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Evaluation of the fungitoxic and fungistatic potential of the root extract of male papaya plant and female papaya plant (Carica papaya L.)

Gowthami Yaram, Chandrasehar Govindan, Deepa Venkataramulu, Darsana Rajaputhran, Chitrikha Thirunavukkarasu and Goparaju Anumolu
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Received 10 February 2015; Accepted 23 March 2015

The fungicidal and fungistatic potential of methanolic root extract of male *Carica papaya* against *Phomopsis vexans* which affects egg plants (*Solanum melongena* L.) leading to the condition called the “Phomopsis blight” was investigated. Result of the experiments revealed that the aqueous extract did not show significant activity, but comparatively the methanol extract of male root demonstrated significant activity against the test fungus. Male methanolic root extract exhibited relatively higher antifungal activity than its female and hermaphrodite form. The extract was also found to be safe on the local algal population, which are one of the most sensitive indicators of toxicity. In summary, the male methanolic root extract can be considered as a potential biocontrol agent in the mitigation of *P. vexans* in the current scenario. There are no biological agents available for the control of *P. vexans* and here the present findings are novel in this regard.

**Key words:** Fungicidal, fungitoxic, *Carica papaya*, *Phomopsis vexans*, methanolic extract, algal.

**INTRODUCTION**

Papaya (*Carica papaya* Linn.) is a unique source of potentially useful compounds with diverse structure and properties (Krishna et al., 2008). Papaya exists in three sexual forms: male, female and hermaphrodite. Papaya flowers are born on inflorescences which appear in the axils of the leaves. Female flowers are held close against the stem as single flowers or in the cluster of 2 to 3 (Chay-Prove et al., 2000). Male flowers are smaller and more numerous and are born on 60 to 90 cm long pendulous inflorescences (Nakasone and Paull, 1998).

Bisexual flowers are intermediate between the two unisexual forms (Nakasone and Paull, 1998; Parle and Gurditta, 2011) female plants and hermaphrodite forms are used for propagation while male plants are discarded considering the fact that they cannot be used for propagation. Hence, in the present study, we attempted to investigate some useful properties that could be obtained from a male papaya plant. In general, papaya plants have been reported to contain biologically active compounds that can be utilized for various purposes (Krishna et al., 2008; Parle and Gurditta, 2011; Chindala et al., 2013). With this background, we tested the fungitoxic potential of the root extract of male papaya plant against the fungi *Phomopsis vexans*, which affects...
brinjal plants (*Solanum melongena* L.) leading to the condition called the “Phomopsis blight” (Figure 1) (Islam and Meah, 2011). Phomopsis blight and fruit rot caused by *P. vexans* has been treated as one of the major constraints to eggplant cultivation in the country (Das and Sarma, 2012).

Chemical control of Phomopsis blight is generally recommended since, at present, there is no biological control strategies that have been developed for this disease. In general, biological control of plant disease is suggested as an alternative to chemical control (Horsfall and Cowling, 2012) and is considered as a cost effective and an environmental friendly technique. Even though a number of mycoparasites have been recognized (Janisiewiz et al., 1988), their role in combating the diseases of common vegetables like brinjal is still scanty. The present study aims to investigate if the root extract of the male papaya plant can be used for controlling the *P. vexans* which infests the plant.

**MATERIALS AND METHODS**

**Source of plant sample**

Healthy mature fresh plant root sample of female and male *C. papaya* were collected from IIBAT Farm, Padappai Tamil Nadu, India.

**Preparation of root extract of *C. papaya***

Extracts of *C. papaya* root were prepared for this study as described:

**Preparation of methanol extract**

Male and female papaya roots were collected, cleaned with water and chopped into tiny bits, and once again rinsed in water to remove fine sand particles, drained for a few hours and oven dried at 45°C for three days to obtain a constant weight. The dried plant material was thereafter blended with the aid of an industrial homogenizer into fine powder. Twenty grams of the female and male root was weighed and extracted with 70% (250 ml) methanol, using Soxhlet apparatus. The extract was dried using Rota-evaporator and weighed.

**Preparation of aqueous extract**

The procedure was repeated with ambient de-ionized water replacing methanol.

**Phytochemical screening**

The extract was tested for the presence of bioactive compounds by using following standard methods by Sofowra (1993).

**Test for alkaloids**

Crude extract of root sample were mixed with 2 ml of 1% HCl and heated gently. Mayer’s and Wagner’s reagent were added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

**Test for phenols and tannins**

Crude extract of root sample were mixed with 2 ml of 2% solution of FeCl₃. A blue-green coloration indicated the presence of phenols and tannins.

**Test for saponins**

Crude extract of root sample were mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

**Test for glycosides**

**Salkowskis test:** Crude extract of root sample were mixed with 2
ml of chloroform. Then 2 ml of concentrated H₂SO₄ was added carefully and shaken gently. A reddish brown color indicated the presence of steroidal ring that is, glycone portion of glycoside.

Detection of cyanide: To aqueous extracts of male and female C. papaya, few drops of potassium hydroxide solution were added to two drops of ferrous sulphate solution and two drops of ferric chloride solution were added and shaken well and heated gently. After heating, the solution was acidified with dilute hydrochloric acid.

Preparation of pathogen inoculum

The pure culture of *P. vexans* (Strain: ITCC No. 7222) was obtained from the Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute, New Delhi. The stock culture was maintained in potato dextrose agar (PDA) slants. The pure culture of test fungus was maintained on PDA slants.

Antifungal assay

Fungitoxicity was assessed by poisoned food technique of Grover and Moore (1962). Seven days old culture of the fungus was used for the preparation of the inoculum disc of 4 mm in diameter. A volume of 0.5 ml from each concentration of the root extract (male and female root) was aseptically poured into Petri plate followed by the addition of 9.5 ml of molten PDA and was swirled gently to achieve thorough mixing of the contents. In the control plates, extract was not added. After solidification of the media, culture disc was placed at the center of Petri plates and incubated at 25 ± 2°C for seven days. The diameter of fungal growth was measured on the 7th day of incubation and percentage of mycelia growth inhibition was calculated using the formula (Rao and Srivastava, 1994):

\[
\text{% of inhibition} = \frac{g_c - g_t}{g_c} \times 100
\]

Where, \( g_c \) = growth of mycelia in control plate; \( g_t \) = growth of mycelia in treated plate.

Antagonistic activity of *C. papaya* extracts against *P. vexans*

Myelia dry weight method

The mycelial growth measurement was determined using liquid media; about 100 ml potato dextrose broth (PDB) was prepared using sterile deionised water. Approximately 100 ml of PDB was uniformly distributed into 250 ml conical flasks and covered with aluminium foil, sterilized by autoclaving. The media was allowed to cool down to room temperature. Root extracts (methanolic and aqueous) of *C. papaya* were aseptically pipetted into each flask. Aseptically, one milliliter (1 ml) of \( 1.006 \times 10^5 \) mycelial fragments/ml of *P. vexans* was pipetted over the surface of PDA plates and spread with glass rod. The plates were incubated for 7 days at 27°C. After incubation, mycelial discs of the pathogen were prepared using sterilized 5 mm cork borer carrying confluent growth of *P. vexans* and inoculated into each flask. The flasks were incubated using shaker incubator at room temperature for 14 days at 250 rpm. On the 14th day, the mycelial mats of the fungal isolate were harvested by filtering on a pre - weighed filter paper after which the filter paper with the mycelia mat were then oven dried at 40°C until constant weight was attained. The weight of the mycelia was determined by subtracting the weight of the filter paper from the total weight of filter paper plus mycelia. Two replicates were maintained for each treatment. Percentage mean growth was calculated as follows:

Mean growth (Abiala et al. 2010) (%): \( W_c - W_t / W_c \times 100 \)

Where: \( W_c \) = weight of mycelial growth of *P. vexans* in the control. \( W_t \) = weight of mycelial growth of *P. vexans* in the treatment

Fungistatic action

The fungistatic action of the extracts of *C. papaya* roots against *P. vexans* was also determined. The Petri plates containing 10 ml of PDA was used to prepare OECD medium disc of *P. vexans* and the test fungus was aseptically placed to each Petri plates and incubated at 25 ± 2°C. After seven days of incubation, the extract was sprayed with the help of a mini sprayer bottle to the plates containing the seven days old test fungi. Control, bavistin and vitavax was used as a positive control and incubated at 25 ± 2°C after seven days. One inoculum disc from each Petri plate was obtained and re inoculated into freshly prepared Petri plates containing the 10 ml of PDA and incubated at 25 ± 2°C after seven days. The average diameter of fungal colonies was measured on seventh day after inoculation and percentage of mycelial growth inhibition was calculated. Three replicates of the Petri dishes containing PDA were used and the colony size was calculated by taking an average of the three.

Effects of root extracts of *C. papaya* on micro algae

As an extension of the findings, we tried to investigate the effects of the extract when it is put to use in the real time. When the extract is sprayed on the brinjal plants as a prophylactic measure, there are all chances that it will reach the surrounding water. Hence, in order to see if the extract has any adverse effects on the organisms that inhabit the surrounding water, we performed the following study. Since algae are the most commonly encountered organisms in the water, the effect of the extract on algae was studied. The green alga, *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) strain SAG 61.81 was used in this study. The primary culture was procured from University of Gottingen, Germany and the culture was maintained in the Department of Ecotoxicology, IIBAT, as per the procedure stated in the Organisation for Economic co-operation and development (OECD) guideline number 201. The nutrient culture medium, OECD TG 201 medium was used for the present study. The deionised water was used to prepare OECD medium throughout the study. All the methanolic root extract formulations were dissolved in the OECD medium without using any solvent. The test item concentrations were fixed for each methanolic root extract formulation. The test item stock solution was diluted with the OECD medium to attain the required test item concentrations. The initial pH of bulk solution of each test item concentration and control was recorded and adjusted within the range 7.63 to 7.92 for test item concentration and 8.04 for control by using 0.1 M NaOH (sodium hydroxide) solution, and final pH was recorded within the range of 6.96 to 7.06 for test item concentration and 7.22 to 7.26 for control at 72 h after incubation. For each treatment and control, 100 ml of test item solution was transferred into sterile 250 ml Erlemeyer flasks. The control and treatment flasks were inoculated with three-day-old preculture of *P. subcapitata* to get an initial cell concentration of about 1 × 10⁷ cells per ml under aseptic conditions.
All the flasks were kept in the shaker incubator for 72 h providing continuous illumination of 6500 to 8000 lux light intensity with temperature ranging from 20 to 22°C and continuously shaken at 110 to 120 rpm. During the experiment, control and treatment flasks were randomly repositioned in the shaker incubator daily to minimize the variation of the light intensity. After 72 h of incubation, alga cell count was recorded in all the flasks using Improved Neubauer’s Haemocytometer under illuminance of the microscope (Satyavani et al., 2012).

RESULT AND DISCUSSION

Root extract yield

The methanolic and aqueous extracts of roots were dried using Rota evaporator and the dry weight was recorded in percentage as follows: Aqueous extract: male (10.33%), female (16.5%). methanolic extract: male (20.8%), female (21.49%).

Phytochemical screening

The dried root extracts of the female and male C. papaya plant were found to contain alkaloids, phenols, tannins and saponins (Doughari et al., 2007). The level of these compounds varies in the fruit, latex, leaves and roots. In addition, plant parts from male and female trees differ in the quantity of these compounds. The quantity of fresh papaya latex and dry latex (crude papain) also vary with sex and age of the tree. Female and hermaphrodite trees yield cruder papain than male trees (Brocklehurst et al., 1985). One interesting observation in the present study was the fact that cyanogenic glycosides were present only in female C. papaya plant roots extract and completely absent in the males, which gives that advantage of the male plants for further investigations. Papaya contains many biological active compounds. Two important compounds are chymopapain and papain, the level of the compounds varies in the fruit, latex, leaves and roots. This effect has been widely studied by Adejuwon et al. (2011), but comparison of phytocconstituents based on sex differentiation has not been studied till now and hence has been addressed in the present study.

Antifungal assay

The results of the antifungal assay are presented in Table 2. It is observed that the methanolic extracts of both male and female are more effective against the test fungal pathogen when compared to their respective aqueous extract counterparts. It has been earlier reported by Tona et al. (1998), Topuriya et al. (1978) and Adejuwon et al. (2011) that the active ingredients vary from one extract to another, which could be due to difference in solubility of marked difference in the performance of the extract against the fungal pathogen can be attributed to the mentioned fact that the active components in the two extracts may differ owing to the solvent used for extraction. It can be observed that while both the extract inhibit the fungal growth completely (100%) at 250 mg/L, the male root extract relatively performs three times better (23.3%) even at the lowest concentration tested (10 mg/L) when compared to the female extract (7.8%). At 50 and 100 mg/L, the male extract performed comparatively more potent than the female (Figure 2). The antifungal activity of papaya plant root has been recorded earlier (Kirtikar et al., 1998); however, as mentioned earlier, there is no sex based studies conducted so far. The present study reports this interesting fact that the male methanolic root extract can be taken as a lead for the search of new antifungal compounds. It needs further exploration to understand the intricacies of the active molecules that are present in the male methanolic root extract so that they can be potentially utilized for therapeutically / microbiologically / biotechnologically important compounds.

Antagonistic activity of C. papaya L. extracts against P. vexans

It has been already established that combining a fungistatic agent with a fungicide will produce antagonism (Adejuwon et al., 2011). Mycelia dry weight method was used to ascertain the in vitro effect of phytoconstituent/plant extract on the mycelial growth of P. vexans in liquid culture media (Abiala et al., 2010). All the root extracts had significant effect on the pathogen (P. vexans), the growth was determined by dry weight method but the observations (Table 1) revealed that the highest mean mycelia dry weight (0.124 mg) was recorded in methanolic control (MC) while the male methanolic extract (MME) had the least (0.097 mg). The results showed MME to be better than other root extracts tested. With 21.77% decrease over control, there was a significant difference between the control and other root extracts. The methanolic root extract of male C. papaya significantly inhibited mycelial growth of P. vexans at higher concentration in the liquid media.

Effects of root extracts of C. papaya on micro algae

P. subcapitata is a unicellular green alga widely distributed in freshwater and soils. Due to its cosmopolitan characteristic, its use is recommended by national and international protocols in eco-toxicity studies. The purpose of this ecotoxicity assessment is to assess the effect of the extract on the microalgae P. subcapitata (Chlorophyceae) (Luciana et al., 2012). Based on 72 h cell count, the percent inhibition for the active components in the various solvents. The biomass (I_b), yield (I_y) and growth rate (I_r) were calculated. The
percent inhibition values of 51.5 (biomass), 51.9 (yield) and 14.9 (growth rate) were recorded in the concentration of 100 mg/L methanolic male extract of papaya. Using statistical tool, EC50 (half maximal effective concentration) value for methanolic male extract of papaya was determined as 107.17 mg/L. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were calculated as 10 and 50 mg/L, respectively using Dunnett’s test. Similarly, percent inhibition values were calculated for methanolic female extract of papaya. The EC50 value for methanolic female extract of papaya was determined as 110.90 mg/L. The NOEC and LOEC were also calculated as 50 and 100 mg/L, respectively. No toxic symptoms were noted for P. subcapitata. However, a physical inhibitory effect was observed due to the high concentration of suspended material in the OECD medium samples.

Conclusions

The search for newer sources of antibiotics is a global challenge pre-occupying research institutes, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs. Plants have the major advantage of still being the most effective and cheaper alternative source of drugs. C. papaya contains many biologically active compounds. Two important compounds are chymopapain and papain, which are supposed to aid in digestion. The level of compound varies in the fruit, latex, leaves and roots. In addition, plant parts from male and female trees differ in the quantity of the compounds. For example, phenolic compounds tend to be higher in male trees than female trees. The quantities of compounds also vary with the sex of the trees and the age of the tree.
Table 2. The results of antifungal assay on root extracts

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentrations (mg/L)</th>
<th>Mycelial growth (cm)*</th>
<th>% Decrease over control</th>
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<tbody>
<tr>
<td>Control (AE)</td>
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<td>10</td>
<td>5.0</td>
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<tr>
<td>Female <em>Carica papaya</em> aqueous root extract</td>
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<td>4.4</td>
<td>12.0</td>
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<td>100</td>
<td>3.9</td>
<td>22.0</td>
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<td>250</td>
<td>2.8</td>
<td>44.0</td>
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<tr>
<td>Control (ME)</td>
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</tr>
<tr>
<td>10</td>
<td>8.3</td>
<td>7.8</td>
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<tr>
<td>Female <em>Carica papaya</em> methanolic root extract</td>
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<td>5.6</td>
<td>37.8</td>
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<tr>
<td>100</td>
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<td>250</td>
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<td>Male <em>Carica papaya</em> aqueous root extract</td>
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<td>10</td>
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<td>Male <em>Carica papaya</em> methanolic root extract</td>
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<tr>
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*Mean of three replications.

In the present study, we found that the methanolic root extract of the male *C. papaya* has inhibitory effect on the fungal pathogen, *P. vexans*. Although there are reports that demonstrate such antifungal activity in the female and hermaphrodite form of *C. papaya* to the best of our knowledge; this is the first report to show that male plant can also possess similar bioactive components. This gives an indication that male papaya plant can be efficiently utilized since they also possess biologically important compounds. The inhibitory effect of the male extracts on the fungal pathogen, *P. vexans* was relatively higher than the female and hermaphrodite counterparts, as evidenced in the present study. Furthermore, the extract did not affect the local algal population, and which indicates it does not contain the toxic properties to non-target species and encourages its potential use as a biological control agent in the mitigation of *P. vexans*.

Conflict of interest

Authors have none to declare.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Balakrishna Murthy Prakhy, Director, International Institute of Biotechnology and Toxicology (IIBAT), Padappai for providing necessary facilities and co-operation during this research work. The authors are also thankful to Dr. Sathya Thiruvathiparam Narasimmabarathy for her co-operation during this research work.

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