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Research Article

Isolation and Identification of *Penicillium citrinum* Strain from the Leaves of *Fragaria* x *ananassa* Cultivated by Nutrient Film Technique

Sowmya RS[1], Warke VG[2], Mahajan GB[2], Satardekar MR[2], Patil TA[2] and Annapure US[1]*

[1]*Department of Food Engineering and Technology, ICT, Mumbai, India*

[2]*HiMedia Laboratories Private limited, Thane, India*

***Corresponding Author:** Annapure US, Department of Food Engineering and Technology, ICT, Mumbai, India.

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Abstract

The study was carried out to identify the fungibiome associated with Nutrient Film Technique (NFT) cultivated strawberry (*Fragaria* x *ananassa*) leaves collected from an indoor hydroponics facility. Sabouraud dextrose agar (SDA) was used for the isolation of associated fungi. After 48 hours of incubation at 37 ± 1 ^{0}C several colonies of fungi were seen. The colonies of fungus ICT-HM-0011S were abundant and grew like pure culture on the SDA plate. The fungus ICT-HM-0011S was characterized and identified by a polyphasic approach based on its macroscopic, microscopic, MALDI-TOF MS, and ITS sequencing. Microscopic investigations revealed that the primary mycelial and spore arrangements resembled with *Penicillium species*. Studies using Bruker MALDI biotyper identification confirmed the genus of the isolate to be *Penicillium*. The ITS sequencing technique confirmed the isolate as a strain of *Penicillium citrinum*. From the results, it was inferred that the organism isolated from infected strawberry leaves was *Penicillium citrinum*. The occurrence of this organism is mainly due to the conditions maintained in the hydroponics facility. This is the first report that concluded the presence of *P. citrinum* in the strawberry (*Fragaria* x *ananassa*) leaves. Based on abundance of this fungi on healthy leaves, we envisage further investigations of this indigenous fungus on strawberry leaves for its potential shielding role, water storage ability enhancement and growth supplement provider.

Keywords: Hydroponics; *P. citrinum; Fragaria* x *ananassa;* MALDI-TOF MS; ITS Sequencing

Introduction

The parts of plants above-ground are normally colonized by various bacteria, yeasts, and fungi. Some microbial species can be isolated almost as a pure culture within plant tissues. In most of the work, phyllosphere microbiology has focused on leaves as a more dominant aerial plant structure [1,2]. There is diverse microbiota in leaves, including many different genera of bacteria, filamentous fungi, yeasts, algae, and very less frequently, protozoa and nematodes. The leaf surface had long been considered a hostile environment for bacterial colonists [1]. The leaf surface is continuously exposed to temperature and relative humidity fluctuations. Also it is well known fact that, many factors may influence the microbial growth on leaves [3].

Strawberry (*Fragaria* x *ananassa*) is an important cash crop worldwide, cultivated organically and conventionally in open fields, and greenhouses or plastic closed tunnels. Strawberry plants and fruits are susceptible to several fungal pathogens, and thus, fungicide treatments are commonly followed to prevent economic losses. Several fungal pathogens infect strawberry plants, mainly Podosphaera (powdery mildew), Phytophthora cactorum (leather rot, crown rot), and *Rhizoctonia spp*. (hard brown rot, Rhizoctonia bud, black root rot, crown rot, web blight, leaf blight, and fruit

rot), *Colletotrichum acutatum* (anthracnose), *Phytophthora fragariae var*. *fragariae* (red stele root rot), *Verticillium albo-atrium* (Verticillium wilt), and *Verticillium dahlia* [4]. Apart from these harmful effects of fungi, there are certain fungal species support growth of fungi in many ways [5]. Moreover during hydroponic cultivation of a plant, the plant is to some extent under an artifial water stress, as water need of every plant is not well reported as such in the literature. This induced water stress has to be overcome. The plant do it by different mechanisms [6]. One natural way, which is often found in mycorrhiza is to form symbiotic association with water stress relieving fungi. In this perspective the plant parts and associated fungi may talk with each other to form a symbiotic association to suistain in water stress condition. For example *Cenangium ferruginosum* [7].

Although studies on the strawberry fungal biota were reported, these reports were either based on isolation from the soil [6,8] grown strawberries or controlled soil environment. Hydroponics is a technology where plants are grown in a controlled environmental condition [9]. However, very few research works have been done on the aerial organs like leaves, flowers, and fruits simultaneously for strawberry species. We report here with an abundantly found and leaf growing fungal species from *Fragaria* x *ananassa* cultivated under a hydroponic system. A polyphasic approach was used for its characterisation. The fungal isolate can be further investigated to check its symbiotic role in its association with *Fragaria* x *ananassa* especially under water stress condition.

Materials and Methods

Cultivation and sample collection

The 90 days old strawberry (*Fragaria* x *ananassa*) plants grown in polythene tray (10 inches x 8 inches x 4 inches) containing cocopeat were collected from K F Bioplants Private Limited-Pune, Maharashtra. These saplings were replanted in a new cocopeat bags before transplanting to the Nutrient Film Technique (NFT) channels of the indoor hydroponic lab- at the research center of the higronics wing of HiMedia laboratories Pvt. Ltd. - Thane, India. After transplanting, the plants were grown under controlled environmental conditions in a well-insulated facility maintained at a temperature between 22-24ºC and 63-65% relative humidity. The plants were observed every day for their growth and deficiency or infection parameters. After 20 days of transplanting, there was a very thin, loose whitish growth seen on the lower surface of the most of the healthy leaves. Those leaves were aseptically plucked and transferred into a sterile glass bottle.

Sample preparation

The faint whitish growth at the edge of the leaves was collected into 10 mL sterile saline, crushed with a sterile glass rod, and vortexed for 60 seconds. Both steps were repeated for three times. The suspensions obtained were further used for the isolation of microbiota, especially fungal population.

Isolation of mycoflora

Sabouraud Dextrose Agar (SDA) (Code: M063-SDA) was prepared according to HiMedia manufacturer's instruction using distilled water. The medium was sterilized using autoclave at 15 lbs pressure at 121°C for 15 minutes and cooled to 45-50°C. Prepared suspensions of the samples were spread on an SDA plate using a sterile glass spreader. The plates were incubated in an upright position at 37 ± 1^0C for 72 hrs. Isolated and well separated fungal colonies were selected for their identification by a polyphasic approach comprising microscopic and macroscopic studies, MALDI-TOF MS, and ITS sequencing studies.

Macroscopic and microscopic study

A macroscopic study was done by observing the growth, color, texture, and appearance of the colonies on SDA and comparing similar data from the literature. Microscopic studies of the isolate were carried out using compound microscopy at 400x and 1000x magnification using ZEISS light microscope.

Identification by MALDI-TOF MS

Generally, the cultivation tubes were inoculated with an isolate from the SDA and rotated in the rotator at 37 ± 1^0C. The tubes were incubated until sufficient fungal growth is observed. The α-cyano-4-hydroxycinnamic acid (HCCA) matrix was prepared before each series of analyses by diluting a saturated solution of HCCA in 250 µl of standard solvent (acetonitrile 50%, water 47.5%, and trifluoroacetic acid 2.5%). The entire solution was vortexed at room temperature until clear. Once removed from the rotator, the cultivation tubes were kept for10 minutes to a setting of sediment. Then filamentous fungi sediment was extracted up to 1.5 ml of standard solvent and transferred to the Eppendorf tube. 1 ml HPLC-grade water was added to the pellet and vortexed for one minute, and then it was centrifuged for 2 minutes at 13,000

rpm. After that, again supernatant was removed carefully. Then 300 µL HPLC-grade water was added to the pellet and vortexed. To that 900 µL EtOH was added to it and vortexed again. Further, it was centrifuged for 2 minutes at 13,000 rpm, and the supernatant was removed. It was centrifuged again for a few seconds and completely removed the residual ethanol. The whole pellet was dried at room temperature for a few minutes. For the dried pellet, 70% formic acid was added in a proportional to the size. The pellet was suspended thoroughly, and the same amount of acetonitrile was added. Then it was centrifuged for 2 minutes at 13,000 rpm. 1 µL of supernatant was pipetted onto a MALDI TOF target plate (Bruker Daltonics TM, Wissembourg, France). Then 1 µl of HCCA solution was overlaid and allowed to dry. For fungal identification, a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik GmbH) was used. The obtained spectra were compared with the Bruker computer database using the compass for flex control version 1.4 and MALDI-Biotyper compass version 4.1 for data analysis. The obtained results were interpreted based on the Vlek., *et al*. (2014) study on multicenter identification by MALDI-TOF MS (http://jcm.asm.org/content/52/8/3023) [10].

Identification of isolated fungi strains by ITS gene sequencing

The identification of the isolated strain based on ITS had been processed, and the report for the same was performed at NCMR-NCCS, Sait Trinity, Pashan, Pune, India. The identification report was generated using NCBI Database, and the gene of a particular isolate of each tissue extract was sequenced and presented in FASTA format. The confidence in identification was limited by both availability and the extent of homology shown by the ~550 bp sequence of the sample with its closest neighbor in the database. Finally, the sequence of isolates was compared with that of other fungus sequences by way of NCBI-BLASTn (http://blast.ncbi.nlm.nih.gov/). The results were compared with the sequence using reference Boratyn., *et al*. (2013) [11].

Results and Discussion

In the present investigation, the *Penicillium cetrinum* was isolated from the fresh leaves of strawberry (*Fragaria* x *ananassa*). The results were confirmed by polyphasic approach including macroscopic features, microscopic features, protemics and genomic way. Figure 1 showed a macroscopic examination of fungal isolates from strawberry plants (*Fragaria* x *ananassa)* cultivated by hydroponics technique. There were four isolates collected for the study from different plants grown by the hydroponics system. Macroscopic studies revealed the typical fungal colonies on SDA agar. The growth of the fungus was rapid, greyish colored with the white periphery, granular powdery colony, and the colony was pale yellow in color on the reverse side of the petriplate. The macroscopic observations matched closely with colonies of typical *Penicillium* species.

Figure 1: Growth of fungal strains isolated from strawberry plants (*Fragaria* x *ananassa*) cultivated by hydroponics technique cultured on SDA medium. 1A and 1B, rear and top view of *Aspergillus* standard ICT-HM-001A. 1C and 1D, rear and top view of *Penicillium* standard ICT-HM-001P. 1E and 1F, rear and top view of *Penicillium* strain isolated from strawberry leaves A1 strain ICT-HM-0011S. 1G and 1H, rear and top view of *Penicillium* spp isolated from strawberry leaves A2 strain ICT-HM-0012S. 1I and 1J, rear and top view of *Penicillium* spp isolated from strawberry leaves B1 strain ICT-HM-0021S. 1K and 1L, rear and top view of *Penicillium* spp isolated from strawberry leaves A1 strain ICT-HM-0022S.

Figure 2 showed a microscopic investigations of fungal isolates from strawberry leaves (*Fragaria* x *ananassa)* cultivated by hydroponics technique. The microscopic features of the organism showed septate, and hyaline hyphae. Conidiophores stipes were

attached to the septate, which are rather long and were biverticillate. Conidia are globose to sub-globose (round to off-round) and have a finely roughened surface. The organism's characteristics were metulae longer than phialides and conidia, both being spherical. The spherical spores' broom-shaped arrangement was well defined, which is typical characteristic of *Penicillium species.*

Using MALDI-TOF MS data, we noted that the isolated fungi from infected leaves were as represented in table 1. The isolates were identified to the species level with a score ranging from 1.73 to 2.10. Based on MALDI-TOF MS results all the four samples from the strawberry leaves were found to be *Penicillium citrinum*.

Sample discription	Sample ID	Closest match of the microbial ID	MALDI-TOF/ MS Score
Penicillium chrysogenum	Reference culture	*Penicillium chrysogenum*	2.36
Aspergillus niger	Reference culture	*Aspergillus niger*	1.81
Strawberry leaves A1	ICT-HM-0011S	*Penicillium citrinum*	1.91
Strawberry leaves A2	ICT-HM-0012S	*Penicillium citrinum*	1.73
Strawberry leaves B1	ICT-HM-0021S	*Penicillium citrinum*	1.75
Strawberry leaves B2	ICT-HM-0022S	*Penicillium citrinum*	2.10

Table 1: Identification of fugal isolates results by MALDI-TOF/MS.

Figure 2: Microscopic examination of fugal isolates from strawberry plants (*Fragaria* x *ananassa*) cultivated by hydroponics technique. Where 2A is Microscopic structure of isolates from ICT-HM-0011S on SDA media (400X), 2B is Microscopic structure of isolates from ICT-HM-0021S on SDA media (400X), 2C is Microscopic structure of isolates from ICT-HM-0011S on SDA media (1000X) and 2D is Microscopic structure of isolates from ICT-HM-0021S on SDA media (1000X)

As all the four samples showed the same outcome upto species level, we further carried out ITS sequence of the strain ICT-HM-0022S sample to understand its closeness to other related species. Results showed the comparison of the top hits from the GenBank database in table 2. It was concluded that the isolate ICT-HM-0022S (A_AUG_18_139.00474) is closest to *Penicillium citrinum.* Using basidiomycete identification, the species level resulted 100% similarity with the reference organism of *Penicillium citrinum*. The sequence of an isolate from strawberry leaves was displayed in the phylogenetic tree in figure 3. The evolutionary history was inferred using the Neighbor-Joining method. NCBI BLAST was performed using ITS sequence generated for sample A_AUG_18_139. First ten closely related sequences were downloaded for construction of phylogenetic tree using MEGA X(ref). The sequences were aligned using Clustal W – multiple sequence alignment tool. The alignment data was converted to MEGA format and a N-J tree was constructed by bootstrap method (n = 500). The optimal tree with the sum of branch length = 0.09824682 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. This phylogenetic analysis showed that the culture has closest match with *Penicillium citrinum* sequence [12].

Culture code	PNR	Sample name	Closest Neighbor	Accession number	% Similarity
ICT-HM-0022S	A Aug18 139	Strawberry leaves	*Penicillium citrinum* NRRL 1841	NR_121224.1	100

Table 2: Identification of fugal isolates results by ITS gene sequencing.

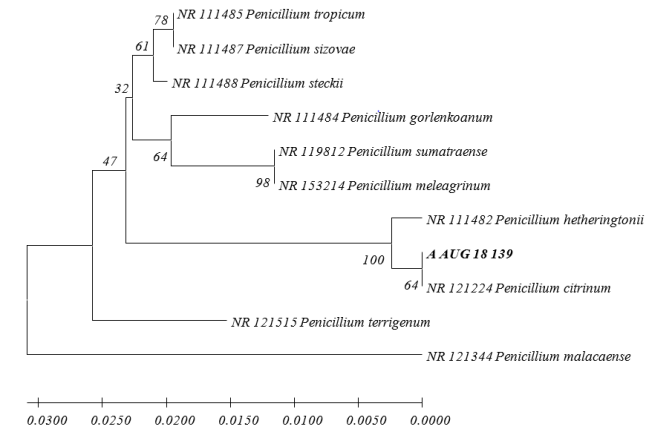

Figure 3: Nucleotide sequences from the genera isolated from strawberry leaves phylogenetic tree.

Results from this study indicated that there was no visible bacterial growth from the plucked healthy strawberry leaves, while in the SDA visible fungus growth occurred. Based on morphological, MALDI-TOF MS, and ITS gene sequencing, the isolates were identified as a strain of *Penicillium citrinum.* Similar result were reported in healthy roots, stems, leaves, and fruits of the *Scoparia dulcis Linn* [13]. The *Penicillium citrinum* is distributed worldwide and isolated from nearly all food types, especially cereals, and can grow up to 37 °C temperature [5]. Dutta., *et al*. (2007) reported *Penicillium citrinum* exists in various conditions including agricultural fields, forest soils, spices, and indoor environments [14].

There were some reports that showed that *Penicillium citrinum* isolates were extracted from wheat, rice, ray, corn, barley, oats, peanuts, amaranth, and soybean [15,16]. Whereas others explained that it was present in leaves, stems, and roots of coffee plants [17], roots of Ixeris repens [18], coconut milk, coffee beans, compost, peanuts [19], dried vine fruits [20], grapes [21,22], cashews, copra, sorghum, soybeans [23,24], dried fish and numerous other food products [5]. Some research work indicated that *Penicillium* produces plant growth factors, and moreover strains of *P. citrinum* were reported to produce a secondary metabolite [25]. The beneficial effect of *Penicillium citrinum* on the plant was its ability to synthesize gibberellins [18], citrinolactones A and sclerotinia C [26], which act as growth promoters. A report states that citrinin induces swarming motility of *Paenibacillus polymyxa* a growth promoting rhizobacterium [27]. Even though this is the first report on isolation and description of *Penicillium citrinum* from hydroponically grown strawberry leaves. There were many studies related to *Penicillium cetrinum* isolation from leaves of multiple shoots, compared to those from callus specially grown in soil under controlled indoor environments. The occurrence of *P. citrinum* and its report by us on healthy strawberry leaves suggests its possible role as water-stress releaver. The exact mechanism need to be explored further.

Conclusion

In conclusion, the fungi isolated from the healthy leaves of strawberry plants are confirmed to be strains *Penicillium citrinum.* The occurrence of this organism was mainly depends on the environment conditions maintained inside the hydroponic chamber. Strains of *P. citrinum* were known to produce a mycotoxin such as citrinin and hydrolytic enzyme like cellulase and endoglucanase, as well as xylulase. These may play role of protecting plant parts from attack by most common fungal species of aspergillus, mucor and Trichoderma. Some strains of the *P. citrinum* are reported to produce a secondary metabolite, having beneficial interaction with the plants that helps in producing gibberellins which act as growth promoters. The study on biological activity of this organism and its potential application as an elicitor in the secondary metabolite production are required.

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