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Development of rapid, efficient and cost effective screening technique for testing arecanut against *Phytophthora meadii* incitant of fruit rot disease

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ABSTRACT

To accelerate identification of disease resistant arecanut germplasm or hybrids against *Phytophthora*, it is very much imperative to develop bioassays which could differentiate resistant and susceptible cultivars efficiently. Here, a cost effective and rapid technique, called the "Detached Leaf Assay", was developed to identify resistant germplasm at the seedling stage itself. Zoospore production in highly virulent *Phytophthora meadii* (P19) was standardized by incubating under a 12 hours light and dark regime. Zoospore suspension was adjusted to 10^5 spores ml^{-1} in Petri plates. Subsequently, surface sterilized arecanut leaves were floated in zoospore suspension and incubated at temperature of 24±1 ˚C. Disease symptoms, including water-soaked lesions, were recorded three days after inoculation. Infection lesion increased from 1 to 7.3 cm^2. The pathogen was re-isolated and confirmed with the original culture. The assay was successfully validated to screen arecanut accessions, wild types and hybrids against *P. meadii*. This technique is the first to be developed, and it is simple, cost-effective, and faster. It also provides consistent infection and could be effectively utilized to screen arecanut germplasm or hybrids against *P. meadii* in the seedling stage itself.

- Developed a cost effective, efficient and rapid screening technique
- The technique was validated to identify resistant arecanut genotypes against *Phytophthora meadii* at the seedling stage.

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	Screening technique, Plant-microbe interaction
Name of your method:	Detached leaf assay screening technique
Name and reference of original method:	Not applicable
Resource availability:	Not applicable

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Background

Arecanut (*Areca catechu* L.; Arecaceae) is a commercially and socially important plantation crop in South and South East Asia. The dry kernel is the main economic product, commonly used as a masticatory, but all parts of the palm can be utilized efficiently through recycling and value addition [1]. The pharmacological properties of nuts have been attributed to alkaloids, and arecanut forms an ingredient in traditional medicines in South and South East Asia [2,3]. Millions of small and marginal farming communities, dealers, and stakeholders rely heavily on the arecanut crop for their livelihood stability and revenue. Arecanut palm is affected by several biotic and abiotic stresses [4]. Among them, *Phytophthora meadii* causing diseases such as fruit rot, bud rot and crown rot, are serious and results in heavy economic losses [5]. Fruit rot disease is the most dreaded disease and causes yield losses up to 90% [6–8]. While, bud rot and crown rot diseases occur frequently in fruit rot affected palms leading to death of palms.

Bordeaux mixture (1%) has been the propitious recommendation for controlling *Phytophthora* diseases of arecanut [9,10]. However, preparation of the Bordeaux mixture is cumbersome, and improper preparation and application can make it ineffective; further, the unavailability of trained climbers to undertake plant protection measures compounds the challenge. Spraying of Bordeaux mixture can cause adverse effects on the environment and biodiversity, such as contamination of soil and groundwater, and significantly impact soil biota [11,12]. Host-plant resistance is considered an eco-friendly, economical and sustainable option to mitigate yield losses due to diseases. In order to accelerate identification of disease resistant arecanut germplasm or hybrids against *Phytophthora*, it is very much imperative to develop bioassays which could differentiate resistant and susceptible cultivars efficiently and effectively.

Since arecanut is a perennial crop, identification of resistant germplasm or hybrids in the seedling stage plays a vital role in order to save time and cost involved in developing a resistant cultivar or hybrid. Here, we developed a simple and rapid screening technique for testing arecanut reaction against *Phytophthora meadii* and it was validated to identify resistant arecanut germplasm or hybrids.

Method details

Selection of Phytophthora meadii *isolate and induction of zoospore production*

Highly virulent *Phytophthora meadii* isolate P19 identified in the previous study [13] and maintained in Plant Pathology Section, ICAR-CPCRI was used for screening process. Here, we subcultured *Phytophthora meadii* (P19) on carrot agar (CA) medium and induced zoospore production by incubating under a 12-hour light and dark regime at 24±1 ˚C in a BOD incubator for eight days. Subsequently, the plates were initially rinsed twice with sterile distilled water, and 20 ml of sterile distilled water was added to each plate and incubated at 4 ˚C for 20 minutes. Then the plates were incubated at room temperature for 20 minutes until the zoospores were released. A zoospore suspension was filtered through a muslin cloth, and the concentration was adjusted to 10^5 spore's ml^{-1} with sterile distilled water using a hemocytometer. Fifteen ml of zoospore suspension was maintained in each Petri plate in order to screen arecanut germplasm. Surface sterilized arecanut leaves (8–9 cm) were floated in zoospore suspension and incubated at temperature of 24±1 ˚C in a BOD incubator (Fig. 1). The observation on infection initiation and diameter of the infection lesion developed were recorded up to five days of incubation.

Preparation of carrot agar medium used for culturing of P. meadii

Materials required: Carrot- 200 g, Dextrose unhydrous (Himedia)- 20 g, Agar agar (Himedia)- 20 g, Distilled water-1000 ml and autoclave for sterilizing the media.

Procedure

- 200 g of carrot is used for preparing one liter of medium. Wash the carrot thoroughly in tap water, peel of the skin and cut into thin slices

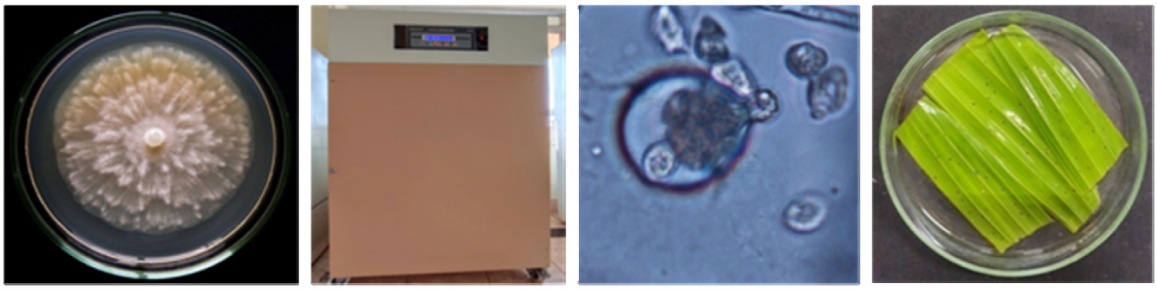

Fig. 1. *P. meadii* cultured on CA medium (A), culture incubated at 12-hour light and dark regime at 24±1 ˚C to induce zoospores (B), production of zoospores (C), detached arecanut leaves floated in 15 ml of zoospore suspension (D).

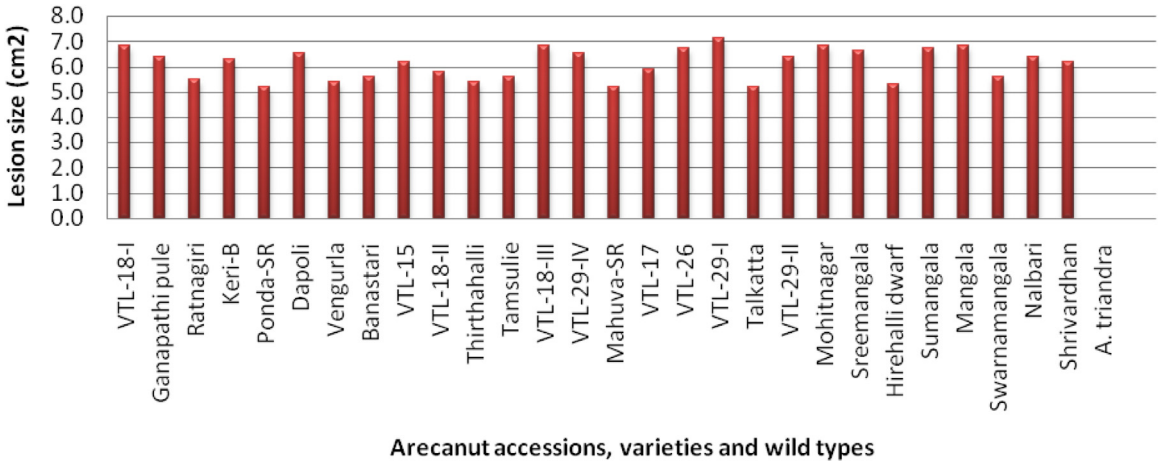

Fig. 2. Graphical representation of disease reaction of arecanut germplasm, cultivars, dwarf mutant and wild type against *Phytophthora meadii* (P19).
[CV= 2.7, CD @ 0.05 = 0.267].

- Boil the carrot pieces in 500 ml of distilled water for 30 minutes and filter through a double layer of muslin cloth
- Add 20 g of dextrose to potato extract
- Add 20 g of agar in 500 ml of water and boil for 30 minutes to dissolve the agar
- Mix thoroughly the molten agar and potato extract and make up the volume to one liter with distilled water
- Dispense 50 or 100ml of medium to conical flasks as per the size. Plug the flasks with cotton and wrap with aluminum foil. Sterilize the medium at 121 ˚C for 20 minutes using Autoclave

Screening of arecanut germplasm, cultivars, wild species and hybrids using detached leaf assay

The assay was validated to screen arecanut germplasm [Thirthahalli, Mahuva-SR, Ganapathipule, Ratnagiri, Dapoli, Vengurla, Talkatta, Keri-B, Ponda-SR, Banastari, Tamsuli, VTL-15, VTL-17, VTL-18-I, VTL-18-II, VTL-18-III, VTL-26, VTL-29-I, VTL-29-II and VTL-29-IV], cultivars (Mangala, Sumangala, Sreemangala, Swarnamangala, Mohitnagar, Nalbari and Shrivardhan), the dwarf mutant (Hirehalli Dwarf) and wild species (*Areca triandra*) and inter-specific hybrids between *A. catechu* and *A. triandra*. Leaves next to the spindle region, which were free from insect and pest damage, were collected from arecanut germplasm, cultivars, the dwarf mutant, the wild species, and inter-specific hybrids, washed in running tap water and surface sterilized. Subsequently, the leaves were cut into into 8–9 cm bits and were floated in sterilized Petri plates containing 15 ml of zoospore suspension (10^5 spore's ml^{-1}) and incubated at temperature of 24±1 ˚C in the BOD incubator. The observation on infection initiation and diameter of the infection lesion developed were recorded up to five days of incubation. Disease reaction was categorized by following severity scale [14] with slight modification. Scale 0 (Resistant): No infection lesion, 1 (Moderately resistant): less than 10% lesion area, 2 (Moderately susceptible): 11–25% lesion area, 3 (Susceptible): 26– 50% lesion area, 4 (Highly susceptible): above 51% lesion area.

Method validation

Phytophthora infection symptoms were manifested as water-soaked brown colour lesions on leaves after three days of inoculation. Infection lesion size increased from 1 to 7.2 cm^2, and the leaves were covered with mycelial growth within seven days of incubation. Among the 25 arecanut germplasm [Thirthahalli, Mahuva-SR, Ganapathipule, Ratnagiri, Dapoli, Vengurla, Talkatta, Keri-B, Ponda-SR, Banastari, Tamsuli, VTL-15, VTL-17, VTL-18-I, VTL-18-II, VTL-18-III, VTL-26, VTL-29-I, VTL-29-II and VTL-29-IV], cultivars (Mangala, Sumangala, Sreemangala, Swarnamangala, Mohitnagar, Nalbari and Shrivardhan), the dwarf mutant (Hirehalli Dwarf) and wild species (*Areca triandra*) screened against *Phytophthora meadii,* except the wild type, *viz*., *A. triandra,* all the remaining accessions showed highly susceptible reaction. Only *Areca triandra* manifested the resistant type of reaction (Fig. 2 and 3).

Screening of arecanut interspecific hybrids aginst Phytophthora meadii *(P19) using detached leaf assay*

Arecanut inter-specific hybrids from two crosses such as *A. triandra* × Shathamangala and Shathamangala × *A. triandra* were screened against *P. meadii* (P19). In *A. triandra* × Shathamangala hybrids (designated as AS 1 to AS 100), 93% hybrids showed resistant reaction (scale 0), while 7% hybrids showed moderate resistance (scale1). In the case of Shathamangala × *A.triandra* hybrids (designated as SA 1 to SA 100), 12% hybrids manifested moderate resistance (scale1), 28% hybrids- moderately susceptible (scale 2), 10% hybrids-susceptible (scale 3), 50% hybrids- highly susceptible (scale 4) type of reaction (Figs. 4 and 5).

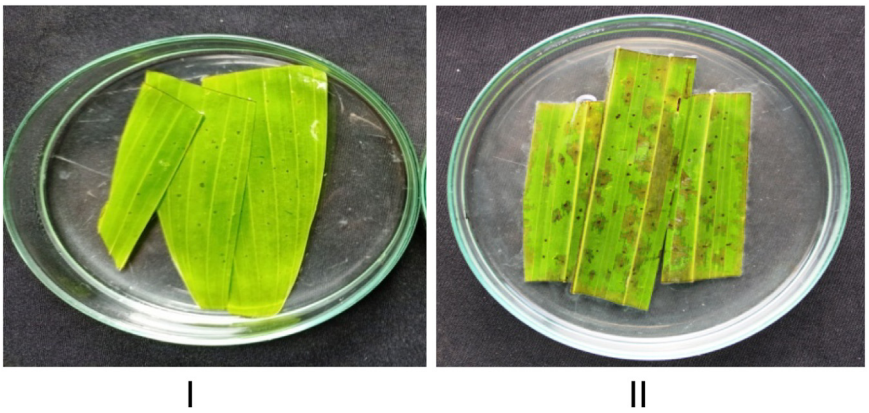

Fig. 3. Resistant reaction of *A. triandra* (I) and highly susceptible VTL 29-I (II) against *Phytophthora meadii* (P19).

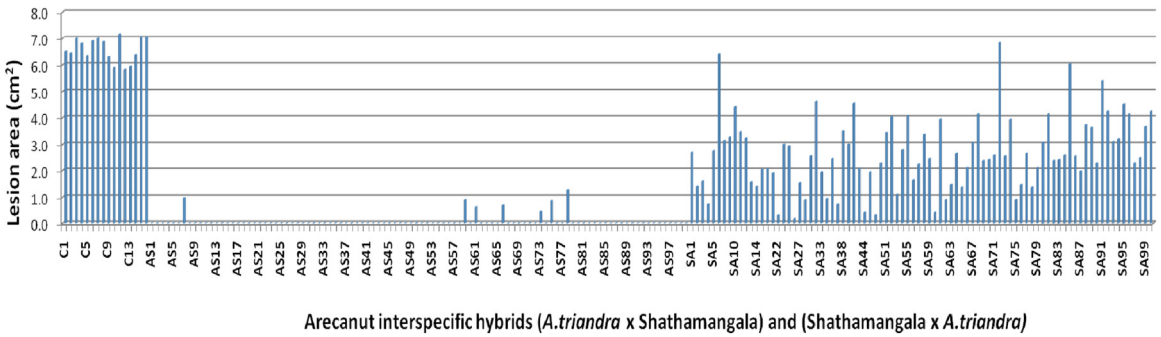

Fig. 4. Graphical representation of disease reaction of arecanut interspecific hybrids against *Phytophthora meadii* (P19).

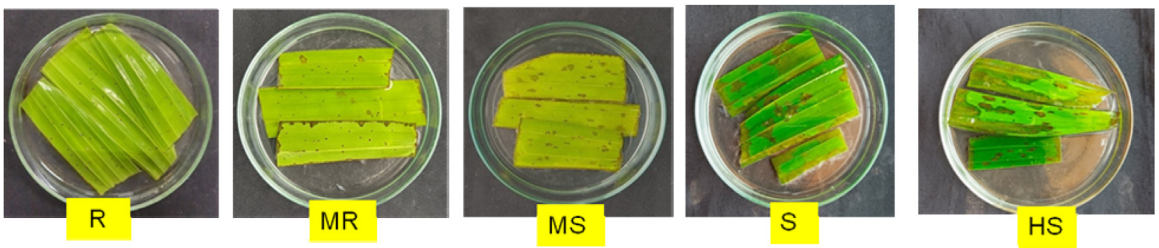

Fig. 5. Categorization of arecanut inter specific hybrids under different reaction types based on infection level.

Discussion and conclusion

Detached leaf assay technique is simple, rapid and cost effective compare to previous screening techniques such as detached arecanut and seedling inoculation method (Prathibha et al. 2015). Where both methods were cumbersome, one needed to inoculate individual nuts or seedlings and also wait until the yielding stage. In addition, sophisticated facilities like plant growth chamber are required and there is delay in expression of disease reaction. Detached leaf assay technique could be used either for proving pathogenicity of *Phytophthora meadii* and also for identification of virulent strains of *Phytophthora meadii.* More importantly, this assay could be efficiently used for rapid screening of hybrids or germplasm to identify resistant genotypes against *Phytophthora meadii* at the seedling stage. This study confirms the great importance of agricultural research and applied science in life as reported before in many scientific papers [15–17].

Limitation: The screening technique requires controlled condition like a temperature of 24±1 °C for inoculum (zoospore) production and expression of symptoms.

CRediT author statement

Conceptualization and methodology: Prathibha V.H., Nararaja N.R., Rajesh M.K., Rajkumar, Validation: Prathibha V.H., Nararaja N.R., Daliyamol, Thejasri, Original draft preparation: Prathibha V.H., Nararaja N.R., Rajesh M.K., Thejasri, Uchoi Anok, Supervision: Vinayaka Hedge. Writing - Reviewing and Editing: Prathibha V.H., Nararaja N.R., Rajesh M.K., Vinayaka Hedge and Daliyamol.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

We have deposited the DNA sequences of *Phytophthora meadii* in NCBI data base and other data presented in this study are available on request from the corresponding author.

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