**Abstract**

A wide range of phytochemicals are present in apple (*Malus domestica* Borkh), their production was accelerated when apple was subjected to biotic stress. These chemicals were measured in the leaf of apple cultivar Shireen, healthy and infected by *Venturia inaequalis* and examined by microscope. Scab-induced metabolic re-programming was analyzed by methanol leaf extract by using Gas Chromatography-Mass Spectrometry, while the mass spectra of the compounds found in the extract were matched with the National Institute of Standards and Technology (NIST) library. The compounds were identified by comparing their retention time and peak area with that of literature and by interpretation of mass spectra. The amount of secondary metabolites increased drastically during the infection that reveals the secondary metabolites play an important role in antifungal activity. GC-MS analysis revealed the presence of near about 261 metabolites. Out of these, 16 metabolites were found to be differentially accumulated in normal and fungal infected leaves in heat map analysis. Phenylalanine, benzoic acid, 4-hydroxyl benzoic acid, protocatechuic acid, p-coumaric acid, ferulic acid, catechene, ursolic acid level were increased whereas phytol and α-Franesene level decreased. It can further help in determining genes responsible for the accumulation of these metabolites and combining this knowledge of metabolomics, genomics can lead to the development of resistant cultivars of apple plant with high resistance towards the pathogen.

**Keywords**

*Malus domestica*, *Venturia inaequalis*, Pathosystem, GC-MS analysis, metabolomics, secondary metabolites

**Introduction**

Apple is deciduous fruits, mostly grown in temperate regions around the world. It is an economically important fruit crop consumed by almost half of the population worldwide due to its phytochemical constituents. Apple fruits have high nutritional values and enormous health-promoting properties (Sarkate et al., 2017). They are a rich source of soluble as well as insoluble fibers, minerals, and vitamins. (Gerhauser, 2008). Apple has free radical scavenging activity due to the phytochemical content rather than that of vitamin C (Eberhardt et al., 2000). Its consumption is related to lower mortality rates due to the presence of dietary flavonoids and phenolics (Vinson et al., 2001). Apple
fruits have good anti-proliferative, antioxidant, gastrointestinal protection from drug injury and cholesterol-lowering properties (Eberhardt et al., 2000). Daily consumption of apple can help in reducing the risk of heart disease and cancer (Gerhauser, 2008).

Most of the top-selling commercial apple cultivars are highly susceptible to apple scab disease, however, few wild apple cultivars are resistant towards scab-infection. Scab resistant cultivars are Shireen, William’s Pride, Prima, Goldrush, Crimson Crisp and susceptible cultivars are Jonathan, Red Delicious, Imperial Gala, McIntosh, and Golden Delicious. Scab disease is caused by fungus Venturia inaequalis (Cooke) G. Winter, which belongs to the Ascomycota. It is a heterothallic fungus including seven chromosomes. The host range of V. inaequalis includes a number of genera such as Malus, Pyrus, Sorbus, etc (Bus et al., 2011). V. inaequalis produces two types of spores, the sexual (ascospore) and the asexual (conidia) spores, both capable of infecting different apple cultivars. Apple scab is one of the most damaging diseases in terms of economic loss. It causes huge economic losses up to a 70 % reduction in apple production. (Gopaljee Jha et al., 2009).

The symptom of scab includes circular velvety olive green, chlorotic or necrotic lesions; spots on pedicels and sepals; and dark-colored brown corky lesions or small black spots on fruits. The maximum radiuses of these lesions are 1 cm from point of infection, and then ceasing to expand (MacHardy, 1996). This phenomenon has been attributed to ontogenic resistance that develops as leaves and fruits mature, and are complete by the time leaves are fully expanded (Gessler and Stumm, 1984; Schwabe, 1979). Scab leads to distortion in fruits, and premature fruit and leaf fall (Jha et al., 2009). The primary source of inoculums is the germination of sexual (ascospore) spores, which causes the initiation of the disease, whereas conidia serve as the secondary source of infections.

Defense strategies of apple against fungus are several, including the production of antifungal chemicals, which are either pre-formed or induced following infection (Grayer and Kokubun, 2001). These compounds are produced and accumulate at a faster rate after infection and toxic to pathogens. (Picinelli et al., 1995; Usenik et al., 2004; Treutter, 2005). Phenolics, flavanols, in particular, play a role in the resistance of apples to V. inaequalis (Treutter and Feucht, 1990a, 1990b).

The accumulation of these metabolites was analyzed by gas chromatography-mass spectrometry technique. Gas chromatography-mass spectrometry (GC-MS) is an emerging technology, routinely used in plant metabolomics (Shuman et al., 2011), especially for facilitating the identification and quantification of the primary metabolites such as amino acids, sugars, organic acids (Schauer and Fernie, 2006) and an wide array of secondary metabolites such as phenolics, flavonoids, and biphenyls (Chizzali et al., 2012). GC-MS has been used to identify and measure metabolites in many plant samples (Bisht et al., 2013; Kumar and Nagar, 2014).

Materials and Methods

Collection of plant material

Apple (Malus domestica Borkh) cultivar 'Shireen were brought from the Central Institute of Temperate Horticulture (ICAR-CITH), Srinagar, India. These plants were maintained under the temperate condition in a micro-climate control greenhouse (temperature 20 - 22°C and relative humidity of 65± 5%) in the Department of
Biotechnology of Indian Institute of Technology Roorkee (Roorkee, India).

**In-Vitro culture of Venturia inaequalis**

The strain of *V. inaequalis* (MTCC No.: 1109) was purchased from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Culturing of *Venturia* was done by the method described by Parker, D. M., Hilber, U. W., *et al.*, [14]. After 3 to 4-week colony of Mycelium of *Venturia inaequalis* was derived from a fungal culture grown on the MEP plate.

**Treatment of leaves by Venturia inaequalis**

Young leaves of the Shireen cultivar of *Mauls* were used for infection by mycelium of *Venturia inaequalis*. Leaves were washed with running tap water for 30 min. with vigorous shaking to remove dust particles and then sterilized in 20 % sodium hypochlorite for 1 to 2 sec., 1 % tween - 20 solutions for 2 to 5 min, 70% ethanol for 5 to 7 min and finally sterilized with distilled water for 5 to 10 min. Following sterilization leaves were dried with filter paper, holes were done by the needle by putting on the PDA plates.

Suspension of mycelium of *Venturia inaequalis* was applied on the adaxial surface of leaves and put the plates were put in dark condition at 26°C temperature for penetration of mycelium into the leaves. After 10 to 15 days visible symptoms appeared on the leaves. The infected leaf of these cultivars was used for microscopy to check the growth of mycelium.

**Microscopy of leaves**

The inoculated leaf sample was cleared in distilled water to remove fungi, and then dry it by tissue paper. The leaves were cut in a rectangular shape having scab and non-scab portion. The small pieces of leaves were cleared in solution A (96% Ethanol + 4% Acetic acid) for 30 min at 75°C temperature, then stained in solution B (70% Ethanol + 29.9% Distilled water + 1% Aniline blue) for 7 min at 75°C temperature. Next, the leaf sample was rinsed three times in solution C (70% Ethanol) at room temperature, and left for at least 30 min. in solution D (50 % Ethanol + 50% Lactic acid) at room temperature. Finally, Pieces of leaves were mounted on microscope slides with lactic acid. The samples were observed using a bright-field light microscope and were examined in the interference blue range (excitation filter 450 – 490 nm, dichroic mirror 505 nm, barrier filter 515 nm).

**Sample preparation for GC-MS analysis of Metabolites**

Infected and non-infected leaves of Shireen were rinsed in distilled water for 10 min with vigorous shaking and then rinse in 70% ethanol for 15-20 min, followed by air drying. 1g dried leaf from both samples was crushed in liquid nitrogen followed by 4ml methanol then pinch of neutral sea sand was added which provide friction so that cells rupture easily. 40µl HCL was added to the mixture. In order to identify extraction efficiency, 5µl phenyl phenol was spiked in the extraction mixture as the internal standard and vortex for 1 min, again 4 ml of methanol was added to the mixture. The homogenized extract was then centrifuged at 12000 g for 15 min at 5°C.

**GC-MS derivatization**

The resultant supernatant was (2ml ) was transferred in 2 ml tube and take 100µl supernatant to concentrate it at 37°C for 1:00 hr. Add 70 µl MSTFA and then put it on dry bath at 37° for 1 hr and in between vertex it after every 5 min. Centrifuge at 15000 rpm for 15 min.
GC-MS analysis

The samples were analyzed on Agilent technologies GC 7890B and MS 5977B system. A DB-5 MS column was used for the separation of compounds with helium as a carrier gas. The injection volume was 1 µL with splitless mode. The injector temperature was set at 280°C.

The oven temperature was initially set at 80°C for 1.5 min, then ramped to 220 °C at a rate of 10°C/min without any hold and further increases at 310°C at the rate of 20°C/min held for 10 min and with a solvent delay of 5 min. The column flow rate was 1 mL/min. The conditions for the operation of mass spectrometers were set as follows: ion source temperature 230°C, MS Quad temperature 150°C, electron energy (70eV) and scanning range of m/z, 25-1000 amu. The compounds were identified by comparing the mass spectrum of the components to that of the mass spectral library from NIST 11.L (National Institute of Standards and Technology).

Metabolite identification

Metabolites present in the cell cultures were identified by matching mass spectra of each compound from the library (3:1 signal to noise ratio) using the NIST-11 mass spectral library (National Institute of Standards and Technology).

For the mass spectra comparison, the matching value of the metabolite identity taken was more than 70. For further confirmation spiking of the sample is done with the corresponding pure standard. In order to check for co-elution, the mass spectra of all peaks were analyzed at three different points, beginning, middle and end of each peak width. No co-elution detected in any of the identified peaks.

Metabolites Analysis via Metabonalyist

The chromatograms obtained were extracted via Agilent ChemStationTM software and were deconvoluted by Automated Mass Spectral Deconvolution and Identification System (AMDIS) using tools available with Mass hunter (Agilent technologies). Metabolite data of infected and non-infected leaves obtained were confirmed with library and metabolites having matching specific fragmentation were selected and data is further converted into .csv (comma separated values) format before uploading. After that, the data were, log-transformed with Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable) followed by normalization before statistical analyses. Multivariate statistical analysis like ANOVA (using Fisher’s LSD method; p-value < 0.05), principal component analysis (PCA) were performed by using interactive online tool Metaboanalyst 3.0 (http://www.metaboanalyst.ca). A heat map was created using the interactive heat map tool of Metaboanalyst 3.0.

Results and Discussion

Penetration of Mycelium into the cultivar of apple

Visible symptoms were appeared on leaf approximately 5 to 10 days after infection. Shireen cultivar contains genetic resistance to apple scab. Disease progression and resistance reactions had been seen on whole leaves. The light microscope was utilized to photograph macroscopic symptoms with increased magnification.

Microscopically, the presence of mycelium on the leaf surfaces was observed at 1-5 dpi, including the development of the germ tubes and appressoria on the leaf surface. A reaction on young leaves could be observed 7
dpi and was characterized by the browning of epidermal cells below and around the penetration site of the pathogen. The zone of browned cells was characterized by light microscopy. After a few days later, the resistance phenotype became macroscopically visible as a depression on the leaf surface characteristic of pinpoint pits. The apple scab resistance found in Shireen leads to the formation of pinpoint pits on the upper surface of young infected leaves. These pits appeared gradually after inoculation mycelia suspension of *V. inaequalis* under optimal conditions.

Browning of epidermal and mesophyll cells surrounding the infection site is associated with the collapse and death of epidermal and mesophyll tissue can be a characteristic of a hypersensitive response. In Shireen, visible browning which is an indication of necrosis of leaf tissue was apparent after 5 dpi and increased by 10 dpi. Resistance reactions were varied including sporulating lesions, hyphae and conidia present (stained blue) in addition to browning of epidermal cells. Nine dpi, the resistance phenotype of Shireen became macroscopically visible without the aid of an amplifying glass as a depression on the leaf surface characteristic of the pinpoint pit phenotype.

**GC-MS based metabolomics analyses of *Venturia inaequalis* treated and non-treated leaves of apple cv. ‘Shireen’**

**Comparative metabolomics of infected and non-infected leaves of Shireen cultivar**

GC-MS based analysis revealed there were changes in the level of the metabolites between treated and normal leaves sample. Near about 261 metabolites were detected and confirmed with NIST 11 and our in house library. Out of these 261 compounds, metabolites having more than 80 % similarity in specific fragmentation patterns and based on their retention time 38 metabolites were confirmed (Table 1). Out of these 38 metabolites, 16 metabolites found to be accumulated differentially in normal and fungal infected leaves (Figure 4).

Representative GC-MS chromatogram (TIC; total ion chromatogram) showing the difference in infected and non-infected leaves is given below in Figure 3.

Figure 4 Graphs showing variations of 16 major metabolites differentially accumulated in Shireen Infected and non-infected leaves with *Venturia inaequalis*. The X-axis indicates the infection status and Y-axis indicates the concentration of metabolite as the Relative percentage area (RPA).

In order to search for any probable discrepancies in the metabolite profiles of infected and non-infected leaves, the 38 identified metabolites were organized and visualized by the Heat Map tool of Metaboanalyst 3.0 (Shen et al., 2015). In heat map analysis, it was found that 16 metabolites are differentially accumulated after infection with fungus *Venturia inaequalis*. Phenylalanine, benzoic acid, 4- hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, ferulic acid, catechine, ursolic acid were highly accumulated in infected leaves whereas phytol and α- Franesene level decreased post-infection with fungus (Fig 4).

Data were statistically analyzed via online statistical tool Metaboanalyst 3.0 which was also confirmed by statistical analysis in metaboanalyst 3.0 after performing PCA heat map was created which show significant differences in accumulation pattern of metabolites in infected and non-infected leaves shown by heat map (Figure 5.)

Figure 5 Heat map showing variations of 38 metabolites (more than 80 % similarity in NIST 11) differentially accumulated in
Shireen Infected and non-infected leaves with *Venturia inequalis*. Total 10 Amino acids were differentially accumulated between infected and normal leaves of Shireen, out of which phenylalanine was highest in infected than normal, which means it helps plants in defense. It is the precursor of coumaric acid and all phenolics and flavonoids compounds, which helps in defense. Sugar alcohols were found, in which mannitol was much more accumulated in infected leaves. Organic acids like shikimic acid and quinic acid concentration increase during infection. Shikimic acid is the precursor of phenylalanine.

Secondary metabolites were detected in leaves, but 4-Hydroxybenzoic acid, protocatechuic acid, benzoic acid, coumaric acid, ferulic acid catechine, were increases after infection but phytol and α-Franesene decreases.

**Table 1** List of 38 identified metabolites from Venturia treated apple cv. Shireen

| Class         | Metabolite     | RT       | NIST id | m/z          | RPA       |
|---------------|----------------|----------|---------|--------------|-----------|
| **Amino Acids** |                |      |         |              |           |
| 1             | L-Alanine      | 5.1405  | 228084  | 233,218      | 1.35435871 | 3.0534753 |
| 2             | L-Valine       | 6.5503  | 144952  | 246,218      | 0.32015376 | 0.0014459 |
| 3             | L-Isoleucine   | 7.5733  | 228158  | 260,232      | 0.31958964 | 0.0008974 |
| 4             | L-Leucine      | 7.8615  | 161853  | 275,260      | 1.53693437 | 1.3550694 |
| 5             | Serine         | 8.4261  | 228085  | 306,218      | 0.37428236 | 0.0641185 |
| 6             | L-Threonine    | 8.7458  | 26149   | 335,291      | 0.62347284 | 0.2963579 |
| 7             | Aspartic acid  | 10.4531 | 62986   | 349,334      | 5.35908355 | 8.4562986 |
| 8             | L-Glutamic acid| 11.6442 | 230760  | 148129       | 0.58267081 | 0.6430073 |
| 9             | L-Phenylalanine| 11.7229 | 35924   | 242,227      | 0.8481384  | 4.1435312 |
| 10            | Tryptophan     | 17.2949 | 221180  | 405291       | 0.63060574 | 2.2348957 |
| **Fatty acids** |                |      |         |              |           |
| 11            | α-Linolenic acid| 16.5704| 333201  | 350,335      | 0.32440528 | 0.0067373 |
| 12            | Linoleic acid  | 17.3095| 12256   | 337,262      | 0.32060234 | 1.8820221 |
| 13            | Myristic acid  | 13.8238| 11232   | 300,285      | 0.32007668 | 0.0027029 |
| 14            | Butanoic acid  | 9.0941 | 228230  | 8873         | 0.34275707 | 0.0170881 |
| **Organic acids** |              |      |         |              |           |
| 15            | Malic acid     | 8.6293 | 46657   | 350,335      | 3.39098534 | 6.459968 |
| 16            | Shikimic acid  | 13.8961| 205502  | 462,372      | 6.86680135 | 8.2878708 |
| 17            | Quininic acid  | 14.2108| 413965  | 552,345      | 0.32183187 | 2.6205762 |
| **Sugar**     |                |      |         |              |           |
| 18            | D-Xylose       | 9.6047 | 228124  | 150,73       | 1.77901339 | 1.0114974 |
| 19            | D-Ribose       | 12.1479| 28267   | 150,73       | 0.32064791 | 0.0006881 |
| 20            | D-Mannose      | 14.5666| 205521  | 438,435      | 1.3672006  | 1.0194719 |
| 21            | D-Glucose      | 14.5666| 205556  | 435,361      | 3.48317508 | 1.0309821 |
| 22            | D-Fructose     | 14.677 | 11338   | 439,437      | 4.10800107 | 1.2015461 |
| No. | Compound               | Rf   | Rr   | RfR  | RrR  |
|-----|------------------------|------|------|------|------|
| 23  | Sucrose                | 19.3741 | 25039 | 451, 437 | 8.98074159 | 6.1861969 |
| 24  | D-Mannitol             | 14.9253 | 229352 | 421, 319 | 1.92989778 | 2.5710062 |
| 25  | D-Sorbitol             | 14.9912 | 46704  | 423, 345 | 0.38415401 | 0.5781112 |
| 26  | Myo-Inositol           | 15.5096 | 7496  | 432, 318 | 0.42204715 | 0.2274036 |
|     | **Sugar Alcohols**     |      |      |      |      |
| 27  | α-Farnesene            | 10.9715 | 9209  | 204135 | 0.3192523 | 0.0006514 |
| 28  | Benzoic acid           | 10.9885 | 183091 | 194, 179 | 0.548034 | 3.1840035 |
| 29  | 4-Hydroxybenzoic acid  | 11.7763 | 190049 | 282267 | 0.32735111 | 1.5762578 |
| 30  | 4-Hydroxybenzenearctic acid | 11.906 | 47018  | 296, 281 | 0.49748042 | 2.3952006 |
|     | **Secondary metabolites** |      |      |      |      |
| 31  | Protocatechoic acid    | 13.8584 | 352467 | 370, 355 | 0.31891183 | 2.3325041 |
| 32  | 4-Coumaric acid        | 15.1373 | 291951 | 308, 293 | 0.37758924 | 4.8621667 |
| 33  | Ferulic acid           | 16.5697 | 234120 | 338, 323 | 0.42922309 | 2.3345868 |
| 34  | Caffeic acid           | 16.8821 | 26654  | 396, 381 | 0.33327407 | 2.226001 |
| 35  | Catechine              | 20.434  | 722951 | 649, 461 | 0.4897089 | 2.9069223 |
| 36  | Oleanolic acid         | 25.1903 | 241951 | 600,482 | 0.32359863 | 5.933836 |
| 37  | Ursolic acid           | 25.8442 | 58500  | 456,248 | 0.33606618 | 0.8641425 |
| 38  | Phytol                 | 17.0533 | 375015 | 296123 | 0.45518709 | 0.2222035 |

Figure 1: Growth of mycelium on the MEP plate and apple leaves
Figure 2 Penetration of mycelium into the leaves of apple. Image A. depicts conidia and hyphae and Image B. depicts hyphae. (Magnification 40X)
Figure 3 Typical GC-MS chromatograms (TIC) infected leaves and normal leaves of *Malus domestica* (Shireen cultivar of Apple)
**Figure 4** Graphs showing variations of 16 major metabolites differentially accumulated in Shireen Infected and non-infected leaves with *Venturia inequalis*. The X-axis indicates the infection status and Y-axis indicates the concentration of metabolite as the Relative percentage area (RPA).

**Figure 5** Heat map showing variations of 38 metabolites (more than 80% similarity in NIST 11) differentially accumulated in Shireen Infected and non-infected leaves with *Venturia inequalis*.
Comparative analysis of normal and infected leaf of scab resistant apple cultivar Shireen was performed after infecting the leaves with the fungal culture of *V. inaequalis*. After 14 days of infection, the infected and non-infected leaves were analyzed by bright field microscopy to see changes at the physiological level and further GCMS analysis was done to detect changes at metabolite level post-infection with fungus.

We analyzed the amino acids, fatty acid, organic acids, sugars, sugar alcohols and secondary metabolites of normal and infected leaf based on the GCMS method. During the infection amino acids such as valine, Isoleucine, leucine, Serine, Threonine level decreases whereas the Alanine, Aspartic acid, Glutamic acid, Phenylalanine, and Tryptophan level increased that reveals the essential amino acid and cell metabolism level decreased during infection and it is strongly confirmed with the decreased level of fatty acid and sugar.

The amount of secondary metabolites increased drastically during the infection that reveals the secondary metabolites play the role of antifungal activity.

Secondary metabolites like Benzoates (benzoic acid, coumaric acid, protocatechuic acid, and caffeic acid) and terpenes like ursoic acid and oleanolic acid level were increased which provide the evidence that the metabolites accumulated post-infection play important role in resistance against the *Venturia inaequalis*.

So by the detailed metabolic study of infected and no infected leaves of Shireen apple cultivar by GC MS we can conclude that metabolites up-regulated or accumulated after infection plays important role in plant defense and do not allow the penetration of fungal hyphae in leaf thus inhibiting the growth of fungus.

The present study in the area of metabolomics of infected leaves gives an insight of regulation at the metabolic level, which can further help in determining genes responsible for the accumulation of these metabolites.

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How to cite this article:
Sonali Kumari, Pratibha, Debabrata Sircar and Rahul Anand. 2020. Metabolic Studies of Malus domestica Leaves Infected and Non-infected with Venturia inaequalis. Int.J.Curr.Microbiol.App.Sci. 9(03): 514-525. doi: https://doi.org/10.20546/ijemas.2020.903.060