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Survey of Phyllosphere & Endophyte Mycoflora Isolated from *Adhatoda vasica* Nees and Estimation of Some Secondary Metabolites

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ABSTRACT

Isolation and enumeration of phyllospheric and endophytic fungal diversity were carried out from the medicinal plant, *Adhatoda vasica* Nees. Occurrence of phyllosphere fungi shows a total number of 3 fungal species (A*spergillus sp.*, *Penicillium sp.*, and *Cladosporium cladosporioides*). A total number of 4 (*Alternaria alternata, Curvularia lunata,* Mycelia sterilia1 and *Aspergillus niger*) and 3 (*Alternaria alternata, Mycelia sterilia* 2 and *Penicillium sp.*) fungi were isolated from leaf and stem respectively. *Alternaria alternata* shows to be the highest colonizer in leaf which was followed by *Curvularia lunata. Mycelia sterilia* 2 represented highest colonization frequency in stem. Production of total phenol, and antagonistic activity were carried out with the dominant endophytic isolates from leaf and stem. *Alternaria alternata* shows the highest production of phenol and highest extent of antioxidant activity. The dominant isolates failed to show any antagonistic activity against the gram positive and gram negative bacteria. The presence of some phytochemical compounds, available in *Adhatoda vasica*, was also tested qualitatively in the vegetative body of the dominant endophytic isolates.

Keywords: *Adhatoda vasica,* Phyllospheric fungi, Gram positive, Mycoflora, Phenol, and Phytochemicals.

INTRODUCTION:

The medicinal plants are of common use in traditional treatment of various diseases (Salie *et al.,* 1996; McGraw *et al.,* 1997). Herbal medicines have been widely practiced trough out the world from ancient time. These medicines are safe and environment friendly. Nearly 80% of the world population depends upon traditional system of health care (Hutchigson *et al.,* 1996). Parts of medicinal plants are traditionally used for the treatment of antifungal, antitumor, anthelminthic, antidiuretic, antiulcerative, diseases of heart, rheumatic pains, chest pain, dyspepsia, fever, diabetes, burning of liver and kidney diseases (Verma *et al.,* 2011). Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flower, leaves, roots that are rich in nutrient and fibers to protect against diseases. Phytochemicals are of two types-primary and secondary, based on their function. Primary phytochemicals comprise common sugar, amino acids, protein and chlorophyll while secondary phytochemicals consists of alkaloid, terpenoid, phenolic compounds, flavonoids; tannins, coumarin, anthroquinone etc act as good radical scavenging activities. They function by either preventing the formation of the free radicals or by inhabiting them before they can damage the cellular components (Igor *et al.,* 2017). Vascular plants in general, harbor phyllosphere and endophytic organisms. The fungi which reside in the internal part of plant tissues are called endophytes and those residing over the leaf surfaces

are recognized as phyllosphere fungi. The phyllosphere is a complex terrestrial habitat that is characterized by a variety of microorganisms including bacteria, filamentous fungi, yeast and algae growing on the surface of leaves (Moitinho *et al.,* 2020).

There are two groups of phyllosphere fungi residents and casuals. On healthy leaf surface residents can multiply, but causal cannot grow. The host remains unaffected in both cases without any adverse effect. It has been estimated, rather roughly that the total area of leaf surface on this earth is about 1 billion square kilometer and it supports about a large population of bacteria along with fungi and actionmycetes and algae. Biochemical environment of phyllosphere is mainly determined by leaf leachates, microbial activity and dusts falling on leaf surface. The composition of the leaf exudates depends on the age of the leaf and plant, therefore microbial population on phyllosphere varies with age. The leachates contain amino acids, organic acids, minerals and carbohydrates (glucose, fructose and sucrose). The phyllospheric microorganism's produce IAA, vitamins, different enzymes, antibiotic substances, nitrogenous substances formed through the fixation of nitrogen and other metabolites. There is an extracellular polysaccharide (EPS) deposited on the leaf surface. EPS causes formation of heterogenous aggregates of different bacterial groups and fungi. The EPS slime provides protection to the microorganisms from desiccation, reactive oxygen species (ROS) and other stress factor. The organisms found on phyllosphere are called epiphytes (Vorholt 2012; Sharif *et al*., 2019; Mazinani *et al.,* 2017).

Phyllospheric microorganisms play an important role in controlling plant disease through antagonistic activity of non-pathogenic microbe against a pathogen. Pathogens present on phyllosphere causes production of elicitors that will induce resistance in plants. The pathogen triggers production of phytoalexins in the plants to create defense. Since phyllosphere microorganisms promotes growth of plants either by restricting pathogenic attack or by providing nutrients or both, the growth and quality of roots will increase. Ever increasing human population has failed to meet their need by plant based medicine exploiting huge amount of plant. To meet this demand researchers are involved in searching alternative means of bioactive compound. Now a days it becomes evident that endophytes produces the bioactive compounds which are used in the treatment of human diseases (Onifade, 2007). About one million endophytic species present in plants (Shekhawat *et al.,* 2010). The endophytic fungi have a symbiotic relationship with host (Shekhawat *et al.,* 2010) and thus it does not cause any harmful effect to the host (Saithong *et al.,* 2010; Wei *et al.,* 2007; Arnold *et al.,* 2003; Selvanathan *et al.,* 2011) found that fungal endophyte provides bioactive stress tolerance of the host plant creating defense against pathogen (Khan *et al* 2010). The fungal endophyte differs depending on geo-graphical location (Fisher *et al.,* 1994; Collado *et al.,* 1999), age of plant and plant parts (Khan *et al.,* 2010; Sahashi *et al.,* 2000; Shahen *et al.,* 2019; Clay & Schardl, 2002).

In medicinal plants fungal endophyte harbor and provide biotic stress tolerance (Zhang *et al.,* 2006; Strobel, 2002; Krishnamurthy *et al.,* 2008). In our current research, the distribution of endophytic fungi was isolated and identified from *A. vasica* Nees. (Acanthacae) stems and leaves. The endophytic fungi are an important source of various secondary meta-bolites. It contains a bioactive compound which is useful for pharmaceutical industries (Strobel 2002; Krishnamurthy *et al.,* 2008; Khan *et al.,* 2010; El-hawary *et al.,* 2020). Extensive researches have been done on the bioactive compounds of fungal endophyte, such as, antitumor agents, Taxol (Stierle *et al.,* 1993; John *et al.,* 2018), antibacterial and antifungal agents plant growth factors, enzymes, insecticidal agents, immune-suppressive compounds and antioxidants (Strobel & Daisy, 2003; Owen & Hundley, 2004; Firoz *et al.,* 2016; Okezie *et al.,* 2020). Therefore the present studies have concentrated to the phylloplane and endophyte Mycoflora of a well-known medicinal plant *A. vasica* Nees to the family Acanthaceae. The experiments with the dominant fungal endophytes and fungal colonizers of phyllosphere isolated from *A. vasica* Nees were carried out along the following lines: Estimation of total phenol; Estimation of antioxidant activity; Determination of antimicrobial activity; Qualitative determination of –

a) Terpenoids; b) Cardiac glycosides; c) Saponins; d) Flavonoids; d) Anthroquinones; e) Coumarins; f) Alkalo

MATERIALS AND METHODS:

Isolation of Phyllosphere Organism and Endophytes.

Phylloshere Organism

Sample collection

Fresh middle aged healthy leaves of the plant *Adhatoda vasica* were collected carefully from our college garden and immediately placed in sterile plastic bags and brought to the laboratory.

Determination of leaf surface area:

The surface area of the leaf was determined by placing the leaf on a graph paper (mm) followed by marking the outline of the leaf. The surface area of the leaf was calculated in cm^2.

Isolation of fungi

Leaves ($60cm^{2)}$ were put into 250ml Erlenmeyer's flasks containing 100ml of sterilized solution (prepared by mixing 98ml distilled water with 2 ml surfactant, tween 20). Three replicates were maintained. The Erlenmeyer's flasks were then put under shaking condition for 2 hours at medium speed (90 rpm.) by using rotary shaker to release the leaf surface micro-organisms into the solution. After 2 hours the three solutions obtained were mixed together by using vortex. The mixture was then diluted to 10^{-2} concentration. 1ml of the diluent was taken and put into the sterilized Petri plate. Followed by pouring of sterilized PDA (Potato Dextrose Agar) media with Streptomycin sulphate (100units/ml) the Petriplates were rotated clock wise, anti-clock wise and moved to and fro for proper mixing. Three replicates were maintained. The Petriplates were then incubated at 30°C for 7 days.

Interim inspection was done after 3 days and 5 days for appearance of the fungal colonies. The colonization frequency of each isolate was determined following Hata & Futai, 1995. From the subculture each isolate are grown in Petri plate containing CDA by placing the inoculum in the center of the Petriplate. For each isolate two Petriplates were prepared-one for study colony characters and the other for microscopic studies. The media in the plate for microscopic study was inserted with sterilized cover slip (3Nos) at 1.5cm away surrounding the inoculum at 1.5 cm distance from the inoculum. The cover slip are taken out on 3rd, 5th and 7th day after inoculation and was placed upside down on a slide containing a drop of lacto phenol and cotton blue mixture. The cover slip was sealed with wax and observed under microscope for identification.

Endophytes

The collected materials were washed with running tap water for 30 minutes and then washed with sterilized distilled water for 2-3 minutes, surface sterilization of the materials was done by 90% ethanol (for 1 minute), followed by 3% NaOCl (8 minutes) and finally by 90% ethanol(30 sec) and then washed with sterilized distilled water (2-3 times). After surface sterilization processes, leaf discs (0.5cm ϕ) were prepared by using sterilized cork borer. Four leaf discs were then transferred to the Petri plates containing sterilized PDA (potato dextrose agar) medium with Streptomycin (100units/ml) to isolate endophytic fungi. Same process was followed in stem (stem was cut into pieces of 1cm). The plates were then incubated at 30◦C for a period of 10days. The organisms that come out of plant materials was isolated and subcultured in slants.

Identification

The isolated phylloplane organism and fungal endophytes were identified based on characteristics and available reproductive structure following (Burnett & Hunter, 1998; Uddin *et al*., 2016; Whatnabe, 2010) and internet information.

Relative colony frequency of phyllosphere organism

The relative colony frequency (RCF %) of a single colony in the plate was calculated by the following formula adopted by Hata & Futai (1995, pp 384-90) after necessary modifications.

$$\text{RCF \% in } 10^{-2} \text{ diluent} = \frac{\text{The no. of individual fungal colony}}{\text{Total no. of fungal colonies}} \times 100$$

Population Frequency of Fungal Isolates in Phyllosphere

Population frequency was estimated by determining number of each fungal isolate/cm^2 of phyllosphere.

Colonization Frequency for Endophyte

The colonization frequency (CF %) for a single endophyte in leaf and stem tissue was calculated by the formula of Hata & Futai, 1995.

$$\text{CF\%} = \frac{\text{The no. of segments colonized by endophyte species}}{\text{Total no of segments}} \times 100$$

Preparation of Fungal Extract

The test fungi were grown separately in 500 ml Erlenmeyer flask containing 250 ml of Czapek's dox broth

(CDB). The flasks were inoculated with test fungus maintaining more or less uniform inoculum potency and incubated at 30◦C for 20 days. The flasks were subjected shaking for 4 hours on a rotary shaker.3 replicates were maintained for each set. The culture filtrates were obtained by filtering through Whatman no.1 filter paper placed on a Buchner funnel under condition of vacuum filtration. After extraction the culture filtrates were used to perform different biochemical tests.

Estimation of Total Phenol

Total phenolic content was estimated in each test sample following the protocol of Bray and Thrope, (1954). A standard curve was prepared using different concentration of catechol as standard. To prepare standard curve, 100 mg catechol solution was dissolved in 100ml of distill water. For working, 2 ml stock solution was diluted 10 times with distilled water. From this working standard, different aliquots viz- 0.2ml, 0.4ml, 0.6ml,0.8ml was taken in separate test tubes, therefore, they contained 20µg, 40µg, 60µg, 80µg of phenol respectively and their volume was adjusted to 3ml for each tube with distilled water. In a separate test tube, 3ml of culture filtrate of unknown strength was added with 0.5ml of Folin ciocalteu reagent. After 2-3 minutes, each test tube was added with 2ml of 20% Na_2CO_3 solution. After thorough mixing, all the test tubes were heated for exactly 1 minute in water bath and cooled at room temperature. The absorbance was measured on a spectrophotometer at 650nm.

Estimation of Total Antioxidant activity

The culture filtrates of each fungus grown in CDB media for 21 days were obtained by filtering through Whatman filter paper (No.1). 20 ml of culture filtrate of each fungus was evaporated and weighed. The residue was mixed with 5ml methanol to use for determining the total antioxidant activity. For this, 1ml sample was added with 2ml reagent solution (prepared by mixing Ammonium molybdate, 4mM; Sodium phosphate, 28mM and Sulphuric acid, 0.6M in a ratio 1:1:1). 3 replicates were maintained for each sample. The reaction mixture was incubated for 60 minutes at 30˚C. The absorbance was then measured on a spectrophotometer at 695 nm. Reducing capacity of the extract was expressed as the ascorbic acid (standard) equivalent.

Antagonistic Activity

Antagonistic activity of the fungal endophytes was determined by antibacterial assay against both Gram positive and Gram negative bacteria using agar cup method. Gram positive bacterium tested was *Bacillus subtilis* and Gram negative bacterium tested was *E. coli*.

Agar cup method

The agar cups (1cm diameter) were prepared by using sterilized cork borer in nutrient agar plate's pre inoculated with bacterial suspension of Gram positive and Gram negative bacteria separately. The cups were then filled with culture filtrate aseptically using 1ml pipette. For each three replicates were maintained in each set. The Petriplates were kept in an incubator for 72 hours at 30˚C and the zone of inhibition (if formed) was measured.

Qualitative Analysis of Bioactive Compounds of *Adhatoda vasica* and Fungal Endophytes

Processing of samples

Leaves of *Adhatoda vasica* plant were properly washed with running tap water for 20 minutes. It was then washed with sterilized distilled water for 2-3 minutes. The rinsed leaves were dried in an oven at a temperature around 60°C to obtain constant dry weight. The dried leaves were pulverized by using a sterile electric blender and stored in airtight glass container, protected from sunlight until required for analysis. The mycelium (of selected dominant endophytes) harvested after 20 days of growth in CDB media, dried at 60˚C until getting constant dry weight. The subsequent steps were similar as above. Bio-chemical tests were done with aqueous ethanolic extract of the powdered specimen by using standard protocol to identify the constituents (Harborne, 1973; Sofoware, 1993; Trease & Evans, 1989).

Test for Terpenoids (Salkowski test)

Extract (5 ml) was mixed with 2ml of chloroform, and concentrated sulphuric acid (3ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results of terpenoids.

Test for Cardiac Glycosides (Keller Killani test)

Extract (5 ml) was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution and

1ml of concentrated sulphuric acid. A brown ring of the inter face was formed to show the results of cardiac glycosides.

Test for Saponins (Frothing test)

Powdered sample (2g) was boiled with 20ml of distilled water in a water bath. The mixture was filtered. 10ml of the filtrate is mixed with 5ml of distilled water in a test tube and shaken vigorously for 15 minutes to develop a stable persistent froth. The froth is then mixed with 3 drops of olive oil. Formation of emulsion suggests the presence of saponins.

Test for Flavonoids

The aqueous filtrate (1ml) was mixed with 5ml dilute NaOH in a test tube. An intense yellow colour was appeared in the test tube. It becomes colourless on addition of a few drop of dilute sulphuric acid indicates the presence of flavonoids.

Test for Anthraquinones

To 1 ml of aqueous plant extract add a drop of benzene and ammonia. A pink colour appears, indicates the presence of anthraquinones.

Test for Coumarins

To 2 ml of aqueous plant extract add 10% sodium hydroxide. A yellow colour appears indicates the presence of coumarins.

Test for Alkaloids

To 5 ml of aqueous plant extract add 10ml methanol, 1% (w/v) HCl and Wagner's reagent (6 drops). A creamish or brownish red or orange precipitate appears indicates the presence of alkaloids.

RESULTS AND DISCUSSION:

Phyllosphere fungal organisms

The fungal organisms isolated from phyllosphere, are *Aspergillus sp.*, *Penicillium sp.* and *C. cladosporioides* (**Table 1**). Among the isolated organism *Penicilluim sp.* shows highest population ($1.66\times10^3/cm^2$). This was followed by *Aspergillus sp.* ($1.16\times10^3/cm^2$) and *C. cladosporioides* ($0.66\times10^3/cm^2$) in descending order. The relative frequency of the isolated phyllosphere fungi was similar to population of organism. Thus *Penicillium sp.* represented highest frequency (47. 61%) and *C. cladosporioides* represented lowest frequency (19.04%) and the intermediate frequency was represented by *Aspergillus sp.* (33.33%).

Fungal endophytes

The endophytes were isolated both from the leaf discs and stem bits using PDA culture plates. Four (4) fungal endophytes were isolated from leaf discs and three (3) from stem bits (**Table 2**). The frequency of fungal endophytes in leaf discs and stems bits is low, which may be due to their inability to grow in PDA media. Among the 4 fungal endophytes of the leaf discs *A. alternata* showed higher colonization frequency (50%). This was followed by *C. lunata* (16.66%) and jointly by *M. sterilia* and *A. niger* in descending order. Among the fungal endophytes from stem bits Mycelia sterilia was found to be dominant as revealed from its colonization frequency (33.33%), which was followed by *A. alternata* (22.22%) and *Penicillium sp.* (11.11%) in descending order. The characteristics features noted in isolated phyllospere fungal organism and fungal endophytes were as follows:

Phyllosphere fungal organisms

***Aspergillus sp.* (Fig. 3)**

Growth restricted (2.9cm/7days), upper surface of the colony is differentiated into central dull white followed by a sporulation zone which is blue green in colour; finally there is a white ring of margin colony adpressed to the media and compact. Reverse brownish white at the center surrounded by a whitish periphery presence of no hyaline margin. Hyphae loosely arranged, no aggregation of hyphae forming tuft. Vesicle club-shaped, sterigmata in 2 whorls, conidiophore smooth surfaced long septate (length 299-356 μm, breadth 4.21-4.93μm), basal sterigmata ranges 14.97μm to 21.32μm and upper sterigmata ranges from 18.93μm to 19.03μm, vesicle length 12.42μm and breadth 9.04 μm. spores round, surface spiny (3.97-4.03μm).

***Penicillium sp.* (Fig. 4)**

Growth restricted (4cm/-7days), upper surface of the colony highly greenish yellow or lemon in colour. Reverse orange to orange yellow in color with hyaline margin, colony compact adpressed to the media. Hyphae loosely arranged hyaline, septate. Conidiophore branched bearing 2 (12.00 to 12.23μm and 11.96 to 12.33μm) to 3 (10.26, 10.91 and 10.56μm), each branch terminating in a whorl of sterigmata (9.36 – 10.33μm), each sterigma bear a chain of conidia. Long conidiophore with septa (51.70 to 52.24μm-length; 2.34 to 2.51μm - breadth). Conidia round (2.32μm) or

oval (length-2.31-2.81µm; breadth-2.58µm), smooth surfaced.

***Cladosporium cladosporioides* (Fresenius) (Fig. 5)**
Growth restricted (3.5cm/13days), upper surface of the mycelia mats adpressed to the media, central inoculum portion elevated, olive-brown in color, uniform outline, velvety and tufted. The edges of the mycelia are olive-grey to white, and feathery. The colonies are diffuse and the mycelia form mats and rarely grow upwards on the surface of the colony. Reverse Dark brown, margin hyaline, compact. Hyphae branched; darkly-pigmented hyphae are not constricted at the septal region. Mature conidiophores are treelike and comprise many long, branched chains of conidia. It produces brown to olive-brown coloured, solitary conidiophores that branch irregularly, forming many ramifications. The sporophores (length- 9.26 to 9.76µm; breadth- 4.31- 4.41µm) are thin-walled and cylindrical and are formed at the end of ascending hyphae. The conidia are small, lemon-shaped and smooth-walled, bi-celled (11.81-12.68µm).

Leaf endophytes

***Alternaria alternate* (Fr.) Keissler (Fig. 6)**
Growth moderate (3.5cm/5days), upper surface of the colony brownish grey followed by narrow greyish margin, extreme margin is more or less uniform in outline, extreme center at the point of inoculum dark brown in color presence of ill-defined concentric zones. Reverse greyish brown with concentric zonation. Colony ad pressed to the media very slightly floccose. Hyphae light brown in color, short septate.

Conidiophore short, septate (length 54.76µm and breadth 4.21µm). Conidia borne single on conidiophore. Conidia brown in color, presence of trans-verse and longitudinal septa. Number of transverse septa is 2-6 and longitudinal septum is 1-2. Conidia club shaped, round at the apex (length 31.141µm-31.22µm and breadth 10.99µm-13.66µm).

***Curvularia lunata* (Walker) Boedijn (Fig.7)**
Growth restricted (4.5cm/5days), upper surface of the colony grey colored compact margin white and narrow extreme margin hyaline with uniform outline. After 10 days, upper surface of the colony is velvety in appearance elevated from the media, greenish grey in color, and presence of concentric rings. Grayish white ring present in between the central and peripheral part. Reverse light grayish brown with a dark narrow zone or ring, margin white or hyaline. The extreme center at the point of inoculum is dark brown in color or grayish brown in color with concentric ring. Hyphae loosely arranged, medium septate. Conidia straight or curved (length- 24.96µm to 26.20µm and breadth- 9.10µm to 9.74µm). Second cell from the tip is large. Conidia 3, septate, brown. Conidiophore short, short septate, smooth (length- 141.38µm to 141.68µm and breadth- 3.84µm to 4.91µm). Conidia are borne at the tip of the conidiophore.

***Mycelia sterilia* 1 (Fig. 8)**
Growth moderate (3.3cm/5days), upper surface of the colony cottony and compact in appearance, marginal part is more compact than the center part, white in color extreme margin thin and hyaline, margin outline is narrow or less irregular. Reverse very light, creamy white. Hyphae thick walled, short septate bearing profuse chlamydospores (length- 7.25 to 7.75µm and breadth- 5.52 to 6.51µm).No other spores were found (breadth of hyphae- 2.96 to 3.55µm).

***Aspergillus niger* van Tieghem (Fig. 9)**
Growth rapid (6.4cm/5days), upper surface of the colony adpressed to the media slightly floccose, central part black, margin broad white to hyaline, margin outline more or less uniform. Reverse whitish at the center which was followed by a hyaline zone, broad margin white in color. Conidiophores (199.35-250µm) septate, smooth, vesicle round and sterigmata present in single whole, sterigmata7-9µm in length. Spores globose, diameter 3.90-4.59µm.

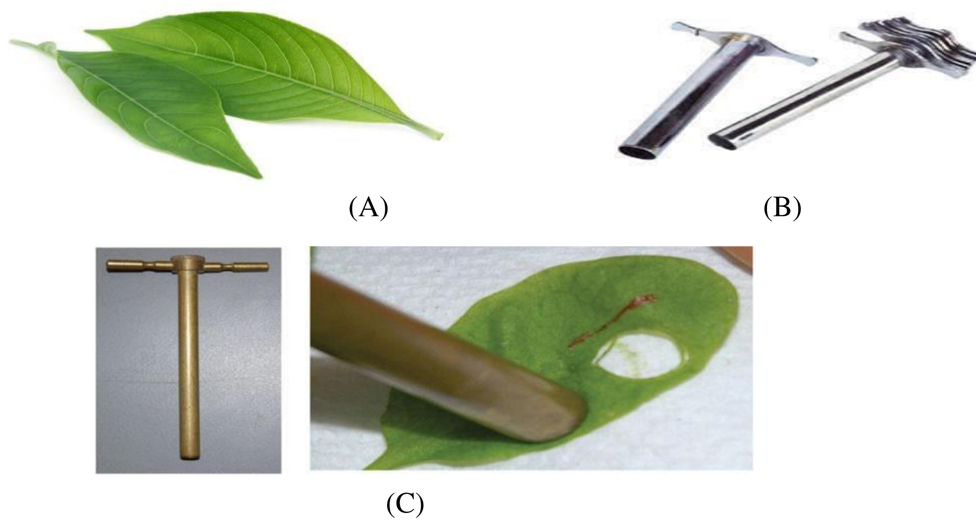
Stem endophytes

***Mycelia sterilia* 2 (Fig. 10)**
Growth very rapid (8.4cm/2days), colony at upper side is hyaline, for a diameter of 3cm followed by cottony white elevated mycelia zone represented by 1-1.2cm zone subsequently hyaline margin with scattered white cottony hyphae. Reverse reddish white, while as the inoculum region is reddish brown. Hyphae loosely arranged thin; no chlamydospore or other spores were found; Breadth of hypha 1.76 to 1.88µm.

***Penicillium sp.* (Fig. 11)**
Growth restricted (2.5/7days), upper surface of the colony slightly elevated from the media, olive green in

color, central inoculum portion slightly elevated and dark in color, margin white, hyaline, margin irregular. Reverse light brown, margin white, hyaline, central inoculum part dark brown in color. Conidiophore (69.86µm) unbranched bearing a whorl of phyallide (10.87, 11.57, 13.21µm) at the tip i.e. monoverticilate. Spores are smooth walled and oval (length- 4.44 to 4.50µm and breadth- 3.14 to 3.32µm). Phyllosphere mycoflora was extensively studies by a number of researchers (Vacher *et al.*, 2016; Chauhan & Navneet, 2015; Angela & Shri, 2016; Nayak & Anandhu, 2017). Occurrence of *Aspergillus sp.* in the phylloplane is considered as very common (John & James, 2017). *Penicillium sp.* in phylloplane was reported by (Chauhan & Navneet, 2015; Pandey *et al.*, 1993; Nayak and Anandhu, 2017; Hilber & Bodmer, 2017). *C. cladosporoides* as phyllosphere mycoorganism was reported by (Hussain *et al.*, 2015). Existence of fungal endophytes have been studied by a number of researchers (Gond *et al.*, 2012; Potshangbam *et al.*, 2017; Ilic *et al.*, 2017; Bhattacharyya *et al.*, 2017). Presence of *Alternaria alternate* as fungal endophyte was reported by (Murthy *et al.,* 2011).

Curvularia lunata as endophyte was reported by (Larren *et al.*, 2002) from Soya bean leaf. Mycelia sterilia was reported by Cai *et al.* (2004). *Penicillium sp.* was reported by John *et al.* (2017). *A. niger* is considered as a very common endophyte (Khan *et al.*, 2010). It is evident from **Table 3** that *A. vasica* contained terpenoids, cardiac glycosides, saponins, flavonoids and alkaloids as revealed from qualitative assay and showed no occurrence of anthroquinons & coumarins. All the endophytes subjected to qualitative test for the above mentioned seven biochemical constituents. It has been found that *A. alternata* and *C. lunata* were able to produce terpenoids, saponins and alkaloids. *A. alternata* alone have the capability to produce cardiac glycosides. *M. sterilia* 2 also produce saponins and alkaloids. As revealed from **Fig. 12** that the total phenol content among the fungal endophytes tested shows highest in *A. alternata* (19.97 mg/100ml), followed by *C. lunata* (7.72 mg/100ml) and *M. sterilia* 2 (3.9 mg/ 100ml) in descending order.



(A) (B)

(C)

Fig. 1: (A) Leaves of *Adhatoda vasica*, (B) Cork borer, (C) Leaf disc preparation.

(A) (B)

Fig. 2: (A) Growth of fungal endophytes from leaf discs, (B) Growth of fungal endophytes from stem bits.

Phenol being antioxidant their production by fungal endophytes has significance in the practical field. Murthy *et al.* (2011) reported efficient products of phenolics by numerous fungal endophytes. Endophytes are closely related with their host plant in stress tolerance through production of phenolics (Huang *et al.,* 2007; White & Torres 2009). Total anti-oxidant activity (**Fig. 13**) of the culture filtrate of fungal endophytes tested was evaluated by Phospho-Molybdate method (Murthy *et al.*, 2011). In culture filtrate, the Mo (VI) is reduced to Mo (V) and as a result of green colored phosphor-molybdenum-(V)-complex is formed, which shows maximum absorbance at 695nm. All the organisms tested showed anti-oxidant activity within a range in between 25.9g equivalent and 8.01g equivalent to Ascorbic acid. The highest level of antioxidant activity among endophytes was found in *A. alternata* which is followed by *C. lunata* (11.41g) and *M. Sterilia* 2 in descending order. It is noteworthy to mention that the organism *A. alternata* which shows maximum phenol content is capable in showing maximum anti-oxidant activity. Similar such positive correlation exists in *C. lunata* In *M. sterilia* 2 however, the phenol content was found to be low while antioxidant activity is high. This lack of correlation indicates that the antioxidant activity is possibly contributed by the other antioxidant constituents. Such positive correlation was also put forwarded by (Murthy *et al.,* 2011; Huang *et al.,* 2007).

Phyllospheric fungal organisms

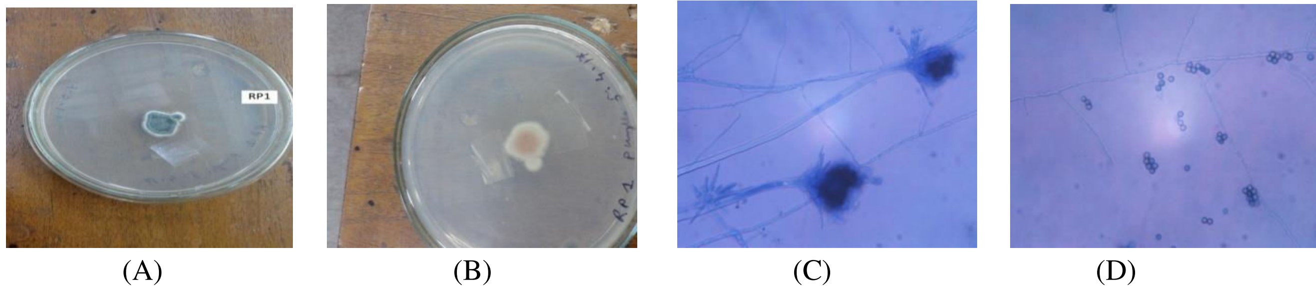


Fig. 3: *Aspergillus* sp. (A) Upper surface of the *Aspergillus* colony, (B) Lower surface of the *Aspergillus* colony (C) Microscopic view *of Aspergillus* Conidia with conidiophores, (D) Microscopic view *of Aspergillus* Conidia.

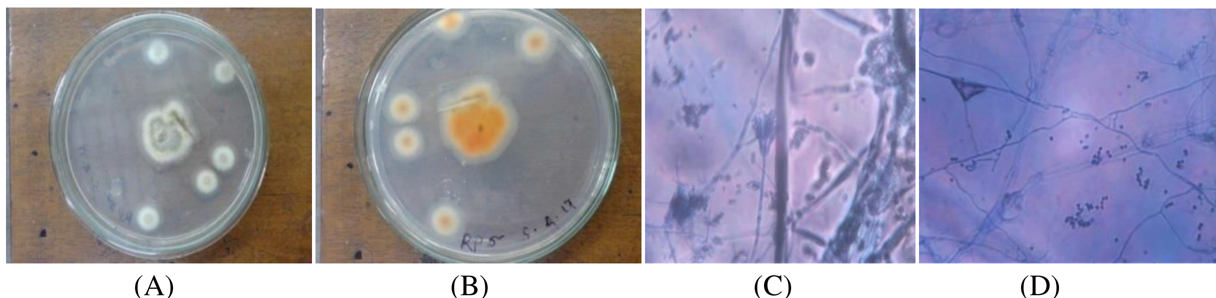


Fig. 4: ***Penicillium*** sp. (A) Upper surface of the *Penicillium* colony, (B) Lower surface of the *Penicillium* colony, (C) Microscopic view *of Penicillium* Conidiophore, (D) Microscopic view *of Penicillium* conidia.

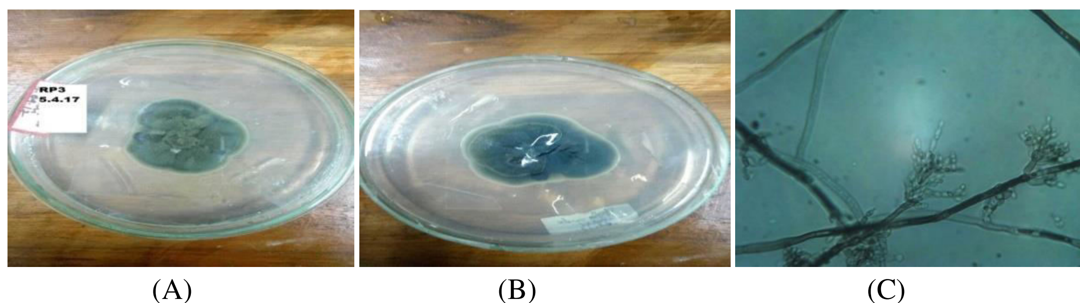
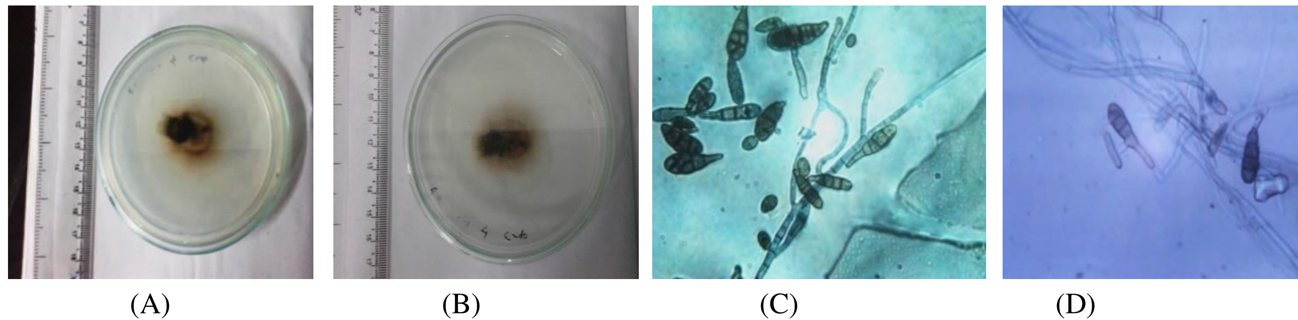


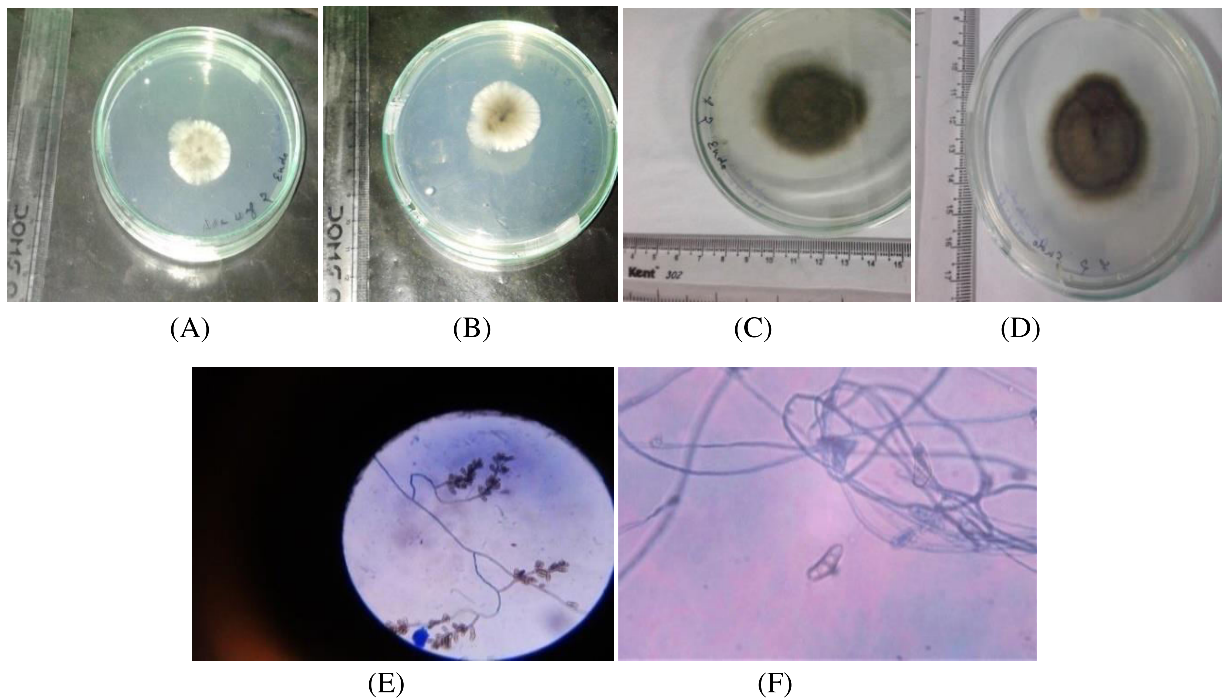
Fig. 5: *Cladosporium cladosporioides,* (A) Lower surface of the *C. cladosporioides* colony, (B) Upper surface of the *C. cladosporioides* colony, (C) Microscopic view *of C. cladosporioides* Conidiophore with conidia.

Fungal endophytes



(A) (B) (C) (D)

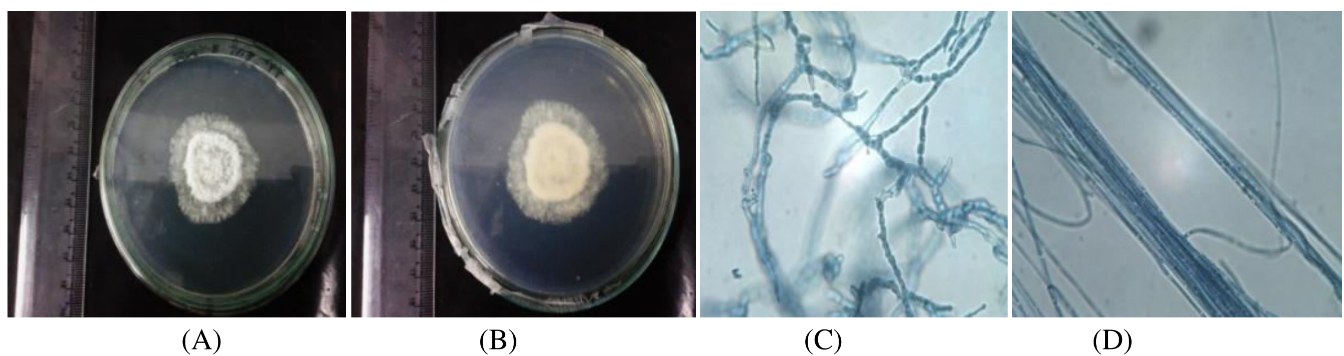
Fig 6: *Alternaria alternate*, (A) Upper surface of the *A. alternata* colony (After 5 days), (B) Lower surface of the *A. alternata* colony (After 5 days), (C) Microscopic view of *A. alternata* Conidia with conidiophores, (D) Microscopic view *of A. alternata* Conidia showing germination.



(A) (B) (C) (D)

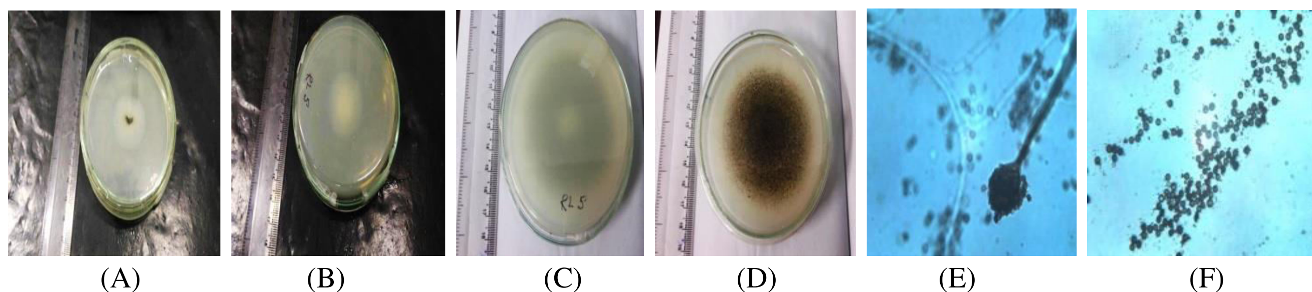
(E) (F)

Fig. 7: *Curvularia lunata*, (A) Upper surface of the *C. lunata* colony (After 5 days), (B) Lower surface of the *C. lunata* colony, (C) Upper surface of the *C. lunata* colony (After 10 days), (D) Lower surface of the *C. lunata* colony, (E) Microscopic view of *C. lunata* conidiophore with conidia, (F) Microscopic view of *C. lunata* conidia.



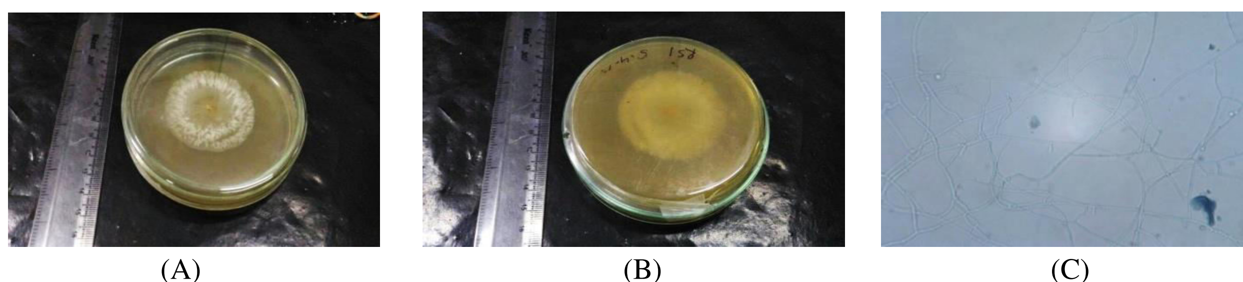
(A) (B) (C) (D)

Fig. 8: Mycelia sterilia 1, (A) Upper surface of the *M. sterilia* 1 colony, (B) Lower surface of the *M. sterilia* 1colony, (C) Microscopic view of formation of Chlamydospores in *M. sterilia* 1, (D) Microscopic view of tuft of hyphae in *M. sterilia* 1.



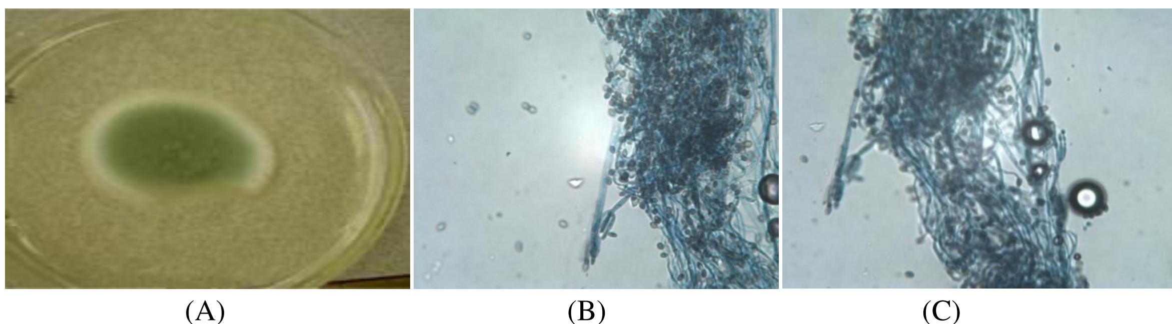
(A) (B) (C) (D) (E) (F)

Fig. 9: ***Aspergillus niger***, (A) Upper surface of the *A. niger* colony (After 5 days), (B) Lower surface of the *A. niger* colony, (C) Upper surface of the *A. niger* colony (After 10 days), (D) Lower surface of the *A. niger* colony, (E) Microscopic view of *A. niger* conidiophore with conidia, (F) Microscopic view of *A. niger* conidia.



(A) (B) (C)

Fig. 10: Mycelia sterilia2, (A) Upper surface of the Mycelia sterilia2colony, (B) Lower surface of the Mycelia sterilia2colony, (C) Microscopic view of the Mycelia sterilia2hyphae.



(A) (B) (C)

Fig. 11: ***Penicillium sp***. (A) Upper surface of the *Penicillium sp.* colony, (B) & (C) Microscopic view of *Penicillium sp.* conidiophore with conidia.

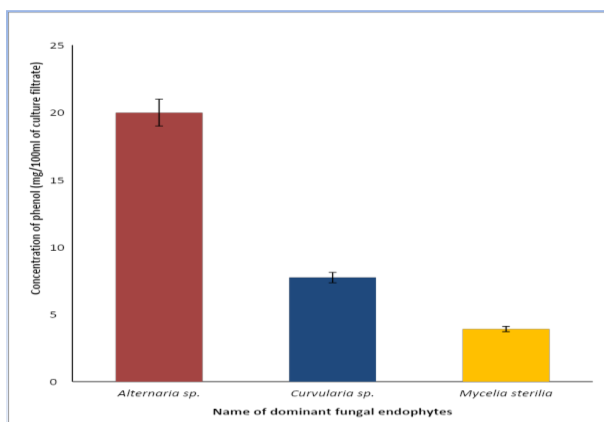


Fig. 12: Total Phenol Content in the Culture Filterate of Dominant Fungal Endophytes.

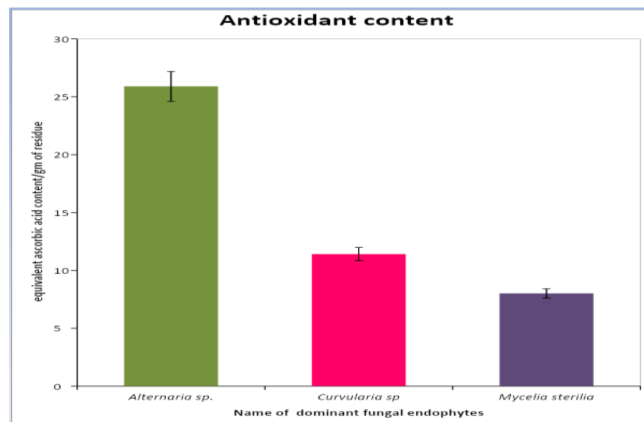


Fig. 13: Total Antioxident Activity of Dominant Endophytic Culture Filterate.

Table 1: Determination of Relative Colony Frequency and Population Frequency of Fungal Organism in Phyllosphere.

Srl no.	Fungal Organism Isolated From The Phyllosphere	Total No Of Fungal Colony	No Of Individual Organism Or Colony	Relative Colony Frequency (%)	Population Frequency Of Fungal Organisms/CM^2 Leaf
1.	*Aspergillus sp.*	21	7	33.33%	1.166×10^3
2.	*Penicillium sp.*		10	47.61%	1.66×10^3
3.	*C. cladosporioides*		4	19.04%	0.66×10^3

Table 2: Colonization Frequency of Fungal Endophytes.

Srl. No	Fugal Organisms Isolated From The Plant	Types of Plant Parts Used	Number of Discs or Bits Used	Number of Discs or Bits Showing Fungal Growth	Colonization Frequency (%)
1.	*A. alternate*	Leaf disc	12	6	50%
2.	*C. lunata*			2	16.66%
3.	*M. sterilia* 1			1	8.34%
4.	*A. niger*			1	8.34%
1.	*M. sterilia* 2	Stem bits	9	3	33.33%
2.	*A. alternata.*			2	22.22%
3.	*Penicillium sp.*			1	11.11%

Table 3: Qualitative Tests for Some Biochemical Constitutes of *A. vasica* and Dominant Fungal Endophytes.

Biochemical constituent	*Adhatoda vasica*	*Alternaria alternata*	*Curvularia lunata*	*Mycelia sterilia* 2
Terpenoids	+	+	+	+
Cardiac glycosides	+	+	_	_
Saponins	+	+	+	+
Flavonoids	+	_	_	_
Anthroquinones	_	_	_	_
Coumarins	_	_	_	_
Alkaloids	+	+	+	+

Table 4: Antagonistic Activity.

Name of the fungal endophytes tested	Zone of inhibition (cm) by agar cup method against	
	Bacillus subtilis	*Escherichia coli*
Alternaria alternata	-	-
Curvularia *lunata*	-	-
Mycelia sterilia 2	-	-

The antagonistic activity of endophytes is tested against *B. subtilis* (Gram positive) and *E. coli* (Gram negative) following Agar cup method. The culture filtrate obtained from the broth culture of endophytes showed no zone of inhibition against the bacteria (both gram positive and negative) (**Table 4**).

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CONFLICTS OF INTEREST:

The authors express no conflict of interest to carry forward this research finding to publish in this journal.

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