp.* and 14 immature females and males were observed. This might be a reason for less population of this plant parasitic nematodes. The pH of the soil sample is 6.9. It is said that pH also has a negligible effect on juvenile development except for the extremely low pH like 2.9. The type of soil is silty clay which has a heavy texture. *Rotylenchulus spp.* is significantly influenced by soil texture wherein they exhibit a decrease in population in increasing median soil particle size. However, it could be noted that the ability of the

nematodes to reproduce and inflict damage was not affected by the soil and plant's nutrient status. The phosphorus harms the nematode reproduction while it has a positive effect on the plant growth (Nakasano, 2004; Warner, 2009)

Conclusions and Recommendations

The most commonly reported plant-parasitic nematodes associated with *A. squamosa* at Rizal were *Criconeimoides*, *Meloidogyne*, and *Rotylenchulus*. Among these, *Criconeimoides* had the highest number which was followed by *Meloidogyne* and *Rotylenchulus*. The collected soil samples have an acidic soil which can be affected by the properties and contents of the soil itself such as the moderately low nitrogen, high phosphorus, sufficient potassium, and the texture of the soil itself, which is heavy. The temperature, concentration of NPK, and the texture of the soil samples showed a relation to the occurrence and count of the plant-parasitic nematodes associated with the *A. squamosa*. These factors serve as a limiting factor to the reproduction and penetrating ability of these plant-parasitic nematodes.

Further studies on its morphological characteristics using high standard equipment is recommended as well as sampling from different locations must be done. Molecular assessment is also recommended for the PPN's to be identified up to their specific epithet. Since this is the preliminary study in the identification of plant parasitic nematodes associated with *A. squamosa*, further detailed analysis and research on this could be a great advancement in the field of Nematology and Plant Pathology in the Philippines.

Acknowledgment

The authors are grateful to *Asst Prof.* Romnick A. Latina of Plant Pathology Department at University of the Philippines, Los Baños, Laguna, their consultant in the study, and for letting them use the Nematology Laboratory in UPLB. They are also grateful to the Commission on Higher Education CHED-DARETO K to 12 Transition Program Cycle II for funding the project.

Conflict Of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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