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

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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. (I1), Penicillium sp. (I2), Fusarium sp. (I3), Rhizopus sp. (I4) and Alternaria sp. (I5) and six bacterial isolates viz., Pseudomonas sp. (I6), Pseudomonas sp. (I7), Bacillus sp. (I8), Bacillus sp. (I9), Staphylococcus sp. (I10) and Micrococcus sp. (I11) 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/cm² was recorded in fruit washing method followed by leaf impression (3.17) and lowest in serial dilution method (2.12).

Keywords
Apple, Epiphytes, Isolation, Methods

Article Info
Accepted: 17 June 2020
Available Online: 10 July 2020

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 $10^2$ to $10^{12}$ 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 ($5.32 \times 10^7$/cm$^2$ leaf area), whereas, the bacterial population was more at physiological maturity ($32.15 \times 10^7$/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 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±2°C for fungi and 26±2°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:

Colonies/cm² = 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±2°C for fungi and 26±2°C for bacteria in an inverted position. The development of colonies was monitored and counted after every 24 hrs. Microbial population/cm² of fruit surface was calculated by applying the formula (Aneja, 2004):

\[
\text{Propagules/cm}^2 = \frac{\text{Total No. of colonies in 0.1 ml} \times 150}{\text{Total area of apple fruit surface}}
\]

\[
\text{(Area of one leaf disc} = \pi r^2, \text{where r is the radius of the disc in cm)}
\]

**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 water 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±2°C for fungi and 26±2°C for bacteria. The development of colonies was monitored and counted after every 24 hrs. Microbial population/cm² of fruit surface was calculated by applying the formula (Aneja, 2004):

\[
\text{Propagules/cm}^2 = \frac{\text{Total No. of colonies in 0.1 ml} \times 100}{\text{Total area of 250 discs} \times 2}
\]

\[
\text{(Area of one leaf disc} = \pi r^2, \text{where r is the radius of the disc in cm)}
\]

**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±2°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
Int.J.Curr.Microbiol.App.Sci (2020) 9(7): 1802-1810

Results and Discussion

Isolation and identification of epiphytes

Five fungal isolates of epiphytes viz., *Aspergillus* sp. (I1), *Penicillium* sp. (I2), *Rhizopus* sp. (I4), *Fusarium* sp. (I3), and *Alternaria* sp. (I5) 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. (I6 and I7), *Bacillus* sp. (I8 and I9), *Staphylococcus* sp. (I10) and *Micrococcus* sp. (I11) 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., I1, I2, I3, I4, I7, I9, I10 and I12 on PDA (0.40 colonies per cm²) and YMA media (0.40 colonies/cm²) were significantly at par, but significantly different from NA media (0.34 colonies per cm²). However, a comparison of different media revealed that the microbial count of I6 (1.03 colonies per cm²) was highest on YMA, of I7 (0.95 colonies per cm²) on NA and of I10 (0.90 colonies per cm²) on PDA. The mean number of colonies was highest in case of I7 (0.92 colonies per cm²) and differed significantly from I9 (0.63 colonies per cm²) and others (0.53 colonies per cm²). The lowest mean numbers of colonies were recorded in case of I3, followed by I4 and I1 (0.02, 0.03 and 0.29 colonies per cm² 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 cm² which was significantly at par with the number of colonies on YMA (0.19 colonies per cm²) but significantly differed from NA (0.41 colonies per cm²). However, a comparison of different media revealed that the microbial count of I6 (0.92 colonies per cm²) was highest on NA media, of I6 (0.42 colonies per cm²) on PDA media and of I7 (0.40 colonies per cm²) on YMA media. The mean number of colonies significantly differed and was highest in case of I6 (0.55 colonies per cm²) followed by I7 (0.50 colonies per cm²) and I8 (0.40 colonies per cm²). The lowest mean numbers of colonies were recorded in case of I3 followed by I2 and I1 i.e. 0.02, 0.10 0.14 colonies per cm² 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 cm². In case of YMA medium, the mean number of colonies of microbial isolates was 0.47 colonies per cm². While as, on NA medium, the mean number of colonies of microbial isolates was 0.44 colonies/cm² of leaf. However, a comparison of different media revealed that the microbial count of I8 (0.98 colonies per cm²) was highest on NA medium, I1 (0.91 colonies per cm²) on PDA medium and 0.81 colonies per cm² on YMA medium. The mean number of colonies

(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).
significantly differed and were highest in case of I₆ (0.86 colonies/cm²) followed by I₉ (0.79 colonies per cm²) and I₁ (0.57 colonies per cm²). The lowest number of colonies were recorded in case of I₃ (0.03 colonies per cm²) followed by I₂ (0.30 colonies per cm²) and I₈ (0.32 colonies per cm²).

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

| Microbial isolates | No. of colonies/cm² | PDA Medium | YMA Medium | NA Medium | Overall mean |
|-------------------|---------------------|------------|------------|-----------|-------------|
| I₁ Aspergillus sp.| 0.42 (0.648)        | 0.35 (0.591) | 0.12 (0.346) | 0.29 (0.538) |
| I₂ Penicillium sp.| 0.54 (0.734)        | 0.41 (0.460) | 0.00 (0.000) | 0.31 (0.556) |
| I₃ Fusarium sp.| 0.06 (0.244)        | 0.00 (0.000) | 0.00 (0.000) | 0.02 (0.414) |
| I₄ Rhizopus sp.| 0.03 (0.173)        | 0.04 (0.200) | 0.03 (0.173) | 0.03 (0.173) |
| I₇ Pseudomonas sp.| 0.86 (0.927)        | 0.97 (0.984) | 0.95 (0.974) | 0.92 (0.959) |
| I₉ Bacillus sp.| 0.00 (0.000)        | 1.03 (1.014) | 0.87 (0.932) | 0.63 (0.793) |
| I₁₀ Staphylococcus sp.| 0.90 (0.948) | 0.00 (0.000) | 0.44 (0.663) | 0.44 (0.663) |
| Mean             | 0.40 (0.524)        | 0.40 (0.464) | 0.34 (0.441) |             |

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/cm² | PDA Medium | YMA Medium | NA Medium | Overall mean |
|-------------------|---------------------|------------|------------|-----------|-------------|
| I₁ Aspergillus sp.| 0.20 (0.447)        | 0.22 (0.469) | 0.0 (0.000) | 0.14 (0.374) |
| I₂ Penicillium sp.| 0.22 (0.469)        | 0.10 (0.316) | 0.0 (0.000) | 0.10 (0.316) |
| I₃ Fusarium sp.| 0.0 (0.000)        | 0.08 (0.282) | 0.0 (0.000) | 0.02 (0.141) |
| I₄ Rhizopus sp.| 0.42 (0.648)        | 0.31 (0.558) | 0.92 (0.951) | 0.55 (0.741) |
| I₇ Pseudomonas sp.| 0.25 (0.500)        | 0.40 (0.632) | 0.86 (0.927) | 0.50 (0.707) |
| I₉ Bacillus sp.| 0.35 (0.591)        | 0.24 (0.489) | 0.61 (0.781) | 0.40 (0.632) |
| I₁₀ Staphylococcus sp.| 0.0 (0.000)        | 0.0 (0.000) | 0.49 (0.700) | 0.16 (0.400) |
| Mean             | 0.20 (0.379)        | 0.19 (0.392) | 0.41 (0.479) |             |

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/cm² |          |          |          | Overall mean |
|--------------------------|---------------------|----------|----------|----------|--------------|
|                          | PDA Medium          | YMA Medium | NA Medium |          |              |
| I₁ Aspergillus sp.       | 0.91 (0.953)        | 0.81 (0.900) | 0.0 (0.000) |          | 0.57 (0.754)  |
| I₂ Penicillium sp.       | 0.47(0.685)         | 0.45(0.670) | 0.0(0.000)  |          | 0.30(0.547)  |
| I₃ Fusarium sp.          | 0.0(0.000)          | 0.11(0.331) | 0.0(0.000)  |          | 0.03(0.173)  |
| I₄ Rhizopus sp.          | 0.73(0.854)         | 0.51(0.714) | 0.0(0.000)  |          | 0.41(0.640)  |
| I₇ Pseudomonas sp.       | 0.67(0.818)         | 0.80(0.894) | 1.13(1.060) |          | 0.86(0.927)  |
| I₉ Bacillus sp.          | 0.0(0.000)          | 0.0(0.000)  | 0.98(0.989) |          | 0.32(0.565)  |
| I₁₀ Staphylococcus sp.   | 0.73(0.854)         | 0.62(0.787) | 1.02(1.009) |          | 0.79(0.888)  |
| Mean                     | 0.50(0.594)         | 0.47(0.613) | 0.44(0.436) |          |              |

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/cm²) from apple phylloplane by leaf impression method

**Figure 2** Graphical representation of microbes (No. of colonies/cm²) from apple phylloplane by leaf impression method

**Microbial isolates**

1. PDA
2. YMA
3. NA
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. (I₁), Penicillium sp. (I₂), Fusarium sp. (I₃), Rhizopus sp. (I₄) and Alternaria sp. (I₅) and six bacterial epiphytes viz., Pseudomonas sp. (I₆), Pseudomonas sp. (I₇), Bacillus sp. (I₈), Bacillus sp. (I₉), Staphylococcus sp. (I₁₀) and Micrococcus sp. (I₁₁) 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.

**Acknowledgement**

I Aqleema Banoo, like to acknowledge Division of plant pathology Skuast-K for providing me the essential necessities that...
were required to conduct this research methodology. I also like to acknowledge the other authors for useful suggestions while preparing the manuscript.

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How to cite this article:

Aqleema Banoo, Efath Shahnaz, Saba Banday, Rovidhia S. Rasool and Taibah Bashir. 2020. Studies on Epiphytic Microflora on Apple (Malus domestica Borkh.) Fruits and Leaves. *Int.J.Curr.Microbiol.App.Sci.* 9(07): 1802-1810. doi: [https://doi.org/10.20546/ijcmas.2020.907.206](https://doi.org/10.20546/ijcmas.2020.907.206)