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Phenotypic Characterization and Primary Metabolite Profiling Of Black Cherry Heirloom Tomato

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ABSTRACT

Tomato (SolanumlycopersiconL.) is an important vegetable crop with numerous uses with a high nutritional value as dietary carotenoids serve as the precursor for vitamin A and prevent several chronic-degenerative diseases. Carotenoid profiling is necessary to understand its importance on human health. In tomatoes, carotenoids are important concerning major breeding traits such as fruit color and human health. In our study, we have selected black cherry and black pear heirloom tomatoes and ArkaVikas as control. To investigate these heirloom lines we have framed our objective in two parts (I) phenotypic characterization of these heirloom lines and (II) metabolite profiling of the lines to understand the development of fruit ripening and which metabolite play an important role in phenotypical character and also levels of primary metabolites by GC-MS. In our study Black Pear, a transition from mature green to red ripe took almost double time. , In the ripening stage of Black Cherry and black pear phytofluene level is completely absent and lycopene level is 3-fold less in comparison to ArkaVikas.

Keywords: Black Cherry, Carotenoid, Primary metabolites, Arkavikas (AV), Black cherry (BC), Black Pear (BP)

I. INTRODUCTION

Tomato (*Solanumlycopersicum*) is one of the most important vegetable crops. In India, its consumption is highest and is next to potato. It is the most widely grown solanaceous vegetable crop grown worldwide under outdoor and indoor conditions. It is consumed as fresh fruit, or cooked and also in processed form. Tomato is grown worldwide with a production of 152 million tonnes [1].Tomato is a rich source of nutrients like lycopene and beta-carotene, the compounds that protect cells from cancer [2]. Tomato is intently modified by plant breeders to improve economically important traits such as yield, fruit size, color, taste, sugar content, and health-related metabolites, lycopene, β-carotene, vitamin C,etc. [3][4].

Tomato is the world's favorite fruit due to its special flavorand high nutritional value. Tomato fruit contains largeamounts of metabolites such as sucrose, hexoses, citrate,malate, and ascorbic acid. There are also many

health-beneficialcompounds such as carotenoids, phenylpropanoids,and terpenoids that accumulate in tomato fruit[5]. The existence of these compoundsestablishes that many basic biosynthetic pathwaysare intact in tomatoes. Therefore, when undertaking metabolicengineering, a limited number of additional genesneeds to be introduced, which can significantly simplifythe engineering process. In addition, substrates such assugars and aromatic amino acids, Therefore, GC-MS can facilitate the identification and robust quantification of a few hundred metabolites within a single plant extract, resulting in fairly comprehensive coverage of the central pathways of primary metabolism [6][7]. Although no single analytical system can cover the whole metabolome, GC-MS has a relatively broad coverage of compound classes, including organic and amino acids, sugars, sugar alcohols, phosphorylated intermediates, and lipophilic compounds [7][8]. The main advantages of this technology are that it has long been used for metabolite profiling and thus there are stable protocols for machine set-up and maintenance, and chromatogram evaluation and interpretation. Furthermore, the advent of faster computers, improved algorithms, statistical software packages, and available databases will likely allow for the exploitation of this method and thus enable the capture of more biologically relevant information.

II. MATERIAL AND METHODS

A. Plant Materials and Growth Conditions

For a study of phenotypical characterization and metabolite profiling of tomatoes, ArkaVikas variety was used as control, and black cherry and back pear heirloom varieties were used as study plant material. The seeds were surface sterilized with 4% (v/v) sodium hypochlorite approximately for five to ten minutes till the seed coat became thinner which was evident by the visibility of the embryo. Thereafter, seeds were washed thoroughly in distilled water till hypochlorite smell goes off and spread on filter papers moistened with distilled water in plastic cups for germination. These cups were kept in the darkroom at a temperature of 25±2°C. After seeds germination (showing radical emergence), seeds were transferred in plastic germination boxes filled with coconut peat. These boxes were kept in a growth room for twenty days then these seedlings were transferred into pots filled with red loam sandy soils in the field.

B. Fruit development and phenotypic characterization

Phenotypic characters such as life morphology, flower morphology, and fruit morphology were observed as well as other characters such as phyllotaxy, intermodal distance, and flower per inflorescence, and fruit per inflorescence were observed. Plants were photographed to observe phenotypic variations.

To determine the age of the fruit, the flowers were tagged day after pollination (DOP) that was considered as zero-day. Pollinated flowers were differentiated by observing floral senescence and the presence of pollen on the stigma. The size of the fruit was periodically measured using software called tomato analyzer version 3.0, also maximum height, maximum width, pericarp thickness were measured.Fruit firmness was measured by Durofel DFT–100 (Agrotech). Two measurements were taken at equatorial positions of each fruit two times and an average was taken into consideration. The firmness unit was expressed in percentage DUR.

With the same tomato analyzer software version 3.0 fruit color of ArkaVikas, black cheery, and black pear were measured at different stages of fruit including mature green, turning and red ripe stage. The color was expressed in L*, a*, b*. L* value relates to lightness (white to black), a* value relates to (green to red), and the b* to (blue to yellow) color of the fruit.

C. Analysis of primary metabolites by GC-MS

GC-MS analysis of ArkaVikas was done in the Red Ripe stage according to Roessner. (2000) for identification of primary metabolites. All experiments were carried out *with* 3 *biological replicates* for each stage. A polar metabolite fraction was extracted from approximately 100mg of powdered fruit tissue in 1400 µl 100% methanol with 60 µl internal standard (0.2 mg ribitol ml^{-1} water). The mixture was extracted for 15 min at 70°C. The extract was vigorously mixed with 1400 µl water and centrifuged at 2200 *g.* Aliquots of the methanol/water supernatant (150 µl) were dried *in vacuo* for 2h. The dried residue was redissolved and derivatized for 90 min at 30°C (in 80 µl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine) followed by a 30 min treatment at 37°C (with 80 µl MSTFA). 20 µl of a retention time standard mixture (F.A.M.E. Mix, Sigma, 1 µg $µl^{-1}$ in hexane) was added beforetrimethylsilylation.

The sample volume of 1 µl was injected in splitless mode. The GC–MS system consisted of an MPS Autosampler (Gerstel), a GC 7890A (Agilent) gas chromatograph, and a Pegasus 4D TOF mass spectrometer (Leco, US). Gas chromatography was performed on a 30 m Rxi-5ms column with 0.25 mm i.d. and 0.25 µm film thickness (Restek). The injection temperature was 230°C, the interface set to 250°C, and the ion source adjusted to 200°C. The carrier gas used was helium set at a constant flow rate of 1.5 ml min^{-1}. The temperature program was 5 min isothermal heating at 70°C, followed by a 5°C min^{-1}oven temperature ramp to 290°C and a final 5 min heating at 290°C. Mass spectra were recorded at 2 scans/sec with an m/z 70–600 scanning range. The chromatograms and mass spectra were evaluated using the ChromaTOF software (Leco, US). The mass spectra of the identified peaks were matched by the NIST library.

Data analysis was done by normalizing all the data by ArkaVikas as the reference sample. All the data was scaled to Pareto-scaling before analysis. Heatmap visualization of the data was done between different replicates of MG and BR and MG and RR stager of using the Pearson distance measure. Principal Component Analysis (PCA) with 95% confidence interval of metabolite data from biological replicate samples was done shows differences between MG, BR, and RR stage. All the statistics were done using Metaboanalyst 3.0.

D. Statistical analysis

All measurements and estimations were done with at least three biological replicates of control ArkaVikas and three experimental replicates at each stage of fruit development. The data were represented in the form of graphs using their mean and standard error values plotted by Sigmaplot software 10.0 version.

III. RESULTS AND DISCUSSION

A. Phenotypic Characterization

Higher plants have evolved several mechanisms by which they can adapt themselves to their surrounding environment. Most prominent of these is the use of environmental stimuli that alters physiological and developmental responses, thus enabling them to cope up with the existing environment. Hereis our observation in table 1.Phenotypic characteristics such as leaf morphology and flower morphology of control as well as two experimental heirloom varieties. Generally,the tomato has compound leaves, a compound leaf is made up of leaflets that are distributed along the leaf rachis, but in the case of black pear we found that there isa decrease in the number of leaflets and potato type leaf morphology as a comparison to ArkaVikas. Here we also observed the other characters such as average internodal distance, average flower per inflorescence, and average fruit per inflorescence but only in case we found black cherry found the difference in flower per inflorescence and fruit per inflorescence.

Tomato fruit attains different colors like green, yellow, pink, light red, and dark red at different time points of the ripening stage. By using tomato analyzer software color of the fruit was measured at red ripe (Table 2). Wild-type fruit at the mature green stage is darker and less dark in red ripe than experimental lines. The Colour of the fruit was measured at the red ripe stage. The tomato analyzer readings showed a decrease in L* value as the fruit reach red ripe from mature green indicating the change in colour of fruit from light to dark and increase in a* value from mature green to red ripe stage indicating the gradual decrease of chlorophyll level and increase in carotenoids level. L*, a*, and b* values were measured at red ripe (RR). Black cherry and black pear significantly lower value of L* at red ripe stage as compared to wild type but in case of black pear l* value comparative more than black cherry. Black cherry and black pear has a significantly high a* value and b* at red ripe stage as compared to the control. L* Lightness,-L* Darkness, +a *Red direction, -a *Green direction, +b *Yellow direction, -b* Blue direction.

Table1.Phenotypic characteristic of AV, BC and BP

LINE	Intermodal distance (cm)	Avg.flower/inflorescence	Avg.fruit/inflorescence	phyllotaxy
ARKA VIKAS	4	2	8	opposite
BLACK CHERRY	7	2.2	9	opposite
BLACK PEAR	3	3	6	opposite

Table.2 Average chromaticity values of AV, BC and BP

Sr.no	Lines	Avg.L* values	Avg.a* values	Avg.b* values
1	Arkavikas	43.66	33.98	43.81
2	Black cherry	22.82	12.24	24.11
3	Black pear	42.15	20.22	3841

B. Fruit development

Physiology of fruit formation includes three phases: Fruit development; characterized by the active growth of fruit until it reaches to mature green stage, where cell division and expansion of fruit almost stops[11] (figure 1). Fruit ripening; follows soon after attainment of mature green stage and that can be characterized by change in colour from green to yellow to orange to bright red and finally senescence; characterized by change in colour from bright red to dull brown and softening of fruit texture. At developmental stages of Arkavikas, black cherry, and black pear fruits we observed to turn from mature green to turning they almost took average 6 to 7 days but to reach the red ripe stage it took almost 18 days in case of black pear rather than control and black cheery.

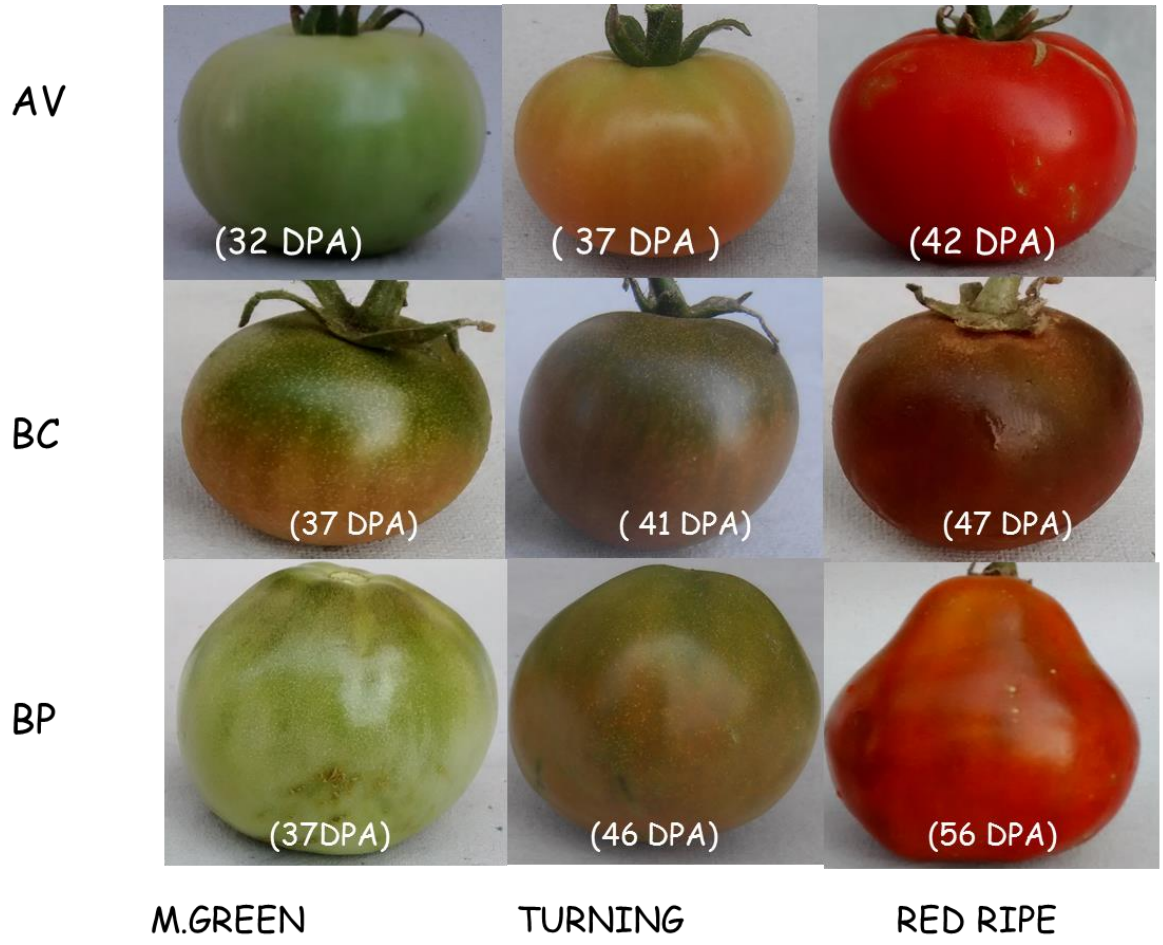

Figure 1: Different developmental stages of AV, BC, and BP

C. Primary metabolite profiling

The most dramatic change in tomato fruit development occurs during the transition to the ripening process that is coupled with the accumulation of numerous secondary metabolites. Moreover, the transition to ripening is accompanied by a massive change to metabolism as a result of the de-greening process, in which the photosynthetically active chloroplasts are differentiated to chromoplasts. The metabolome is defined as the total small-molecule complement of a cell, and metabolomics is therefore the study of all the low molecular-weight molecules or metabolites of a cell or organism[9][10].

Metabolite profiling is a rapidly expanding technology that aims to quantify the entire metabolome of biological samples. Gas Chromatography-Mass Spectrometry (GC-MS) is one of the most widely used analytical tools for profiling highly complex mixtures of primary metabolites, such as organic and amino acids, sugars, sugars alcohols, phosphorylated intermediates, and lipophilic compounds[9].

Primary metabolite profiling study was done in a red ripe stage of AV, BP, and BC. In this primary metabolite study, we found we identified around 72 different metabolites. (Table 3). The hierarchical clustering was done between different replicates of ripened fruit tissue using two parameters- similarity measure and clustering algorithms. Each sample can be treated as one cluster. Thus depicting the pictorial relationship among the replicates of ripened fruit samplesbased on identified metabolites. Further, Principal Component Analysis was done. It tells about the variance in the sample clusters. We found that AV, BC and BP formed three different

clusters which were indicative of these lines behaving distinctly in terms of the concentration of metabolites present.

A heat map from the clustering data of the identified metabolites was generated for detailed analysis (Figure 4). Several metabolites were identified with the significant increase in concentration in varieties of tomato analyzed. In the case of primary metabolite analysis, we identified, Black cherry and Black pear havea high concentration of amino acid and low concentration of organic acid as compared to Arkavikas and low content of organic acid.

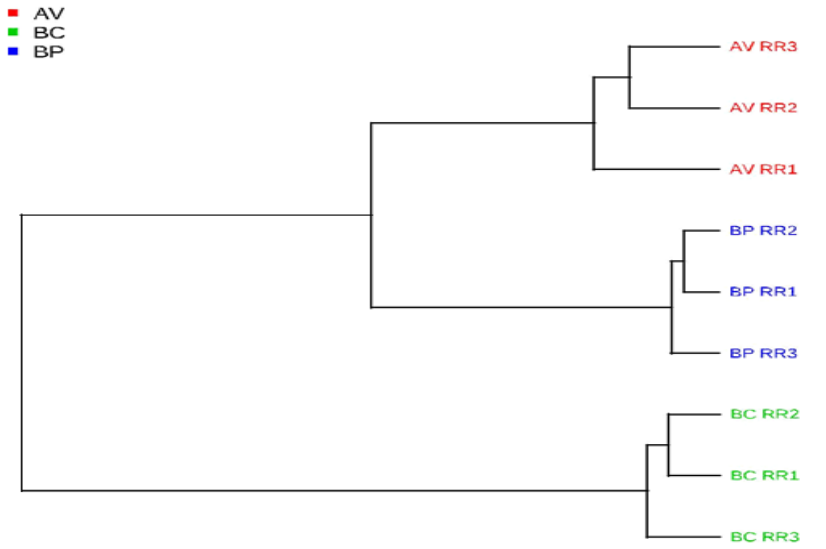

Figure 2: Dendogram showing hierarchical clustering of replicates of ripened fruit of AV, BC and BP plant.

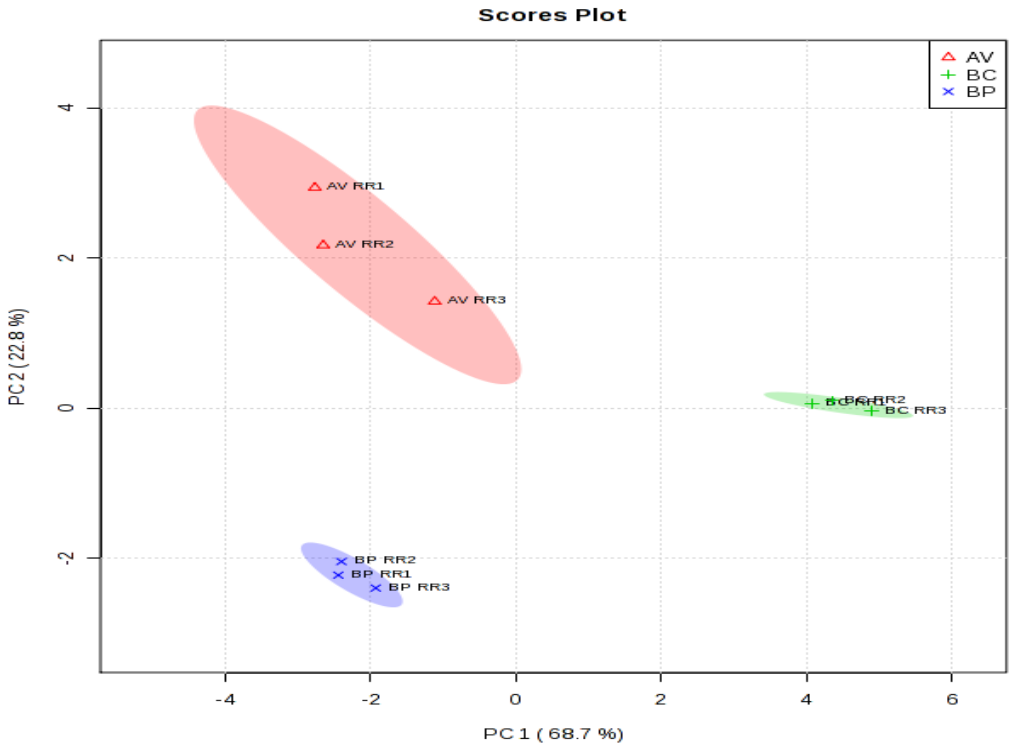

Figure 3: PCA analysis of replicates of ripened fruits of AV, BC and BP plant.

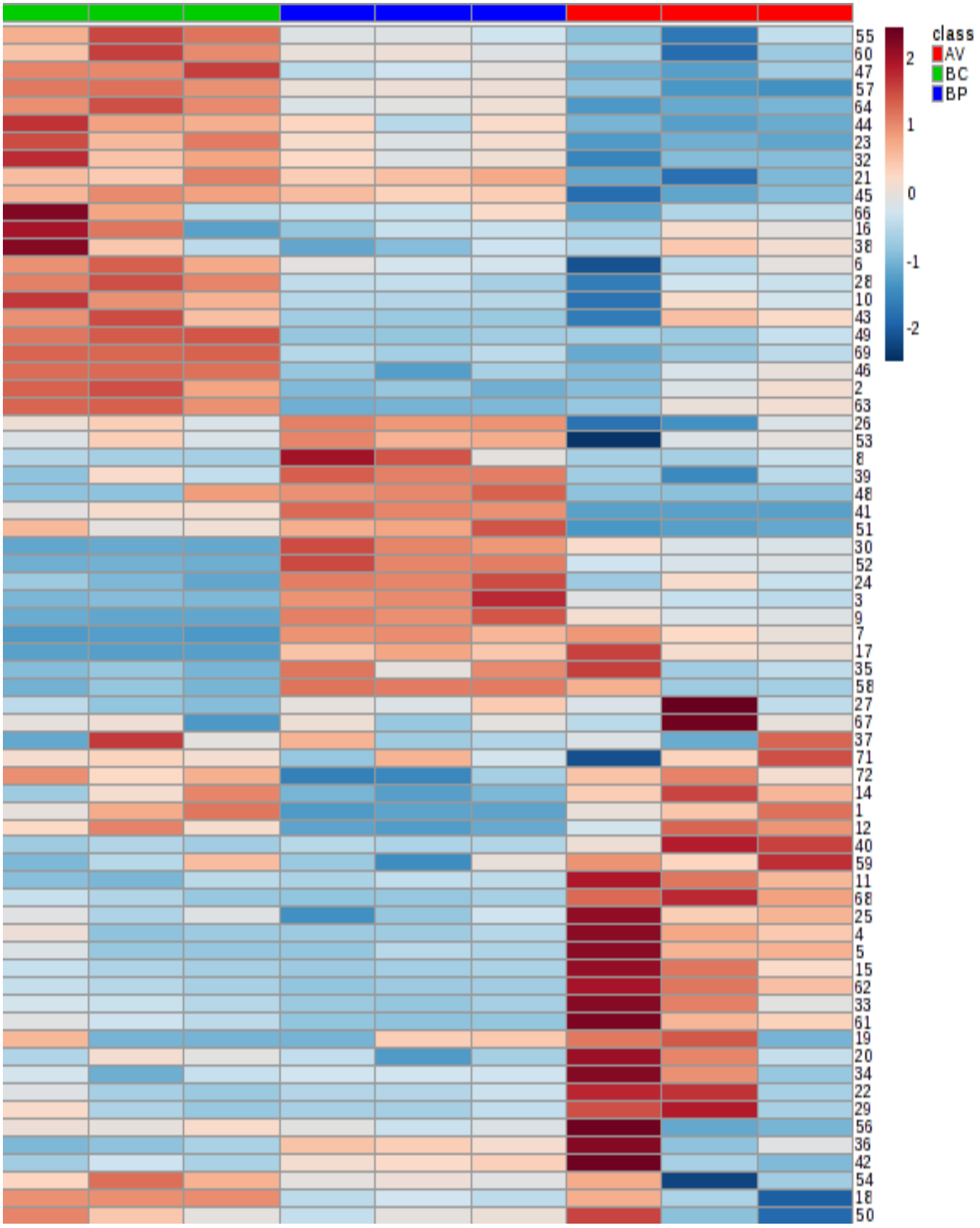

Figure 4: Heat map of the identified metabolite. Heat map visualization of the data was done between different replicates of red ripe stages of fruits using the Pearson distance measured.

Table 3: List of metabolites significantly present at high concentration in AV, BC.BP

Sr. No	LINES	METOBOLITES
1	ARKA VIKAS	Putrescine, L-Tryptophan, Decanoic acid, Succinic acid , Lactic Acid, 3-Hydroxybenzoic acid, 4-Hydroxybenzoic acid, Fumaric acid, Malic acid , Nicotinic acid , Maleic acid .
2	BLACK CHERRY	L-Alanine, L-Glutamic acid, Asparagine, Pipecolic acid, Dodecanoic acid, Pyroglutamic acid, L-Glutamine, L-Asparagine, L-5-Oxoproline, 2-Imidazolidone-4-carboxylic acid, Asparagine, Alanine, 3-cyano, Glutamine, Ornithine, L-Threonine.
3	BLACK PEAR	L-Leucine , L-Valine , d-Mannose, L-Serine, myo-Inositol-2-phosphate , cis-Aconitic acid, Itaconic acid, Methyl maleic acid, Hydroxylamine, cis-Caffeic acid, Octadecanoic acid, Galactaric acid, Palmitic Acid, Adenosine, L-Cysteine, D-Xylose, Arabinose methoxyamine , Glucose methoxyamine , Sucrose , Citric acid , 2,3-Butanediol, Myo-Inositol.

IV. CONCLUSION

In this study, phenotypic characterization and metabolite profiling of black cherry and black pear was done. We found that in the case of Black Pear, a transition from mature green to red ripe took almost double time in comparison to control. At developmental stages of Arkavikas, black cherry, and black pear fruits we observed to turn from mature green to turning they almost took average 6 to 7 days but to reach the red ripe stage it took almost 18 days in case of black pear rather than control and black cheery. In the case of primary metabolite analysis, Black cherry and Black pear havea high concentration of amino acid and a low concentration of organic acid.

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