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Nemato-toxic potential of Betel (*Piper betle* L.) (Piperaceae) leaf

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ABSTRACT

In vitro and *in-planta* trials were conducted to determine the nemato-toxic potential of aqueous leaf extracts of Betel (*Piper betle* L.) against the root-knot nematode species, *Meloidogyne incognita*, at five concentrations, standard extract (S) [1:2 (w/v] and its four dilutions, 20%, 40%, 60%. 80% of S. In the *in vitro* trials, second-stage juveniles (J_2s) and eggs of *M. incognita* were directly exposed to the extract while in the *in-planta* trials, the effect of the extract was evaluated as a root dip treatment using tomato plants. The findings indicated that the extract was lethal to J_2s but also inhibited egg hatch. The seedling dip treatments reduced root infestations in terms of gall formation, egg production and J_2 population densities in soil while simultaneously enhancing the growth of tomato plants. All these effects varied in a dose-dependent manner. Based on the LC_{50} value, the eggs were found to be less sensitive to the extract than J_2s. One hundred percent of mortality of J_2s was recorded at four concentration levels, 40%, 60% and 80% of S and S, while 100% egg inhibition was only recorded at S. The highest reductions in gall formation (i.e., number of galls/root system), egg production and J_2 population were all recorded at S as 83%, 87% and 84% compared to the untreated water control. The maximum growth enhancement, which was 235% of the control, was detected in the root length of treated tomato plants. It appears the aqueous leaf extract of *P. betle* shows promise as a sustainable eco-friendly nematicide for the management of *M. incognita*.

1. Introduction

Root-knot nematodes (*Meloidogyne* spp.) cause serious yield losses on a broad range of agricultural crops (Stirling and Stirling, 2003; Sahebani and Hadavi, 2008), in particular, in tropical and subtropical regions, worldwide (Kiewnick and Sikora, 2006). The infective second-stage juveniles (J_2s) initiate infection by penetrating roots adjacent to the tips in susceptible host plants. Once inside the roots, the J_2s migrate to the vascular cylinder and establish permanent feeding sites resulting in characteristic gall formation, which negatively affects absorption of water and nutrients (Trudgill and Blok, 2001). *Meloidogyne incognita* is extremely devastating and is commonly encountered not only in tropical and sub-tropical, but also temperate areas of the world (Sasser, 1989). To date, in Sri Lanka, six *Meloidogyne* species, i.e., *M. incognita* (Kofoid and White) Chitwood, *Meloidogyne javanica* (Treub) Chitwood, *Meloidogyne arenaria* Chitwood, *Meloidogyne hapla* Chitwood, *Meloidogyne brevicauda* Loss and *Meloidogyne graminicola* (Golden and Birchfield) have been reported on a number of economically important crop species (Ekanayake and Toida, 1997), of which *M. incognita* is the most widespread and predominate species particularly in vegetable production (Ekanayake and Toida, 1997; Premachandra et al., 2007). Since Sri Lanka drives towards sustainable agriculture, the development of nematode management strategies which are safer to farmers, consumers as well as the environment are urgently encouraged. In this context, plant-based strategies have been proven to be effective (Chitwood, 2002).

Piper betle (L.) (Piperaceae) (Betel) is a perennial dioecious creeper, widely cultivated in tropical and sub-tropical countries particularly in south and south-east Asia. People use Betel leaves for chewing either alone or together with other condiments like areca nut (*Areca catechu* L.; Arecaceae). Betel is widely cultivated in Sri Lanka and considered an economically and medicinally important plant. Previous studies indicate the derivatives of *P. betle* leaves, i.e., oils and alcoholic extracts, possess insecticidal (Mohottalage et al., 2007), anti-fungal (Mohamed et al., 1996) and anti-bacterial (Evans et al., 1984) properties. However, to date, only few studies have been focused on plant parasitic nematodes (Mackeen et al., 1997;

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Premachandra and Gunasekara, 2008; Wiratno et al., 2009). The objectives of the present study were to determine the effects of aqueous leaf extracts of *P. betle* on egg hatching and J_2s mortality of *M. incognita* and their efficacies as root dips for the management of *M. incognita* at five concentrations. This research is part of a larger initiative that seeks to promote the commercialization of plant-based nematicides in Sri Lanka.

2. Materials and methods

2.1. Nematodes

A population of *M. incognita*, originally collected from field grown tomato *Lycopesicon esculentum* Mill (Solanaceae) roots, was propagated on potted tomato plants cv. "Thilina" in a screen house at the Department of Zoology, University of Ruhuna, Matara, Sri Lanka. The galled tomato roots with mature egg masses were washed gently to remove the adhering soil. Subsequently, the egg masses were isolated under a stereo-microscope and rinsed with sterile distilled water (SDW). After transferring into 0.5% sodium hypochlorite solution (NaOCl), they were agitated for 4 min to dissolve the gelatinous matrix. Thereafter, the eggs were washed thrice in SDW on a mesh sieve with 26 μm pore size and stored in SDW until used for the experiments (less than 1 h). The J_2s were obtained by placing the extracted eggs on modified Baermann funnels at the ambient temperature (30 °C ± 2).

2.2. Betel extract

Mature fresh *P. betle* cv. Common Betel, were collected in the close vicinity of the University of Ruhuna. Leaves (850 g) were thoroughly washed and macerated in 1700 ml SDW [i.e., at a ratio of 1:2 (w/v)] in a kitchen blender. The macerated suspension was then left at ambient temperature for 24 h. After 24 h, the suspension was stirred well and centrifuged at 3000 rpm for 30 min. The supernatant was separated as a clear solution and was designated the standard extract (S) from which four dilutions were made to obtain final concentrations of 20%, 40%, 60% and 80% (v/v) by adding SDW.

2.3. Experiment 1: effect on survival of J_2s - in vitro

One hundred recently hatched (within 24 h) was collected and stored in 2 ml of SDW. They were exposed to 2 ml of the test extract at five concentrations, i.e., 20%, 40%, 60% and 80% of S and S, in 5 cm diameter glass Petri dishes. The Petri dishes with 4 ml of SDW alone, served as controls. All the test extracts and controls were replicated five times, arranged in a complete randomized design and stored at an ambient temperature for 48 h. After exposure, the Petri dishes were observed under a stereo-microscope at x 240 and the numbers of mobile and immobile J_2s were recorded. The immobile J_2s were transferred to SDW and left for 24 h. Thereafter, the juveniles were re-checked and any immobile were assumed to be dead. If any J_2s regained mobility, the effect was considered as nematostatic (paralysis). In addition, the shape of the dead juveniles was recorded. The experiment was repeated once.

2.4. Experiment 2: effect on egg hatching - in vitro

Fifty mature eggs of *M. incognita* in 2 ml SDW was added to 2 ml of test extract at five concentrations, 20%, 40%, 60%, 80% of S and S. After thorough shaking, the Petri dishes were covered with the lid and incubated at the ambient temperature. The control Petri dishes received only 4 ml of SDW. Hatching was observed after seven days and the numbers of J_2s emerged were recorded with respect to each concentration. The experiment was performed twice with five replications per each treatment and untreated controls which were arranged in a complete randomized design.

2.5. Experiment 3: effect as a root dip in-planta trial

The seeds of tomato, *L. esculentum* cv. "Thilina" were surface sterilized with 0.1% NaOCl for 1 min and rinsed three times with SDW. They were sown in cells of plastic module trays, each cell containing 62 g of steam-sterilized compost mixture (cattle manure 50%; paddy straw 20%; green manure 15%; poultry litter 10%: coconut coir dust 5%) and the trays were placed in a screen house. Four-week old tomato plants were carefully uprooted and gently agitated in water to remove the adhering soil. The root systems were immersed in freshly prepared aqueous leaf extracts of Betel at concentrations of 20%, 40%, 60%, 80% of S and S for 1 h. They were subsequently transplanted into 18 cm diameter plastic pots containing 2 Kg of steam-sterilized compost mixture. Tomato seedling roots dipped in SDW served as untreated controls. Five days after transplanting, pots were inoculated with 2000 J_2s of *M. incognita*. All the treatments and untreated controls were replicated five times and arranged in a randomized block design within the screen house. Plants were watered daily and fertilized fortnightly with 5 g of Baur's fertilizers for vegetables (N 12%; P_2O_5 9%; K_2O 8%). Sixty days post-inoculation, the tomato plants were uprooted carefully washed free of soil and the lengths and fresh weights of the shoots were measured. The numbers of galls and egg masses were counted on all root systems. In addition, galled roots over total number of secondary roots were recorded and the percentages of galled-roots per root system were calculated. The dry weights of the shoots were determined by keeping them in a drying oven at 70 °C for three days. Finally, the soil in each pot was homogenized and J_2s from a 200 cm^3 sample were extracted using a modified Baerrmann funnel technique.

2.6. Statistical analysis

In the *in vitro* experiments, the percentages of J_2 mortality and egg hatching of *M. incognita* were normalized using arcsin square root transformation, prior to the analysis. The data for the repeated trials were subjected to Brown and Forsythe's test for homogeneity of variance and then combined for the further analyse if the assumptions were not violated. The LC_{50} values were calculated for J_2 mortality and egg hatching using Probit analysis. In the root dip treatment, the data on percentage galled roots were arcsin transformed prior to the analysis while the shoot and root length, shoot dry and fresh weight and number of galls, egg masses/root system and J_2 population in soil were subjected to square root $(X+1)$ transformation. Dunnett's test was used to compare different treatments with the untreated control while ANOVA was performed to ascertain the effects among the different treatments. Means were compared using Tukey's range test. All analyses were performed using SAS (SAS Institute, 1999) at the significance level of 0.05.

3. Results

3.1. Experiment 1 – effect on survival of J_2s -in vitro

Since there was no variance heterogeneity between the two trials, the data were pooled over the concentrations. At all the concentrations of *P. betle* aqueous extracts tested, significantly higher ($P < 0.0001$) percentages in J_2 mortality were recorded compared with the untreated water control in which no mortality was detected, after 48 h of exposure (Table 1). At 20% of S, 87% mortality of J_2s was observed and the remaining 13% showed