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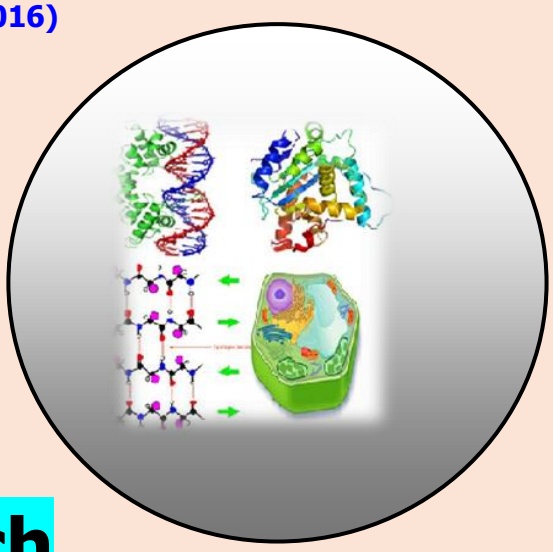

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# Plant Growth Promotion and Antimicrobial Study of Endophytic Fungi Isolated from *Limonia acidissima*

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**ABSTRACT**

***From the Limonia acidissima plant parts (root, stem bark and leaves) 31 isolates were obtained and out of that 3 endophytic fungi (Aspergillus flavus FR14, Aspergillus fumigatus FS11 and Aspergillus flavus FL3) were studied. These fungi were subjected to plant growth promotion activity (PGPA) and preliminary phytochemical screening. From the results, it was found that they have promising effect in the growth of plant by producing plant growth hormone Indole acetic acid (IAA) and ammonia and also has potential to produce various secondary metabolites having ability to control human and plant pathogens. The outcome of this entitled study, offers promising information for the potential utilization of these remarkable endophytic fungus and its components in the biotechnological, medicinal, and agricultural industries.***
***Keywords: Limonia acidissima Endophytic Fungi, Plant Growth Promotion, Antibacterial, Antifungal and Phytochemical.***

## INTRODUCTION

Endophytic microorganisms produce natural bioactive compound and have ability to kill or inhibit a wide range of harmful disease causing agent including fungi, viruses, phytopathogen and other bacteria and protozoa that affect human and animals (Muzzamal *et al*., 2012). The association between endophytes and plant is often mutualistic. In response to environmental stress, co-evolution may exist between endophytes and their host. Endophytic microorganisms can be isolated from sterilized surface of plant tissue. Primarily, endophytes enter into plant tissue through root zone. Aerial portion of plant such as flowers, stems and cotyledons may also be used for entry by endophytes. Inside a plant, endophytes may either become localized at the point of entry or spread throughout the plant Munif, 2012). Fungal endophytes like *Ascomycota* or their anamorphs are the colonizers for plants and algae and those often being identified as *Orchid mycorrhizes* (Rungjindamai, 2008). In an oil palm, *Elaeis guineensis* which is a Thai plantation found to be positive for *Basidomycetous* harbouring in healthy leaves, petioles and rachis of the plant. Now for the first-time fungi such as *Tetraploa aristata, Phoma tropica, Monodicyts castaneae, Acremonium terricola* and *Penicillium glandicola* were reported as prominent fungi as endophytes (Bezerra *et al*., 2012). Fungi as an endophyte being classified as per phylogeny and life history traits as *Clavicipitaceous (C)* which generally infects grasses and the *non Clavicipitaceous endophytes* (*NC-endophytes*) which can grow in asymptomatic tissues of non-vascular plants, conifers, ferns and allies and angiosperms has also been reported (Rodriguez *et al*., 2009). At the present moment human is putting intense focus on the natural products, natural medicines so that artificial drugs should be replaced (Malviya *et al*., 2012). As it is known that either whole plant or parts of it has a good source of medicinal, therapeutic or savoury qualities, it is advisable to promote such possible medicinal plants as a source of new drugs. In a

response now, whole world is exploring hidden potential of plants world (Daniyanand Muhammad, 2008). In Indian plant, *Limonia acidissima* considered as one of the vital medicinal plants with potential to carry new drug like properties to control number of diseases. *Limonia acidissima* is a member of family Rutaceae, is a large tree imparts number of medicinal properties such as antimicrobial, antifungal, astringent and also the anti-inflammatory activities. In addition, whole plant parts or its preparation also has been useful as insecticides, antirodent in animal husbandry in some cases (Shermin *et al*., 2012). Our previous study shows that almost all the parts of *L. acidissima.* Plant was found to be rich in phytochemicals when tested with non-polar and polar solvent (Petroleum ether, chloroform, acetone and methanol) extracts of root, stem bark and leaves. The prominent presence of alkaloids, carbohydrates glycosides, proteins, Saponins, Phytosterols, fixed oils and fats, Phenolic compound and flavonoids, gum and mucilages were reported (Pranita *et al*., 2014). Studies aimed at investigate endophytic fungi from *L. acidissima* for the expression of PGPA, bioactive compounds and its related antimicrobial activity when tested under *in vitro* conditions.

## MATERIAL AND METHODS

**Plant collection**

In an order to detect endophytes plant parts such as root (R), stem bark (S) and leaves (L) of *L. acidissima* were collected from Nagpur region (Maharashtra, India) and brought to the laboratory.

**Isolation of bacterial and fungal endophytes from plant**

In the present study, promising endophytic fungi were isolated from the plant, *L. acidissima* root, stem bark and leaves parts. In a process, freshly collected plant was washed in running water and checked for any injury as an exclusion criterion. In a process, plant parts were diced into small pieces and then treated with surface sterilizing agent such as 70% ethanol for 30 seconds and washed with sterile distilled water two times, it was then treated with 0.1% mercuric chloride for 2 minutes and once again washed with distilled water for 3 times. To confirm the disinfection protocol, final rinse of 0.5ml water used as an inoculum on Potato dextrose agar (PDA). All other sterilized plant parts then collected in sterile Petri plates separately. Plant parts were then cut it into small transverse sections and plated on the plates and incubated at 22$^{o}$C for 4-5 days for fungal growth. After incubation, all isolates were sub-cultured to purify on the same fresh medium

**Morphological characteristics**

Microscopic examination of fungal isolates was carried out by using lactophenol cotton blue staining.

**Plant Growth Promoting Activity (PGPA)**

After successful isolation of fungi on the PDA medium; all isolates were checked for the possible plant growth promoting activity by screening them with three tests such as Indole acetic acid, Ammonia production and Phosphate solubilisation and promising isolates were selected further.

**A. Indole 3 acetic acid (IAA) production test**

Fungal isolates were allowed to grow on potato dextrose broth supplemented with 200µg/ml of L-tryptophan, at 30$^{o}$C for 48 hours. After incubation, growth was centrifuged at 3000 rpm for 10mins and in 2ml of supernatant two drops of ortho phosphoric acid was added. It was then mixed and added with 4ml of the Salkowsaki's reagent (50ml, 35% of perchloric acid, 1ml of 0.5M $FeCl_3$ solution). In a result, progression of pink colour in tube indicated positive result. Formation of pink colour was measured as 535nm by using spectrophotometer along with the standard (Bric et al 1991).

**B. Phosphate Solubilisation**

Fungal isolates were separately inoculated centrally on the Pikovskaya's medium and plates were incubated at 30$^{o}$C for 3-6 days. The clear zone around the colony considered as positive test for phosphate solubilisation ability (Gaur 1990).

**C. Ammonia Production**

Fungal isolates were tested for the production of ammonia. In requirement, peptone water (10ml) was inoculated with fresh culture and incubated at 30$^{o}$C for 48-72 hours. In a peptone water 0.5ml of Nessler's reagent was added and checked for brown to yellow colour which has indicated positive test for ammonia production (Cappucino and Sherman, 1992).

**Phytochemical analysis by endophytic fungi**

In this study, loopful agar culture of fungi was inoculated in Potato dextrose broth (PDA) and incubated for 2-4 days at 22$^{o}$C.

After incubation, 1ml of the medium of fungi were inoculated to 10ml Czapek Dox broth and incubated for 2-3 days at 27°C. After incubation, 1ml from this medium was transferred to 250ml of Czapek Dox broth and samples kept for growth on a rotary shaker at 27°C at 150rpm. Optical density was measured every 2 hours at 620nm. Similarly change in pH was also recorded for the culture media three times in 24 hours. During study, peak point optical density was considered as the stage of life cycle where maximum Phyto-active compounds may get synthesized and hence for every sample peak point was considered as a stage of sample selection and withdrawal. These samples were further tested for preliminary phytochemical analysis and antimicrobial study also been done especially against plant and human pathogens.

**Phytochemical analysis**

Phytochemical analysis of fermented broth was also tested to detect Alkaloids, carbohydrates, Glycosides, Proteins and amino acids, Saponins, Phytosterols, fats, Phenolic compounds, flavonoids, gums and mucilages proposed by N. Raaman (Raaman, 2006). Extracts were then subjected to antibacterial and antifungal assays.

**Antimicrobial activity of the fungal filtrates against human and plant pathogens**

In a protocol, the broth culture of the promising isolates was centrifuged at 3000 rpm to obtain cell free supernatant.

**Antibacterial and Antifungal activity**

In an antibacterial and antifungal activity of filtrate, Czapek dox agar medium was used. Further upon solidification 0.1ml of 0.5 O.D. McFarlands standard (1.5x $10^8$ CFU/ml). Inoculum was inoculated on medium and spread evenly on the medium. Thereafter, 10mm wells were punctured with the help of sterile borer. In an empty well 100µl of cell free supernatant of each sample was inoculated and allowed to diffuse for 2 hours without disturbing in refrigerator. Plates were then incubated at 35±2°C for 24 hours and zone of inhibition was recorded in milli-meter.

In an order to screen the effect of the fungal broth of *L. acidissima* of root, stem bark and leaves against human pathogens *E. coli* (NCIM- 2065), *Bacillus subtilis* (NCIM- 2063)*, S. aureus* (NCIM- 5345)*, Shigella boydii* (NCIM- 5644)*, Pseudomonas aeruginosa* (NCIM- 2200) and *Salmonella abony* (NCIM- 2257) and in case of fungi it was *Aspergillus brasiliensis* (NCIM- 1106) and *Candida albicans* (NCIM- 3471) were considered. In addition, plant pathogens such as *Xanthomonas campestris 1, Xanthomonas campestris 2, Rhizoctonia bataticola* and *Scetrotium rolfsii* was also tested by well diffusion method in Czapek dox agar medium.

## RESULT

**Isolation of fungal endophytes from plants**

Presence of endophytic microorganisms was investigated when surface sterilized *L. acidissima* root, stem bark and leaves part were inoculated on to the potatoes dextrose agar. After incubation, numbers of colonies were appeared to be emerging out of the plant part. It is clear that fungal isolates which were endophytic in nature are prominently remained present in the plant parts. For the repeatability and reproducibility of isolates, all these isolates were subjected to sub cultured on the same media and were considered as endophytes.

**Morphology study**

Plant *L. acidissima* also found to be prominent for fungal endophytes which were successfully evidenced by their morphological features and available diversity. As per study, root part showed the presence of 13 isolates; likewise stem part was detected with 10 fungal isolates and leaves part with 8 fungal isolates

**Plant growth promoting activity (PGPA)**

Total 31 isolates of fungi were retrieved from for root, stem bark and leaves were tested for PGPA features.

As per IAA test, only one isolate from each part such as root, stem bark and leaves was found to be promising for IAA value as FR14- 0.16, FS11- 0.05 and FL3- 0.10 respectively as shown in Graph 1, 2 and 3.

In a test of phosphate solubilisation it has been observed that all fungal isolates (n=31) found to be negative for the said features and ammonia production was positive for all the isolates.

Overall result indicated that only three fungal isolates were found to be promising with PGPA features and hence those only tested further. Those were identified as FR14, FS11 and FL3.

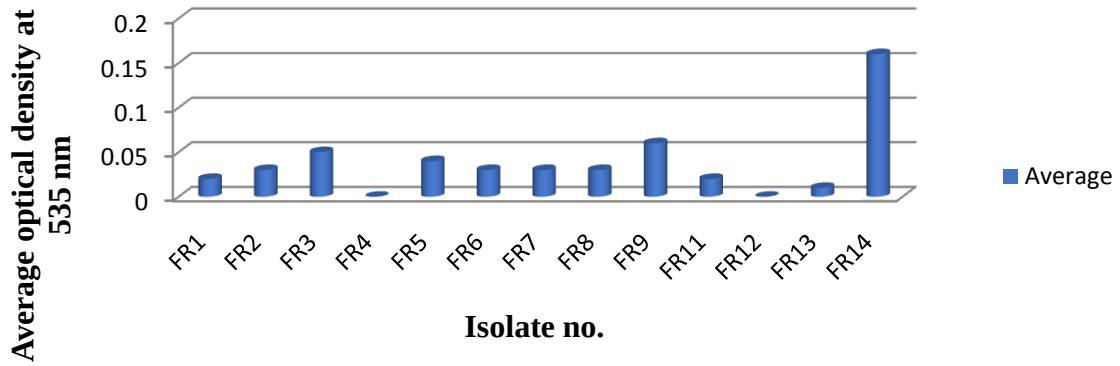
**Identification of fungal species**

As per morphological analysis and staining results fungal species were identified as under,

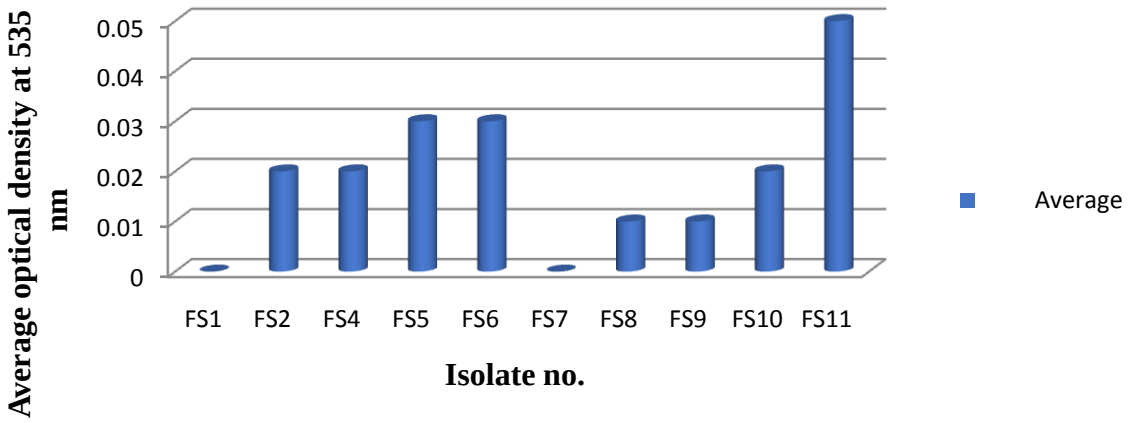
FR14- *Aspergillus flavus*; FS11- *Aspergillus fumigatus* and FL3- *Aspergillus flavus*

Herafter, FR14 referred as *Aspergillus flavus* FR14; FS11 as *Aspergillus fumigatus* FS11 and FL3 as *Aspergillus flavus* FL3.

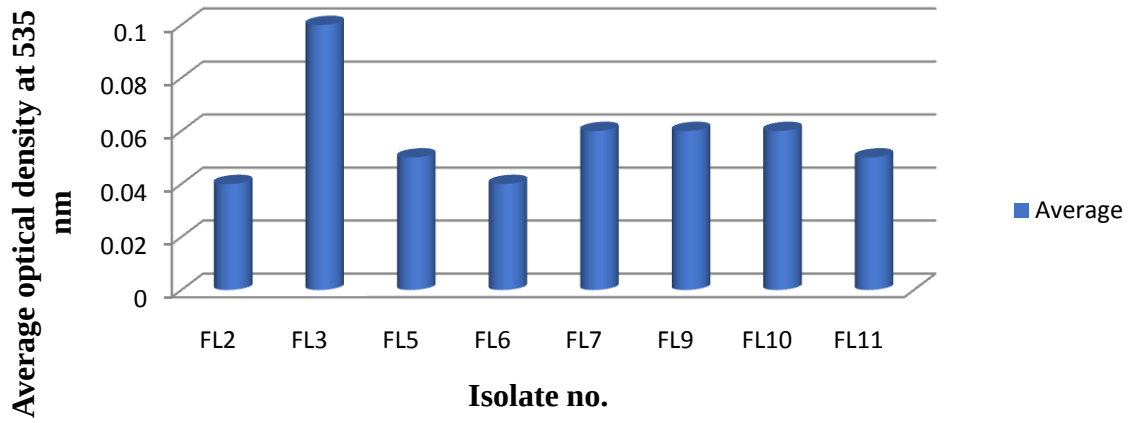
**Graph 1 - Indole Acetic Acid Production by Endophytic Fungal of Roots**




**Graph 2 -Indole Acetic Acid Production by Endophytic Bacteria of Stem bark**




**Graph 3 -Indole Acetic Acid Production by Endophytic Bacteria of Leaves**



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**Fungal Growth analysis**

Fungal growth of isolates was recorded up to seven days using colorimeter at 620nm to get the maximum growth optical density (O.D). During investigation it has been observed that isolate *Aspergillus flavus* FR14 was found to be at its peak point growth on 5$^{th}$ day with 0.53 (O.D). Other two fungal isolates *Aspergillus fumigatus* FS11 and *Aspergillus flavus* FL3 was found to be maximum at its growth on day six with 0.31 and 0.65 O.D. respectively.

**Phytochemical analysis**

Ability of fungal species those have been promising for PGP activity were tested further for the production of bioactive Phyto-chemical compounds when allowed to grow Czapek Dox broth. As per phytochemical test, fermented broth of each fungal isolate was recorded for the features as shown below: -

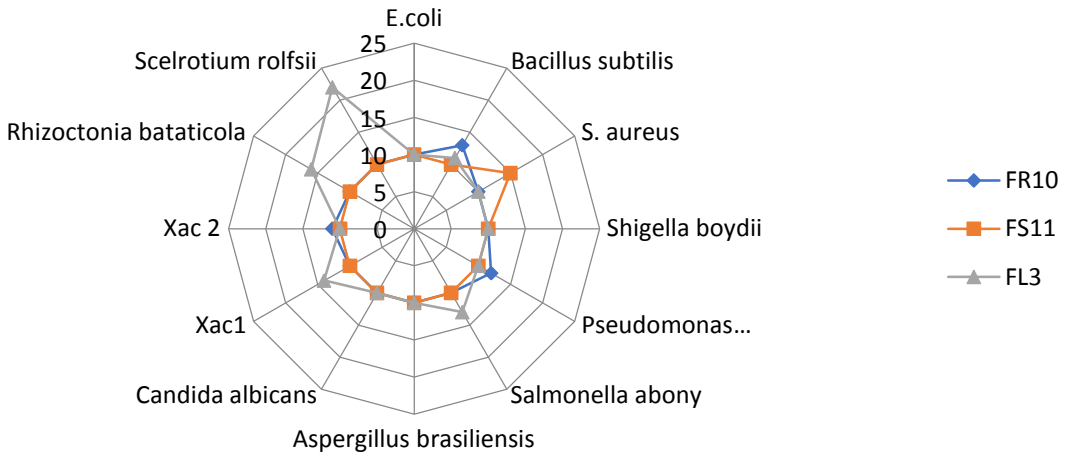
| Sr. No. | Phytochemical test | *Aspergillus flavus* FR14 | *Aspergillus fumigatus* FS11 | *Aspergillus flavus* FL3 |
|---|---|---|---|---|
| 1 | Alkaloids | ++ | + | ++ |
| 2 | Carbohydrates | +++ | +++ | +++ |
| 3 | Glycosides | - | - | - |
| 4 | Proteins and amino acid | + | + | ++ |
| 5 | Saponins | + | + | + |
| 6 | Phytosterols | + | + | + |
| 7 | Fats | +/- | +/- | +/- |
| 8 | Phenolic compound and flavonoids | ++ | + | + |
| 9 | Gum and Mucilages | + | - | + |

Foot note: "+" positive result; "_" negative result; -/+: not clear for result; Increasing + number indicate higher degree of reaction.

**Antimicrobial activity of fungal filtrate**

Ability of fungi to produce number of bioactive compounds as an antibacterial and antifungal agent has been systematically investigated in Hi-sensitivity agar medium against six human bacterial pathogens such as *E. coli, Bacillus subtilis, S. aureus, Shigella boydii, Pseudomonas aeruginosa* and *Salmonella abony* and in case of fungi it was *Aspergillus brasiliensis* and *Candida albicans.* In other approach plant pathogens like bacteria *Xanthomonas campestris 1, Xanthomonas campestris 2,* along with fungi as *Rhizoctonia bataticola* and *Sclerotium rolfsii* were tested successfully for the antimicrobial effect.

**Graph 4 - Antimicrobial activity of filtrate of endophytic fungal isolated from root, stem bark and leaves of *Limonia acidissima* against human and plant pathogens**



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**Human pathogen antimicrobial activity**

Human bacterial pathogens when tested with culture broth by a well diffusion assay it has been observed that *S. aureus* remained intermediate susceptible to the *Aspergillus fumigatus* FS11 with 15mm zone of inhibition. *Salmonella abony and Bacillus subtilis* was intermediate sensitive to the *Aspergillus flavus* FL3 filtrate with 11mm and 13mm zone of inhibition, respectively. Other than these isolates, *E. coli, Shigella boydii Pseudomonas aeruginosa* and fungus *Aspergillus brasiliensis* and *Candida albicans* found to be resistant and no growth inhibition was evident as shown in Graph 4.

**Plant pathogen antimicrobial activity**

Effect of endophytic fungal filtrate when tested on the plant's bacterial and fungal pathogens, filtrate of fungi *Aspergillus flavus* FL3 found to be the most effective as it has been controlling the growth of bacteria *X. campestris* 1 and 2 and fungi *R. bataticola* and *S. rolfsii* with 14mm, 10mm, 16mm, and 22mm zone of inhibition, respectively. In another set, filtrate of fungi *Aspergillus flavus* FR14 inhibited the growth of *S. rolfsii* intermediately as shown in Graph 4.

## DISCUSSION

In the present study, fungal endophytic presence has been confirmed in *L. acidissima* by using specific medium for their growth such as potatoes dextrose agar (for fungi). In a Number of methods where endophytes are sampled from plants has been cultured on potato dextrose agar (O kubo *et al*., 2009). Since, we are able to culture most of the endophytes by sub culturing on the given media.

In the present study, once the growth of colonies recorded on media, we confirmed that endophytes are prominently present in *Limonia acidissima* stem bark, leaves and root as fungi but their exact count diversity cannot be confirmed as many probably remained uncultured.

In the present study with the successful isolation of endophytes ability to provide essential growth factors/nutrients to the parent plant has been investigated.

In set of PGP study, isolated fungi (31 isolates) were successfully investigated for PGPA. Here one isolates *Aspergillus flavus* FR14, *Aspergillus fumigatus* FS11 and *Aspergillus flavus* FL3 from roots, stem bark and leaves found to be better IAA producer. In similar report Waqas *et al*., (2012) reported the promising effect of fungi as gibberellins and indole acetic acid producer when isolated as endophytes. They identified fungi as *Phoma glomerata LWL2* and *Penicillium sp. LWL3* able to host rice plant. Khan *et al*., (2012) reported another fungal strain *Paecilomyces formosus LHL10* able to produce phytohormone indole acetic acid and resultant significant enhancement in cucumber shoot length was recorded when inoculated. Hoffman *et al*., (2013) reported that endophytic fungi *Pestalotiopsis aff. neglecta, Xylriales* able to produce IAA and its expression enhances in presence of bacterium*, Luteibacter sp., xanthomonodales* which remain dependent on L-tryptophan dependent pathway.

All fungi found to be negative for phosphate solubilization while, ammonia production recorded positive in all isolates. In a similar report, Hassan (2017) reported the fungi like *Penicillium chrysogenum* and *Penicillium crustosum* able to produce ammonia along with indole acetic acid and could promote the growth of medicinal plant *Teucrium polium L*. In a similar way, number of fungi reported to be the producer of ammonia such as *Lecanicillium psallioate* (Senthil Kumar *et al*., 2018); *Purpureocillium lilacinum* (Cavello *et al*., 2015) *Variovorax boronicumulans CGMCC 4969* (Lin *et al*., 2013); *Trichoderma harzianum* (Cai *et al*., 2013); and *Trichoderma gamsii (NFCCI 2177)* (Rinu *et al*., 2014).

Overall result with fungal PGPA showcased that three fungal isolates present its importance (FR14, FS11, FL3) and used in the study successfully for further analysis.

In the present study three fungal isolates *(Aspergillus flavus* FR14, *Aspergillus fumigatus* FS11 and *Aspergillus flavus* FL3) as endophytes of *L. acidissima* were identified.

*Aegle marmelos* also remain positive for fungus *A. flavus L7* as an endophyte able to produce flavonoids and phenolic as major bioactive compounds. They were found to be 65.77mg GAE/ml and 158.33mg quercetin/ml of crude extract, respectively. These compounds reported to involve in antibacterial activity along with antifungal features (Patil *et al*., 2015). Li *et al*., (2012) confirmed the *A. flavus* as an endophytic fungus of *Melia azebarach* and able to produce thirty-nine fungal metabolites including two new alkaloids, 3-hydroxyfumiquinazolin A and 12β-hydroxy-13α-methoxyverruculogen TR-2. These compounds found to be broad spectrum antifungal in nature and able to control *Botrytis cinarea*, *Alternaria alternata, Colletotrichum gloeosporioides, Fusarium solani* and others.

Khan *et al.,* (2011) reported that *A. fumigatus sp. LHO2* isolated as endophytic fungus of rice can produce Gibberellins. Upon testing by co-inoculation on Waito-C rice plant growth promoting activity of fungus has been confirmed. The results were more promising as significant increased shoot length, leaf area, shoots fresh and dry biomass, chlorophyll content was recorded.

In a next set of study, fungi were allowed to grow in Czapek dox medium and its overall growth pattern has been understood successfully. On the basis of data, *Aspergillus flavus* FR14 showed the peak point on the 5$^{th}$ day and on the 6$^{th}$ day *Aspergillus fumigatus* FS11 and *Aspergillus flavus* FL3 showed the peak point.

Study further recorded the potential of fungal extract filtrate producing phytochemical, antibacterial and antifungal compounds.

In the culture broth of endophytic fungi of roots, stem bark and leaves as *Aspergillus flavus* FR14*, Aspergillus fumigatus* FS11 and *Aspergillus flavus* FL3, presence of alkaloids, carbohydrates, proteins and amino acid, saponins, Phytosterols, fat, phenolic compounds, gums and mucilage were observed. Although glycoside was found to be present only in broth of endophytic fungi of stem bark. Scientist Wu *et al*., (2012) also been reported the saponin producing endophytic fungi sampled from *Aralia elata* capable of controlling *S. aureus*, and product has been confirmed through HPLC.

Culture broth of endophytic fungi also been tested for the antibacterial as well as antifungal activity against human and plant pathogens. Here filtrate *Aspergillus fumigatus* FS11 inhibited *S. aureus*; filtrate *Aspergillus flavus* FL3 inhibited *Salmonella abony* and *Bacillus subtilis* growth. On a similar platform endophytic fungi of conifers found to be positive for antimicrobial activity, anti-inflammatory activity, and recorded for many bioactive compounds (Stierle and Stierle, 2015). *X. campestris* 2 was found to be unaffected whereas, *Scelrotium rolfsii* found to be sensitive against *Aspergillus flavus* FL3 filtrate.

## CONCLUSION

In recent time with the concept of sustainable development isolation, identification and characterization of potent plant endophytes and its use in commercial aspect remain the top priority. In addition, here we proposed that, plant also posses' number of fungal endophytes producing several bioactive compounds which was guaranteed to control the growth of human or plant's pathogens. These isolates also assured to be imparting plant growth promoting features by producing Indole acetic acid and ammonia production, making them complete plant growth supporting microbes.

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**Competing Interests**

The authors declare that they have no competing interests.

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