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METABOLOMIC ANALYSIS OF CITRUS INFECTED WITH HUANGLONGBING

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ABSTRACT

Metabolic profiling is the measurement of low-molecular weight metabolites and their intermediates in any biological function that directs the dynamic response to genetic modification, physiological responses, pathophysiological responses, and/or developmental stimuli. The measurement and interpretation of the endogenous metabolite profile from a biological sample (typically urine, serum, or biological tissue extract) have provided many opportunities to investigate the changes induced by external stimuli (e.g., drug treatment) or to enhance the knowledge of inherent biological variation within sub-populations. This article will focus on the basic principles of metabolic profiling and its tools (nuclear magnetic resonance [NMR], liquid chromatography-mass spectrometry [LC-MS]) which can be applied in toxicological and pathological pathways in a biological system of citrus infected with Huanglongbing. Citrus Huanglongbing (HLB) is the most destructive disease of citrus worldwide. It is a fast-growing, alphaproto-bacterial disease, which can spread within a tree in one year. The major symptoms of HLB include yellowing leaves with vein yellowing, inedible, small fruits, which remain green. HLB-infected fruits drop from the affiliated trees. Seeds are usually aborted, and fruit is bitter in taste. Metabolomic techniques are based on extraction, separation, and quantification methods were developed to find potential HLB biomarkers in leaves from orange trees from commercial groves. Flavonoids and their derivatives such as naringenin, hesperidin and quercetin, as well as the amino acid, L-proline were significantly increased in HLB-affected citrus trees. Conversely, sesquiterpenes β-elemene, (-) trans-caryophyllene, and α-humulene were significantly decreased in HLB samples when compared to healthy and zinc deficient trees.

Keywords: Metabolomics, LC-MS, NMR, Huanglongbing, Flavonoids

INTRODUCTION

Metabolites are the major end product of any regulatory cellular process in any biological system responsible for the genetic or environmental changes in an environment. Many of the metabolomics analysis are used for profiling of specific selected compounds, or alternatively fingerprinting metabolic changes is used to determine different metabolic level individually. For the reliable metabolomics analysis of plant tissue, careful procedure applied for the extraction of plant tissue, sample preparation, data acquisition and data mining (Albrecht & Bowman, 2012).

There are two types of metabolomics analysis, *i.e.* targeted analysis and untargeted analysis. Targeted analysis focuses on a specific chemical compound in a sample which requires a higher level of purification. The selective metabolites extract in targeted analysis. Conversely, untargeted analysis of metabolites, extraction and identification of as many as metabolites is possible which results in fingerprints without the identification and quantification of specific compounds (Allwood et al., 2008; Cevallos-Cevallos et al., 2009; Monton & Soga, 2007).

Analysis of metabolites comprises several steps, i.e. preparation of sample, extraction of metabolites, derivatization, and separation of metabolites (through GC, CE & LC) (Mumtaz et al., 2017; Servillo et al., 2017; Wishart, 2008a), their treatment, detection (NMR & MS) and a treatment of data (Cevallos-Cevallos et al., 2009; Della Sala et al., 2017). Proper grinding during sample preparation improves the extraction of metabolites. The “Freeze-drying” is a sublimation/dehydration process and lowers the conceivable differences in metabolites due to the removal of water content and other solvent from the sample. The initial and most critical step is the extraction method, which minimizes the amount and concentration of the sample. The nature of compound of interest is usually unknown in untargeted metabolites. Derivatization is potentially used before the qualitative analysis through Gas Chromatography (GC), which increases the volatility of analytes. Separation and detection of metabolites are the most important step in metabolic profiling and different techniques have been used such as, High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography UPLC, Capillary Electrphoresis (CE), Gas Chromatography (GC), Mass Spectrophotometer (MS), Nuclear Magnetic Resonance (NMR) (Killiny et al., 2017), Near Infrared Spectroscopy (Munir et al., 2019) etc.

The major technological change in metabolomics occurs due to its dynamic range in analytical procedures, i.e. the highest to lowest concentration of metabolites detected in an instrument which itself response from its linear analyte concentration. These dynamic ranges depend upon the debris compounds and the specific compounds in the samples. This is the most difficult step in metabolomics analysis. The dynamic ranges in the metabolomics analysis vary from 104-105 M or mM for the individual compound in a sample, but this can be reduced when other interfering chemical compounds are present in a sample (Southam et al., 2007).

To summarize, many other chemical compounds present in an analyte may interfere in the analysis of specific compounds; which limited the reliability and ranges of this specific compound to be profiled. For example, when primary metabolites are profiled (such as sugars), many secondary metabolites interfere with the primary metabolite analysis; these may be flavones, flavonoids, etc. (Sumner et al., 2003).

The extraction of metabolites is the most crucial step from plant samples. Metabolite analysis possess changes in gene expression level that control metabolite fluxes, and which can be measured by the proteomic analysis, as well as by transcriptomic analysis. Metabolite analysis is done to study the genetic modification in plants, their plant-host interactions as well as the environmental responses of the plant. Study of metabolites is widely used to discover the byproducts of transgene expression in evaluation of genetically modified plant tissues and potential adoption of the plant crops in the environment. Studies have been conducted on physiological growth of plant tissues under stress conditions, which have been induced a variety of small molecules such as: bacterial lipopolysaccharide, yeast elicitors and methyl jasmonic acid. Plant-host interactions symbolized challenging and complex biochemical situations that were measured by different metabolites approaches (Allwood et al., 2008; Matsukawa et al., 2017).

Huanglongbing is a devastating disease which drastically affects citrus industry world-widely (Ikpechukwu, 2012). Metabolic profiling supports our understanding in toxicological and pathologic changes which occurs in healthy or diseased cells or tissues and helps in control of bacterial and viral diseases through identification of potential biochemical markers. Additionally, after identification of potential biomarkers, compound profiles can be identified through worldwide databases (MzCloud, METLINE, MetExplore etc.) which helps in screening of compounds in the rate of diseases and helps whether to choose or removed these compounds from the plants (Xu et al., 2017). Metabolite analysis provides a very clear picture of what changes actually occur in biological pathways, as well as at the genetic (transcriptional) and protein levels in any biological system (Lu et al., 2017). Many tools have been proposed to study the low molecular weight metabolite (flavonoids: flavone, flavanol, flavanone *etc*.) such as, NMR, LCMS, HPLC, GCMS, PAGE, Gene Chip and RIA (Clarke & Haselden, 2008; Zandalinas et al., 2017).

The classes of flavonoids contain at least 6000 different types of flavonoids which include flavones, flavanoles, aurones, isoflavanoid and anthocyanin. Flavonoids play a role in fertility, UV scavenging, and disease resistance as phytoanticipin and pytoalexin, besides their function as pigmentation in flower and pollinator attraction in fruit. Citrus plants are the important source of flavones, flavanols and polymythoxylated flavones, which helps in disease resistance against pathogens. The most important flavonoids present in citrus are tangerine, sinensitin and hesperidin (Ballester et al., 2013a; Hwang et al., 2017).

Analysis of metabolites from *C*Las-infected citrus cultivars

Rutaceae is the family of tropical and temperate region with, 150 genera and 1600 species. *Rutaceae* is very important family due to their economic value. There was a phytochemical investigation of *Rutaceae* family indicated many biologically active compounds present in citrus family, these are coumarins, alkaloids, flavonoids and limonoids in *Citrus limon* Linn. (var. Jatti Khatti), *Citrus reticulata* Blanco (var. kinnow), *Citrus limetta* Risso (var. mitha) and *Citrus aurantifolia* Swing (var. kaghzi nimboo) (Nogata et al., 2006). The class of flavonoids contain at least 6000 different types of flavonoids include flavones, flavanoles, aurones, isoflavanoid and anthocyanin. Flavonoids include in fertility, UV scavenging and disease resistance as phytoanticipin and pytoalexin, besides their function as pigmentation in flower and pollinator attract in fruit. Citrus are the important source of flavones, flavanols and polymythoxylated flavones which helps in resistance the plants to diseases against pathogens. The most important flavonoidsinclude in citrus are tangerine, sinensitin and hesperidin (Ballester et al., 2013b).

The flavonoid content was modified byMassenti et al.(2016) from HLB-affected Valencia oranges and; determined higher level of hesperidine, narirutin, tangertin, didymin and nobiletin from the HLB-affected juice, fruit peel and pulp of the citrus fruit through LC. Different quality components such as peel color, level of citric acid and total soluble salts (TSS) were also analyzed through asymptomatic and controls where they revealed a smaller symptomatic fruit in size, containing less and bitter juice, having higher acidity level, peel color was half yellow and half green and lower sugar level than the controls in their results. The flavonoids in the peel were decreased in level with the maturity of fruit and in pulp and juice, were observed higher in symptomatic than controls and asymptomatic citrus plant. Secondary metabolism and physical properties were affected in symptomatic citrus plants where at the maturity level where citrus greening was at its peak level and was observed the loss of fruit in HLB infected citrus fruit due to the higher level of flavonoids in pulp and peel of fruit.

Chemical analysis showed lower soluble solids and higher acidity. The level of Limonin and nomilin level of *C*Las-infected citrus plants were decreased in their concentration than healthy juices. The chemical and sensory differences were tested through electronic tongue differentiations of blends of juices of citrus (Raithore et al., 2015).

A study from state of Florida observed the bitter taste and off-flavor citrus fruit infected with Huanglongbing. They found higher level of sugar and pectin content with low pH level in HLB-affected fruit. They revealed in their study that the sonicator increased the yield of DNA 86% by using homogenization of cells compared to DNA extraction by pestle and mortar. This study particularly analyzed the method of detection of *C*Las-infected citrus plants (Bai et al., 2013).

Limonin and nomilin are two biter chemical compounds found in citrus fruits and usually activated in off-flavor of *C*Las-infected citrus fruits. Limonin and nomilin with the sugar (Sucrose) and citric acids level from *C*Las-infected citrus juice compared to control and found lower level of limonin (4.0 mg/l) than the nomilin (5.4 mg/l). The addition of sucrose to the orange juice decreased the level of bitterness including nomilin and limonin. All citrus varieties are susceptible to *C*Las-infection, but lemon showed reduced susceptibility (Dea et al., 2013).

Analysis of metabolomic was also checked by inoculation of *Penecillium* to *C*Las-infected navelate orange and found that metabolites and their derivatives present in phenyl propanoids pathway play significant role in stimulating the citrus fruits infected with *C*Las by using HPLC-Photo Diode Array detectors and quadrupole time-of-flight mass spectrometry (HPLC-PDA-QTOF–MS) and found higher level of compounds in flavedo than albedo. The flavonoids componentswere analyzed and observed that drupanin aldehyde and citrusnin A was higher in elicited fruit (Ballester et al., 2013b).

Increased concentration of maltose and α amylase affected the *C*Las-infected leaves but surprisingly, decreased level of β amylase found with the time in *C*Las-infected leaves. Moreover, higher level of fructose and glucose and lower level of maltose were observed in *C*Las-infected grapefruit, Hamlin and Valencia late. But it was noted that maltose level was increased in January in freezing temperature in mature *C*Las-infected leaves (Cancalon, 2012). Higher concentration of narirutin and hesperidin with higher level of antioxidant capacity and phytochemical activity was also observed in *C*Las-infected citrus plant (Ramful et al.,2011). Decreased level sucrose and starch level in flavedo portion of HLB symptomatic fruit than asymptomatic and healthy fruit. Decreased level of ethylene with increased level of IAA and ABA were analyzed in *C*Las-infected hypodermal cells of flavedo portion citrus fruit than asymptomatic and healthy (Rosales &Burns 2011).

Volatile and non-volatile compounds from orange juice chemically taken from the *C*Las-infected citrus trees through HPLC and GC-MS. 62% decreased level of Brix/acid from symptomatic and asymptomatic juices. Surprisingly, a very low level of flavanone, flavanone and neohesperidosides which were main components of bitterness of CLas-infected fruit, observed in citrus juices of HLB-affected fruits. Limonin, rates of terpenes, alcohols and linalool were found higher; and average rate of ethyl butanoate, total ester content, total aldehydes content and total sesquiterpenes content were found in symptomatic juice as compared to control. The researchers found that off-flavor of *C*Las-infected fruits were due to lower concentration of carbohydrates and higher the concentrations of acids in that fruits which increased the compounds responsible for the bitter taste of citrus fruit (Dagulo et al., 2010).

Citrus fruit is rich source of vitamin C and responsible for major component of various metabolic changes as) worked on juice flavor, chemistry through triangle test in order to experience the flavor of citrus juice (Plotto et al., 2008). Test was performed with juice after or before filtration of pulp. Major differences observer after filtration between the smell and taste of that juice compared to unfiltered juice. HLB-positive filtered juice was sweet and had decreased level of acids and higher level of aldehydes and soluble solids to acid ratio than HLB-negative filter juice, which was the major cause of sweeteners. They also observed volatile organic compound analysis and found higher concentration of Methanol, 2 methyl propanol and α pinene of *C*Las-infected juice.

CONCLUSION

Metabolomics has shown to be an important tool for the progress of the main biological science areas. This study demonstrated the metabolomics analysis, its types and tools to detect the secondary metabolites in citrus plants infected with HLB. The objectives of this current study are to study the earliest significant difference of metabolites in leaves of citrus infected with HLB and healthy leaves.

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