

Original Research Article

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## Studies on Epiphytic Microflora on Apple (*Malus domestica* Borkh.) Fruits and Leaves

Aqleema Banoo\*, Efath Shahnaz, Saba Bandy, Rovidha S. Rasool and Taibah Bashir

Division of Plant Pathology, Sher-e-kashmir University of Agriculture Science and  
Technology of Kashmir, Shalimar Campus, Srinagar 190025, India

\*Corresponding author

### ABSTRACT

The present study was undertaken with an objective to estimate predominant epiphytes from leaves and fruits of apple by using different methods on different media. In all, eleven epiphytes were isolated using potato dextrose agar, nutrient agar and yeast maltose agar media. Amongst them, five fungal isolates viz., *Aspergillus* sp. (I<sub>1</sub>), *Penicillium* sp. (I<sub>2</sub>), *Fusarium* sp. (I<sub>3</sub>), *Rhizopus* sp. (I<sub>4</sub>) and *Alternaria* sp. (I<sub>5</sub>) and six bacterial isolates viz., *Pseudomonas* sp. (I<sub>6</sub>), *Pseudomonas* sp. (I<sub>7</sub>), *Bacillus* sp. (I<sub>8</sub>), *Bacillus* sp. (I<sub>9</sub>), *Staphylococcus* sp. (I<sub>10</sub>) and *Micrococcus* sp. (I<sub>11</sub>) were predominantly noticed under all the three methods (leaf impression, serial dilution and fruit washing) and were hence, used for further studies. The highest average colony count of 3.62 colonies/cm<sup>2</sup> was recorded in fruit washing method followed by leaf impression (3.17) and lowest in serial dilution method (2.12).

#### Keywords

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### Introduction

Apple (*Malus domestica* Borkh.) is one of the important temperate fruit crops in world. It belongs to family Rosaceae and sub family Pomoidae and has originated in temperate region between Black Sea and Caspian Sea (Bal, 2006). Plant associated fungi and bacteria isolated from rhizoplane and phylloplane surfaces are called as epiphytes

(Andrews and Harris, 2000). The epiphytic microorganisms are the microorganisms that are naturally present on the surfaces of fruits and vegetables, and can be isolated to be used for the control of postharvest pathogens as naturally occurring antagonists (Janisiewicz, 1987; Sobiczewski *et al.*, 1996). Bacteria are the dominant microbial inhabitants of the phyllosphere, occurring in concentrations of 10<sup>2</sup> to 10<sup>12</sup> cells/gram of leaf, although

archaea, filamentous fungi and yeasts may also be important (Whipps *et al.*, 2008). The epiphytic microorganisms existing on fruits and leaves of fruit trees were the object of studies conducted by Janisiewicz and Roitman (1988), Wisniewski and Wilson (1992), Sobiczewski and Bryk (1995). Janisiewicz *et al.*, (2001) reported the feasibility of using mixtures of bacterial and yeast antagonists for the control of *P. expansum* on apples and observed an increment in the control of blue mould by mixtures of yeasts with different nutritional profiles. He also reported that the mechanism by which microbial antagonists suppress the postharvest pathogens is still unknown, but competition for nutrients and space, production of antibodies, direct parasitism, and induced resistance are most widely accepted modes of their action. In another research, Manso and Nunes (2011) isolated epiphytic microorganism (*Metschnikowia andauensis*) from the surface of leaves of different pome and citrus fruits. Studies on the phylloplane micro flora of safflower by Mandhare and Suryawanshi (2009) revealed that the bacterial population was more than that of fungi in both the cultivars of Bhima and NARI-NHH-2 at various growth stages *viz.*, seedling, flowering and physiological maturity stages of crop. He also reported that the mean fungal population was highest at flowering stage ( $5.32 \times 10^2/\text{cm}^2$  leaf area), whereas, the bacterial population was more at physiological maturity ( $32.15 \times 10^2/\text{cm}^2$  leaf area). Fungal and bacterial populations were highest on phylloplane of cultivar 'Bhima'. studies on phylloplane micro-organisms as bio-control agents for postharvest disease management is lacking. Hence, keeping in view the above facts, present investigation shall be undertaken with the following objectives: To estimate predominant epiphytes from leaves and fruits of apple by using different methods on different media.

## **Materials and Methods**

### **Estimation of predominant epiphytes from leaves and fruits of apple**

#### **Collection of leaf and fruit samples**

For the estimation of epiphytes, an orchard at Harwan, which had not been treated with fungicides for the last five years was selected. A group of seven trees, centrally located in the apple orchard was sampled. Fifty leaves were collected randomly from each tree, bulked, composited and taken immediately to the laboratory in sterilized perforated polythene bags for further studies. By using leaf area meter (Systronics leaf area meter 211) surface area of each leaf was measured and the average leaf area was expressed in  $\text{cm}^2$ . Similarly, fifty healthy blemish-free apple fruits were collected randomly from each apple tree in sterilized perforated polythene bags and brought to the laboratory for further studies. Length and diameter of collected healthy fruits were measured with the help of a digital Vernier caliper and an average fruit length and diameter were expressed in centimeters (cm).

#### **Isolation of epiphytes by leaf impression method**

The composited and selected leaves were washed with sterilized 70 distilled water in order to remove dirt and dust and were air dried. Dorsal and ventral surface of each leaf was pressed momentarily against the surface of potato dextrose agar plates at three places separately. The plates were properly labeled and incubated in an inverted position at  $24 \pm 2^\circ\text{C}$  for fungi and  $26 \pm 2^\circ\text{C}$  for bacteria for 3 days. The similar process was repeated with nutrient agar media and yeast agar media plates. The emergence and development of colonies was counted after every 24 hours (Aneja,2004). Colonies of bacteria,

actinomycetes and fungi per centimeter square of leaf was calculated by using the formula:

$$\text{Colonies/cm}^2 = \text{Total No. of colonies/Leaf area}$$

### **Isolation of epiphytes by serial dilution method**

Five discs, each of 5mm diameter were cut from every leaf using a sterile 5 mm cork borer. A total of 250 leaf discs were obtained, transferred to 100 ml of sterilized distilled water and stirred for 20 minutes till a suspension was obtained. The contents were shaken for uniform distribution of the cell/spores and 0.1 ml of aliquots from  $10^{-1}$  and  $10^{-2}$  dilutions using a sterile pipette were transferred to each sterile plates containing potato dextrose agar, nutrient agar, yeast maltose agar medium using L-shaped spreader. The plates were properly labeled and incubated at  $24\pm 2^\circ\text{C}$  for fungi and  $26\pm 2^\circ\text{C}$  for bacteria in an inverted position. The development of colonies was monitored and counted after every 24 hours for three days (Mukhtar *et al.*, 2010). Microbial population/cm<sup>2</sup> was calculated by applying the formula (Aneja, 2004):

$$\text{Propagules/cm}^2 =$$

$$\frac{\text{Total No. of spores in 0.1 ml} \times 100}{\text{Total area of 250 discs} \times 2}$$

(Area of one leaf disc =  $\pi r^2$ , where r is the radius of the disc in cm)

### **Isolation of epiphytes by fruit washing method**

For isolation of epiphytes from fruits, selected fruit were dipped individually in a container of 150 ml sterile water and shaken for 20 minutes on a mechanical shaker at 250 rpm,

to break free the micro-organisms. Each fruit was washed twice. The contents were shaken for uniform distribution of the cell/spores and 0.1 ml of aliquots from  $10^{-1}$  dilutions using a sterile pipette was transferred to sterile plates containing potato dextrose agar medium, yeast maltose agar medium and  $10^{-2}$  dilution was transferred to nutrient agar medium. Same procedure was repeated for second washing. The plates were properly labelled and incubated at  $24\pm 2^\circ\text{C}$  for fungi and  $26\pm 2^\circ\text{C}$  for bacteria. The development of colonies was monitored and counted after every 24 hrs. Microbial population/cm<sup>2</sup> of fruit surface was calculated by applying the formula (Aneja, 2004):

$$\text{Propagules/cm}^2 =$$

$$\frac{\text{Total No. of colonies in 0.1 ml} \times 150}{\text{Total area of apple fruit surface}}$$

(Area of apple fruit surface =  $4\pi r^2$ , where r is the radius of the disc in cm)

### **Identification**

The fungal colonies obtained were studied for colony characters, such as colony colour, margin, aerial growth and microscopic observations with respect to nature of mycelium, spore bearing structure, spore shape etc. The features of all the isolates were compared with the description in the standard manuals (Chowdhry *et al.*, (2000); Barnett and Hunter 1972).

The bacterial colonies were grown at  $26\pm 2^\circ\text{C}$  for 24 hours on specific media *viz.*, NA and King's B medium slants or plates. The colony morphology was studied on plates after streaking a loop full of isolated colony. The bacterial isolates were Gram stained and slides observed under Gaynor microscope at 100X magnification. Cell shape, size, Gram reaction was observed and photographed

(Gerhardt 1981). The bacterial cultures were examined for various morphological, biochemical and physiological characteristics as per the procedure described in Bergey's manual of Determinative Bacteriology (Holts *et al.*, 1994).

## Results and Discussion

### Isolation and identification of epiphytes

Five fungal isolates of epiphytes *viz.*, *Aspergillus* sp. (I<sub>1</sub>), *Penicillium* sp. (I<sub>2</sub>), *Rhizopus* sp. (I<sub>4</sub>), *Fusarium* sp. (I<sub>3</sub>), and *Alternaria* sp. (I<sub>5</sub>) were isolated from leaf and fruit surface using three different methods (Plate 1) and isolates were identified on the basis of their morphological characteristics which were compared with authenticated descriptions (Chowdhry *et al.*, (2000); Burnett and Hunter, 1972). Six bacterial epiphytes *viz.*, *Pseudomonas* sp. (I<sub>6</sub> and I<sub>7</sub>), *Bacillus* sp. (I<sub>8</sub> and I<sub>9</sub>), *Staphylococcus* sp. (I<sub>10</sub>) and *Micrococcus* sp. (I<sub>11</sub>) were isolated. For identification purposes, morphological and biochemical characters of these isolates were studied as per the procedures described by Holt *et al.*, (1994).

### Estimation of predominant epiphytes by leaf impression method

The mean populations (Table I) of all microbial isolates *viz.*, I<sub>1</sub>, I<sub>2</sub>, I<sub>3</sub>, I<sub>4</sub>, I<sub>7</sub>, I<sub>9</sub>, I<sub>10</sub> and I<sub>12</sub> on PDA (0.40 colonies per cm<sup>2</sup>) and YMA media (0.40 colonies/cm<sup>2</sup>) were significantly at par, but significantly different from NA media (0.34 colonies per cm<sup>2</sup>). However, a comparison of different media revealed that the microbial count of I<sub>9</sub> (1.03 colonies per cm<sup>2</sup>) was highest on YMA, of I<sub>7</sub> (0.95 colonies per cm<sup>2</sup>) on NA and of I<sub>10</sub> (0.90 colonies per cm<sup>2</sup>) on PDA. The mean number of colonies was highest in case of I<sub>7</sub> (0.92 colonies per cm<sup>2</sup>) and differed significantly from I<sub>9</sub> (0.63 colonies per cm<sup>2</sup>)

and others (0.53 colonies per cm<sup>2</sup>). The lowest mean numbers of colonies were recorded in case of I<sub>3</sub>, followed by I<sub>4</sub> and I<sub>1</sub> (0.02, 0.03 and 0.29 colonies per cm<sup>2</sup> respectively).

### Estimation of predominant epiphytes by serial dilution method

Perusal of the data presented in Table II revealed that the mean number of colonies of microbial isolates on PDA media was 0.20 per cm<sup>2</sup> which was significantly at par with the number of colonies on YMA (0.19 colonies per cm<sup>2</sup>) but significantly differed from NA (0.41 colonies per cm<sup>2</sup>). However, a comparison of different media revealed that the microbial count of I<sub>6</sub> (0.92 colonies per cm<sup>2</sup>) was highest on NA media, of I<sub>6</sub> (0.42 colonies per cm<sup>2</sup>) on PDA media and of I<sub>7</sub> (0.40 colonies per cm<sup>2</sup>) on YMA media. The mean number of colonies significantly differed and was highest in case of I<sub>6</sub> (0.55 colonies per cm<sup>2</sup>) followed by I<sub>7</sub> (0.50 colonies per cm<sup>2</sup>) and I<sub>8</sub> (0.40 colonies per cm<sup>2</sup>). The lowest mean numbers of colonies were recorded in case of I<sub>3</sub> followed by I<sub>2</sub> and I<sub>1</sub> *i.e.* 0.02, 0.10 0.14 colonies per cm<sup>2</sup> of leaf.

### Estimation of predominant epiphytes by fruit washing method

A perusal of the data presented in Table III revealed that the mean number of colonies of microbial isolates on PDA medium was 0.50 per cm<sup>2</sup>. In case of YMA medium, the mean number of colonies of microbial isolates was 0.47 colonies per cm<sup>2</sup>. While as, on NA medium, the mean number of colonies of microbial isolates was 0.44 colonies/cm<sup>2</sup> of leaf. However, a comparison of different media revealed that the microbial count of I<sub>8</sub> (0.98 colonies per cm<sup>2</sup>) was highest on NA medium, I<sub>1</sub> (0.91 colonies per cm<sup>2</sup>) on PDA medium and 0.81 colonies per cm<sup>2</sup> on YMA medium. The mean number of colonies

significantly differed and were highest in case of I<sub>6</sub> (0.86 colonies/cm<sup>2</sup>) followed by I<sub>9</sub> (0.79 colonies per cm<sup>2</sup>) and I<sub>1</sub> (0.57 colonies per cm<sup>2</sup>). The lowest number of colonies were

recorded in case of I<sub>3</sub> (0.03 colonies per cm<sup>2</sup>) followed by I<sub>2</sub> (0.30 colonies per cm<sup>2</sup>) and I<sub>8</sub> (0.32 colonies per cm<sup>2</sup>).

**Table.1** Microbial count of major epiphytes from apple phylloplane by leaf impression method

Microbial isolates		No. of colonies/cm <sup>2</sup>			
		PDA Medium	YMA Medium	NA Medium	Overall mean
I <sub>1</sub>	<i>Aspergillus</i> sp.	0.42 (0.648)	0.35 (0.591)	0.12 (0.346)	<b>0.29 (0.538)<sup>c</sup></b>
I <sub>2</sub>	<i>Penicillium</i> sp.	0.54 (0.734)	0.41 (0.460)	0.00 (0.000)	<b>0.31 (0.556)<sup>d</sup></b>
I <sub>3</sub>	<i>Fusarium</i> sp.	0.06 (0.244)	0.00 (0.000)	0.00 (0.000)	<b>0.02 (0.414)<sup>a</sup></b>
I <sub>4</sub>	<i>Rhizopus</i> sp.	0.03 (0.173)	0.04 (0.200)	0.03 (0.173)	<b>0.03 (0.173)<sup>b</sup></b>
I <sub>7</sub>	<i>Pseudomonas</i> sp.	0.86 (0.927)	0.97 (0.984)	0.95(0.974)	<b>0.92 (0.959)<sup>g</sup></b>
I <sub>9</sub>	<i>Bacillus</i> sp.	0.00 (0.000)	1.03(1.014)	0.87 (0.932)	<b>0.63 (0.793)<sup>f</sup></b>
I <sub>10</sub>	<i>Staphylococcus</i> sp.	0.90(0.948)	0.00 (0.000)	0.44 (0.663)	<b>0.44 (0.663)<sup>e</sup></b>
<b>Mean</b>		<b>0.40 (0.524)<sup>B</sup></b>	<b>0.40(0.464)<sup>B</sup></b>	<b>0.34(0.441)<sup>A</sup></b>	

CD(P≤0.05) Isolate (I) = 0.004, Media (M) = 0.002 Isolate × Media = 0.007  
 Figures in brackets are square root transformed values.

**Table.2** Microbial count of major epiphytes from apple phylloplane by serial dilution method

Microbial isolates		No. of colonies/cm <sup>2</sup>			
		PDA Medium	YMA Medium	NA Medium	Overall mean
I <sub>1</sub>	<i>Aspergillus</i> sp.	0.20 (0.447)	0.22 (0.469)	0.0 (0.000)	<b>0.14 (0.374)<sup>c</sup></b>
I <sub>2</sub>	<i>Penicillium</i> sp.	0.22(0.469)	0.10(0.316)	0.0(0.000)	<b>0.10 (0.316)<sup>b</sup></b>
I <sub>3</sub>	<i>Fusarium</i> sp.	0.0(0.000)	0.08(0.282)	0.0(0.000)	<b>0.02(0.141)<sup>a</sup></b>
I <sub>4</sub>	<i>Rhizopus</i> sp.	0.42 (0.648)	0.31(0.558)	0.92 (0.951)	<b>0.55(0.741)<sup>g</sup></b>
I <sub>7</sub>	<i>Pseudomonas</i> sp.	0.25(0.500)	0.40 (0.632)	0.86(0.927)	<b>0.50 (0.707)<sup>f</sup></b>
I <sub>9</sub>	<i>Bacillus</i> sp.	0.35(0.591)	0.24(0.489)	0.61(0.781)	<b>0.40 (0.632)<sup>e</sup></b>
I <sub>10</sub>	<i>Staphylococcus</i> sp.	0.0(0.000)	0.0(0.000)	0.49(0.700)	<b>0.16 (0.400)<sup>d</sup></b>
<b>Mean</b>		<b>0.20(0.379)<sup>A</sup></b>	<b>0.19(0.392)<sup>A</sup></b>	<b>0.41(0.479)<sup>B</sup></b>	

CD(P≤0.05) Isolate (I) =0.003 Media (M)=0.002 Isolate × Media= 0.006  
 Figures in brackets are square root transformed values.

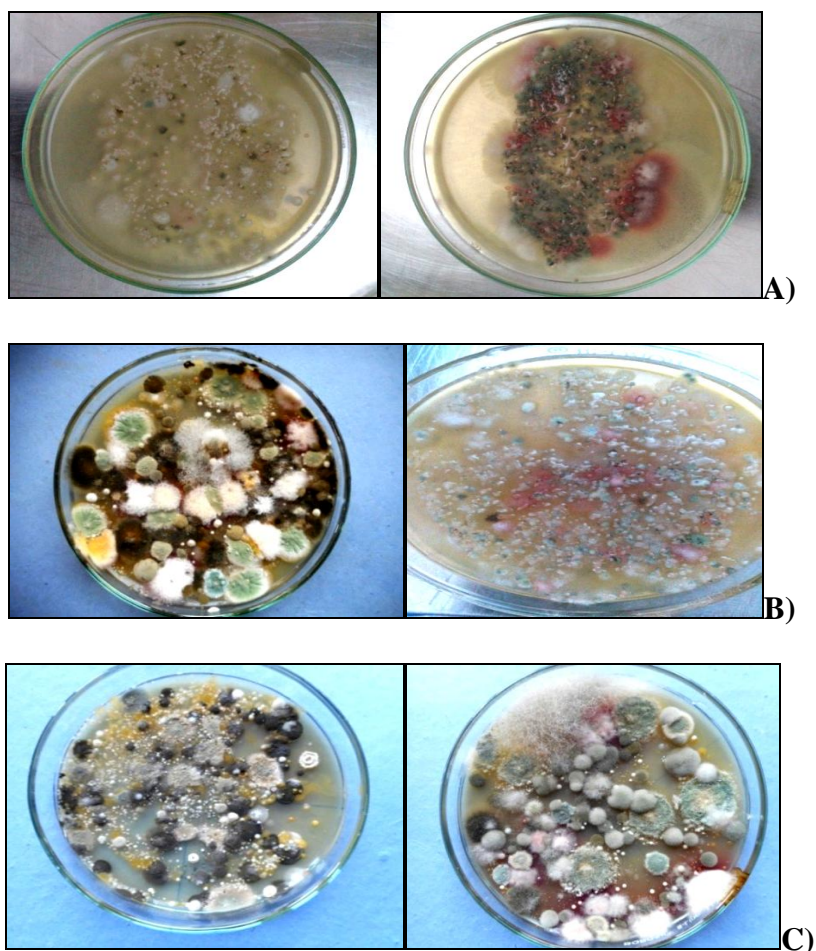


**Table.3** Microbial count of major epiphytes from apple fruits by fruit washing method

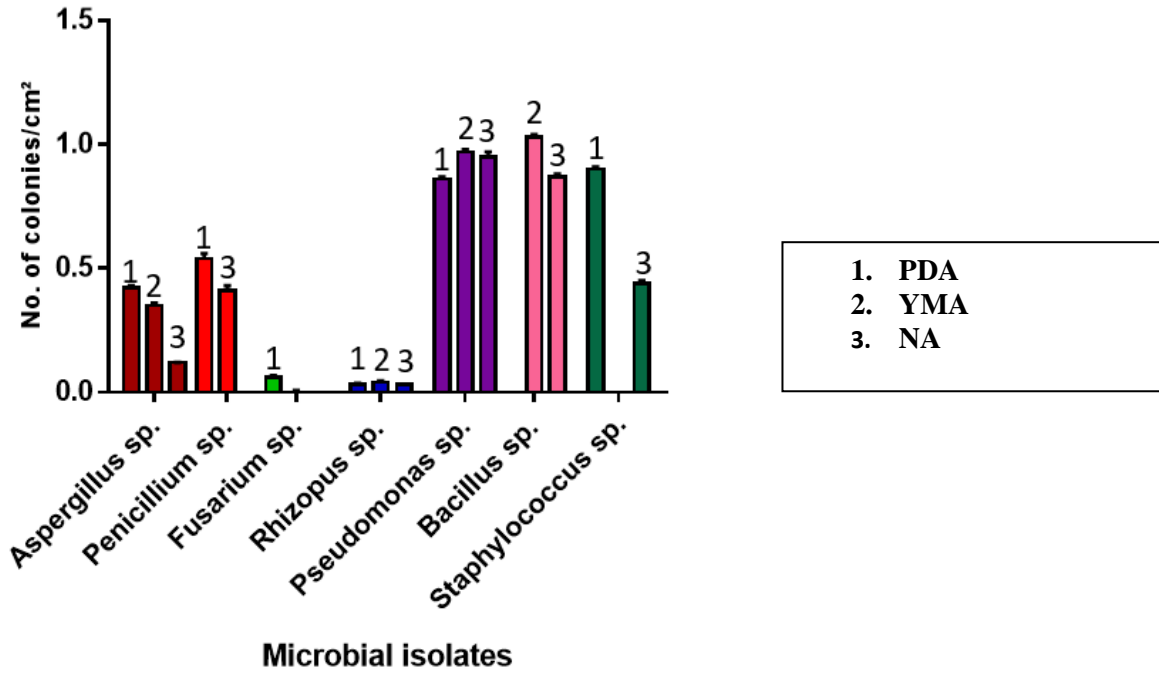
Microbial isolates		No. of colonies/cm <sup>2</sup>			
		PDA Medium	YMA Medium	NA Medium	Overall mean
I <sub>1</sub>	<i>Aspergillus</i> sp.	0.91 (0.953)	0.81 (0.900)	0.0 (0.000)	<b>0.57 (0.754)<sup>e</sup></b>
I <sub>2</sub>	<i>Penicillium</i> sp.	0.47(0.685)	0.45(0.670)	0.0(0.000)	<b>0.30(0.547)<sup>b</sup></b>
I <sub>3</sub>	<i>Fusarium</i> sp.	0.0 (0.000)	0.11(0.331)	0.0(0.000)	<b>0.03 (0.173)<sup>a</sup></b>
I <sub>4</sub>	<i>Rhizopus</i> sp.	0.73(0.854)	0.51(0.714)	0.0(0.000)	<b>0.41(0.640)<sup>d</sup></b>
I <sub>7</sub>	<i>Pseudomonas</i> sp.	0.67(0.818)	0.80(0.894)	1.13(1.060)	<b>0.86 (0.927)<sup>g</sup></b>
I <sub>9</sub>	<i>Bacillus</i> sp.	0.0 (0.000)	0.0(0.000)	0.98 (0.989)	<b>0.32(0.565)<sup>c</sup></b>
I <sub>10</sub>	<i>Staphylococcus</i> sp.	0.73(0.854)	0.62(0.787)	1.02(1.009)	<b>0.79(0.888)<sup>f</sup></b>
<b>Mean</b>		<b>0.50(0.594)<sup>B</sup></b>	<b>0.47(0.613)<sup>C</sup></b>	<b>0.44(0.436)<sup>A</sup></b>	

CD(P≤0.05) Isolate (I) =0.003 Media (M) =0.002 Isolate × Media =0.006  
 Figures in brackets are square root transformed values.

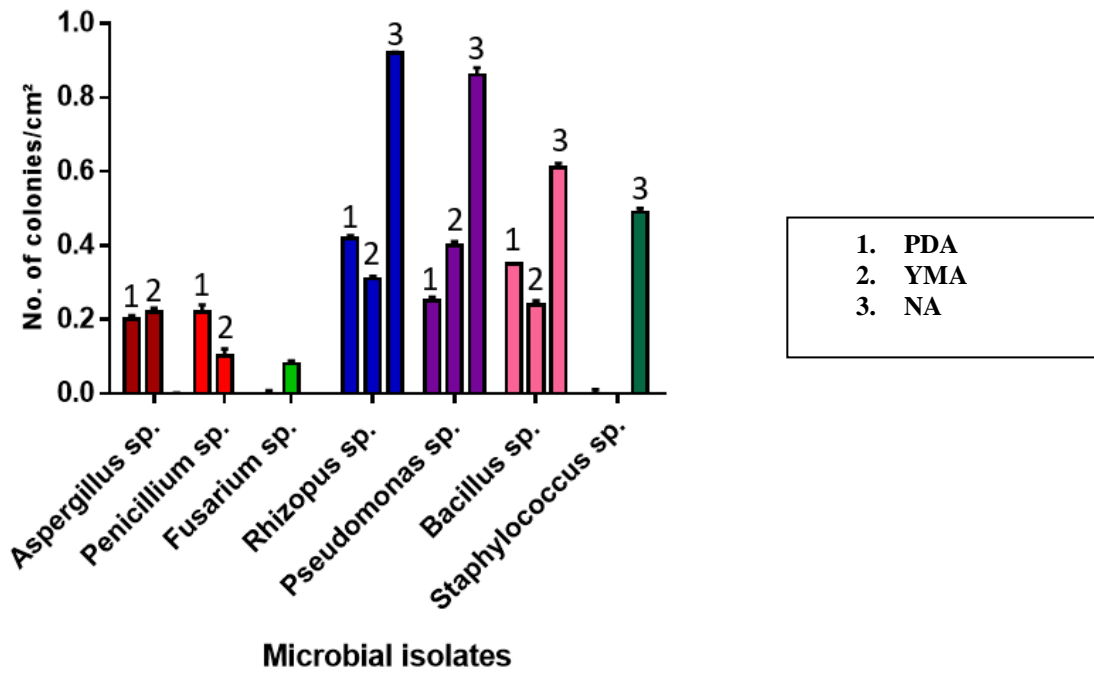
**Plate.1** Isolation of epiphytes by using different methods



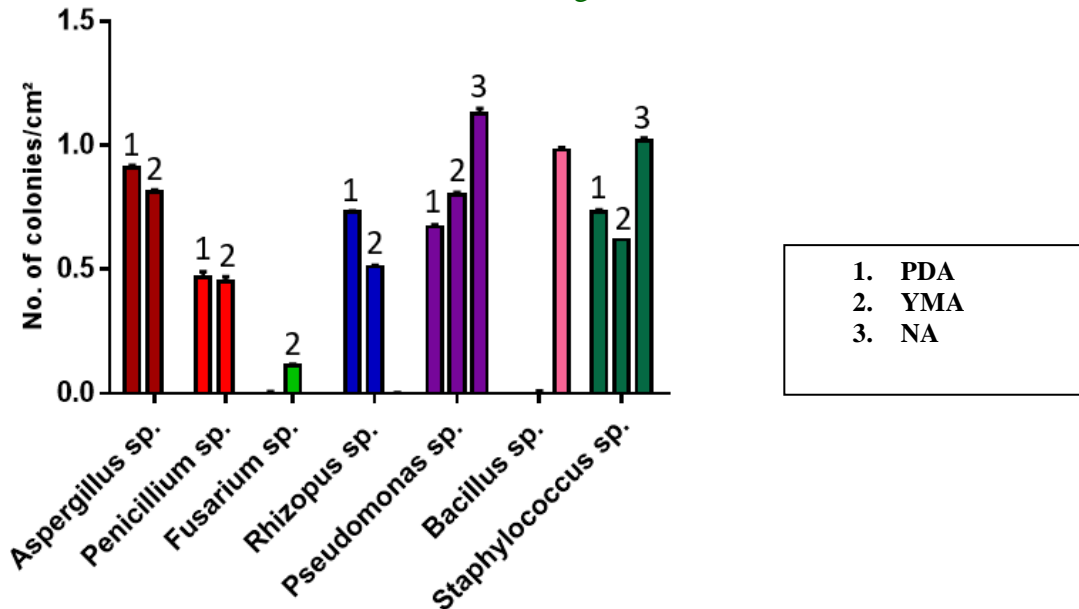
**Figure.1** Graphical representation of microbes (No. of colonies/cm<sup>2</sup>) from apple phylloplane by leaf impression method



**Figure.2** Graphical representation of microbes (No. of colonies/cm<sup>2</sup>) from apple phylloplane by leaf impression method



**Figure.3** Graphical representation of microbes (No. of colonies/cm<sup>2</sup>) from apple phylloplane by fruit washing method



**Microbial isolates**

There are very few studies on the epiphytes of apple and their role in the management of postharvest diseases of apple. The present investigation was, therefore, aimed at isolation and estimation of predominant epiphytes from apple leaves and fruits.

Five fungal epiphytes viz., *Aspergillus* sp. (I<sub>1</sub>), *Penicillium* sp. (I<sub>2</sub>), *Fusarium* sp. (I<sub>3</sub>), *Rhizopus* sp. (I<sub>4</sub>) and *Alternaria* sp. (I<sub>5</sub>) and six bacterial epiphytes viz., *Pseudomonas* sp. (I<sub>6</sub>), *Pseudomonas* sp. (I<sub>7</sub>), *Bacillus* sp. (I<sub>8</sub>), *Bacillus* sp. (I<sub>9</sub>), *Staphylococcus* sp. (I<sub>10</sub>) and *Micrococcus* sp. (I<sub>11</sub>) were isolated using different techniques viz., leaf impression, serial dilution and fruit washing methods on three different media (potato dextrose, nutrient agar and yeast agar media). The various methods used in this study to quantify the number of propagules/colonies of microbes are only a rough means of estimating the presence of micro-organisms on leaves (Leben, 1971). It takes into account only those microbes which can be washed away from the leaves and grown on media. Similarly, Ogwu and Osawaru (2014)

estimated microfloral isolates on healthy leaves of mature okra and examined eight genera of fungi (*Rhodotorula*, *Saccharomyces*, *Mucor*, *Trichothecium*, *Cladosporium*, *Rhizopus*, *Aspergillus* and *Penicillium*) and six genera of bacteria (*Bacillus*, *Pseudomonas*, *Micrococcus*, *Proteus*, *Staphylococcus*, *Serratia*).

From all the three epiphytes isolation methods highest number of microbial colonies observed was *Bacillus* followed by *Pseudomonas*. This investigation shows that apple phylloplane is inhabited by various fungal and bacterial epiphytes. Most predominant microflora were *Bacillus* and *Pseudomonas* sp. This is in conformity with Bryk *et al.*, (2004) who isolated two strains of *Pseudomonas* sp. (B194 and B224) from apple leaves.

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