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BIOCHEMICAL PROFILING OF CITRUS INFECTED WITH CITRUS GUMMOSIS DISEASE

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A B S T R A C T

This research aims at the estimation of total soluble phenols, total soluble sugars, antioxidants and minerals i.e. zinc (Zn), iron (Fe) and manganese (Mn) in healthy and gummosis infected plant tissues. In this study, the mean of total soluble phenols were higher in healthy (6mg/ml and 3 mg/ml in leaves and bark, respectively) than infected plants tissues (2mg/ml and 1mg/ml in leaves and bark, respectively). The total soluble sugar in healthy plant tissue (5mg/ml, 3 mg/ml and 10 mg/ml in leaves, bark and fruits, respectively) was greater than in infected plants tissues (2mg/ml, 1 mg/ml and 3 mg/ml in leaves, bark and fruits, respectively). The antioxidant enzymes was present in higher quantity in infected plant tissues (43mg, 15mg and 50mg SOD, POD and CAT, respectively) than healthy plant tissues (35mg, 10mg and 40mg SOD, POD and CAT, respectively). The mineral elements significantly reduced in infected plants (51mg/kg, 48mg/kg and 30mg/kg Fe, Zn and Mn, respectively) as compared to healthy one (116mg/kg, 78mg/kg and 74mg/kg Fe, Zn and Mn, respectively). The biochemical characterization of gummosis disease would be helpful in sustainable disease management.

Keywords: Biochemical analysis, Gummosis, Profiling, Antixodants, Soluble suagrs, Soluble phenols.

INTRODUCTION

Citrus is the 2nd major fruit crop cultivated commercially in almost 50 countries worldwide (Naqvi, 2000). Foot decay/gummosis caused by *Phytophthora citrophthora*, is a destructive disease that incites huge qualitative and quantitative losses (Mekonen *et al*., 2015). It assaults citrus plants in nurseries causing damping-off to stringy root decay, crown decay, pre-mature defoliation, foot decay, and gum oozing from trunk in developed plantations (Shekari *et al*., 2012). Phenolic antioxidants have a vital position in defending organisms against the adverse causes of reactive oxygen species (Roginsky and Lissi, 2005). The phenolic contents may differ in various germplasm that is influenced by agro-technical procedures, climatic situations, pre and postharvest handlings (Vinson *et al*., 2001). Citruses possess many antioxidants and phenols that manages the devastating effects of biotic stresses (Lin *et al*., 2014). The antioxidant enzymes activate the plant defense system against pathogenic attack (Das *et al*., 2010). The attack of *Phytophthora* produces many ROS, the harmful effects of which are mitigated by the enzymatic antioxidants (Elkahoui *et al*., 2005). Total soluble sugars function as structural component of plants and take part in growth and development (Loreti *et al*., 2005). It regulates the plant metabolic pathway under stress conditions and responds to the adverse signals (Tran *et al*., 2007). Plants undergo significant reduction in quantity and frequency of photosynthesis resulting in serious physiological and biochemical changes due to sugar deprivation in sink tissues (Yu, 1999). Sugars interact with plant hormones to boost growth and development factors (Stokes *et al*.,

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2013). The sugars signals to stimulate plant defense responses against fungal pathogens and oomycetes (Morkunas *et al*., 2011). Pathogens interrupt with host metabolism by uptake of sugars and other metabolites for their own needs (Chen *et al*., 2010).

Mineral nutrients play an important role in the growth, development and defense of plants. Iron (Fe) is the integral part of chlorophyll and photosynthesis that is affected by the pathogenic attack resulting in decrease of photosynthetic frequency and chlorophyll quantity (Kobyashi *et al*., 2019). Nutrients are the first line of defense against the biotic and abiotic stresses (Walters and Bingham, 2007). Zinc (Zn) is the major part of plant immune responses and act as a cofactor of many enzymes (Hojyo and Fukada, 2016). Manganese (Mn) contributes in the manufacture of phenolics and stimulates plant defense responses (Fernando *et al*., 2009).

Due to the extreme significance of antioxidants, phenols, sugars and minerals in the defense mechanism, growth and development of the plants, the overall objective of this study was to assess the effect of gummosis disease on total soluble phenol, total soluble sugar, minerals and antioxidant enzymes in healthy and infected plants tissues.

MATERIALS AND METHODS

Sample preparation: The survey of different citrus orchards was conducted for the collection of leaves & bark samples both from diseased and healthy citrus plants. The sample collection from diseased plants was accomplished on the basis of characteristic symptoms i.e. gum formation on the base of trunk. The leaves and bark were washed with tap water to remove the dust particles followed by surface sterilization and crushed with liquid nitrogen later on. The powder of leaves and bark was collected separately in test tubes and 20ml ethanol was added in 2.5 gram of powder and placed in shaker for 24 hours. The supernatant was collected after 24 hours for further procedure.

Determination of total soluble phenols: According to Folin-Ciocalteu reagent (FCR) method, the sum of total phenolics in extracts was calculated. The standard solution was prepared by mixing 10mg of gallic acid in 10ml of methanol followed by addition of 5ml of 10% FCR, and 4ml of sodium carbonate. The mixture was subjected to water bath at 45°C and absorbance was recorded at 765 nm. The total phenol contents was calculated by using following formula (Singleton *et al*., 1999).

$$C = cV/m$$

C= total phenols mg GAE (Gallic acid equivalents)/g dry extract, concentration of gallic acid mg/ml and m = mass of extract in grams

Determination of total soluble sugar: Total soluble sugar (TSS) was determined by homogenizing 100 mg plant sample in 10 ml of 80% ethanol and centrifuged at 4000 rpm for 20 minutes. The supernatant was collected and the residue re-extracted with 10 ml of 80% ethanol and centrifuged again at 4000 rpm for 20 minutes followed by mixing of supernatant together. Using a standard curve prepared from the graded glucose concentration, the amount of total soluble sugar present in the extract was calculated.

Determination of antioxidant enzyme: The plant samples were chopped, homogenized in liquid nitrogen and stored at -80°C until assay. The enzyme extract for SOD, POX and CAT was prepared by grinding frozen tissue with 20 ml extraction buffer. The extract was centrifuged for 20 min at 15,000×g and the supernatant was used for enzymatic assay. Assay of SOD activity (unit of SOD min1 g1 FW) was based on formation of blue coloured formazone by nitro-blue tetrazolium chloride dye and O_2 which absorbs at 560 nm (Dhindsa*et al*., 1981). POX activity (mmoltetraguaiacol min1 g1 FW) was assayed as an increase in optical density due to the oxidation of guaiacol to tetraguaiacol (Castillo *et al*., 1984). CAT (mM H_2O_2 reduced per min/g FW) assay was based on the absorbance at 240 nm on UV spectrophotometer; a decrease in absorbance was recorded over a time period as described by Aebi (1984).

Mineral analysis: The collected healthy and infected leaves were carefully washed separately with distilled water followed by drying in direct sunlight for 3 days and in oven at 40°C. These samples were powdered in a grinder and 2 g powder was added in 10 ml of nitric corrosive. The solution was heated on hot plate at 95°C for 15 minutes. The solution was cooled and 5 ml of concentrated nitric corrosive was added and warmed for extra 30 mins at 95°C. The example was cooled again and 2 ml of water and 3 ml of 30% hydrogen peroxide was added and then sample was analyzed in atomic absorption spectrophotometer (Jarosova *et al*., 2014).

RESULTS

Effect of citrus gummosis disease on total soluble phenols in citrus plants: In this study, the results of phenol analysis in healthy leaves & bark were significant (*P<0.05*). In the case of diseased leaves & bark results

were not significant (*P<0.05*). The total amount of phenols on healthy leaves & bark was higher than infected leaves & bark. The value of cv in healthy leaves & bark was 3.45 and in diseased leaves & bark was 6.99 (Figure. 1). In healthy leaves the amount of total soluble phenols was more than 5 mg/ml that is reduced to 3 mg/ml in case of diseased leaves. The healthy bark gave almost 3mg/ml of total soluble phenols which decreased to 1mg/ml due to gummosis disease as evident from the respective samples.

Effect of citrus gummosis disease on total soluble sugars in citrus plants: In total soluble sugar analysis, the results of sugar analysis in leaves, bark and fruit of both healthy and diseased samples were significant (*P<0.05*). The total amount of sugar in healthy leaves, bark and fruit was higher than diseased counterparts. The value of cv in healthy leaves, bark and fruit was 47.62 and the value of cv in diseased leaves, bark and fruit was 79.5 (Figure 2). The maximum quantity of total soluble sugars was recorded from fruits followed by leaves and bark both in healthy and diseased samples.

Effect of citrus gummosis disease on antioxidant enzymes in citrus plants: In the present research, the total amount of antioxidant enzymes (SOD, POD, CAT) in diseased leaves was higher than in healthy leaves. The following trend of enzymes in healthy leaves was CAT> SOD> POD. The following trend of enzymes in diseased leaves was CAT> SOD> POD. The CV value of healthy leaves of all enzymes was 37.32. The CV value of diseased leaves of all enzymes was 36.48 (Figure 3).

Effect of citrus gummosis disease on mineral nutrients in citrus plants: The mean values of different minerals in healthy and infected plant samples are presented (Table 1). The elements like Zn, Fe and Mn have been determined by atomic absorption spectrophotometer. The order of concentration of elements in healthy and diseased samples showed the following trend: Fe > Zn >Mn.

In the present research, iron deposition in healthy and diseased plants was found to be higher than in the other minerals. The content of Zn a biologically active metal was found to be higher in healthy leaves when compared with infected leaves of citrus gummosis. The content of Mn is less than Fe and Zinc but the healthy leaves were found to be higher than the infected leaves.

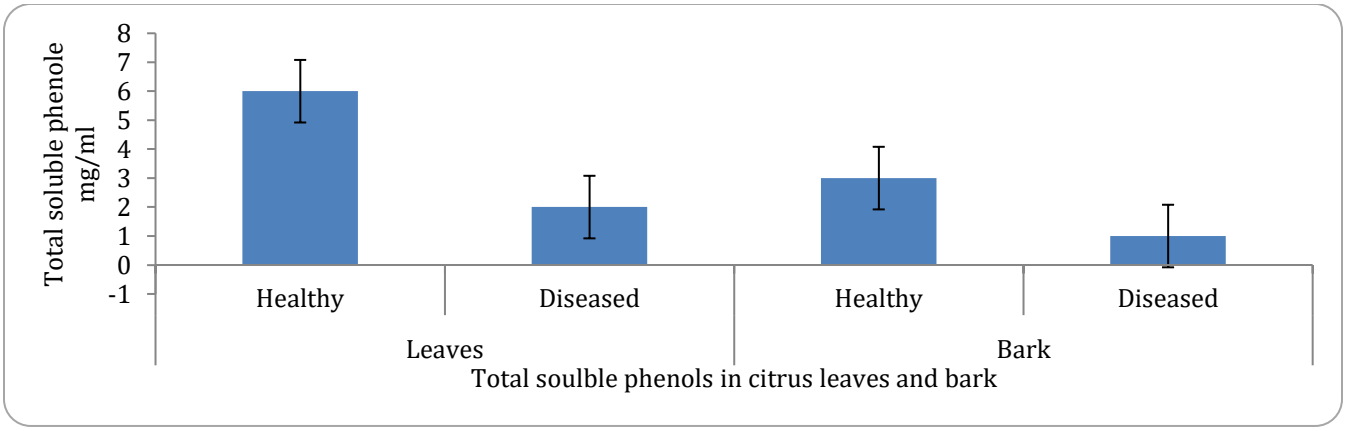

Figure 1.Effect of citrus gummosis disease on total soluble phenols in leaves and bark of citrus

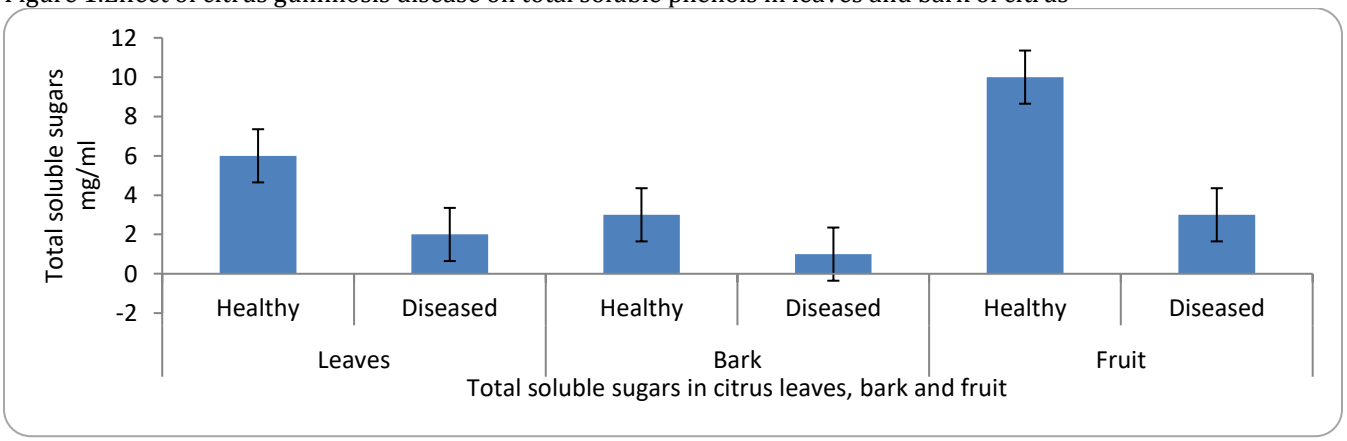

Figure 2.Effect of citrus gummosis disease on total soluble sugars in leaves, bark and fruits of citrus

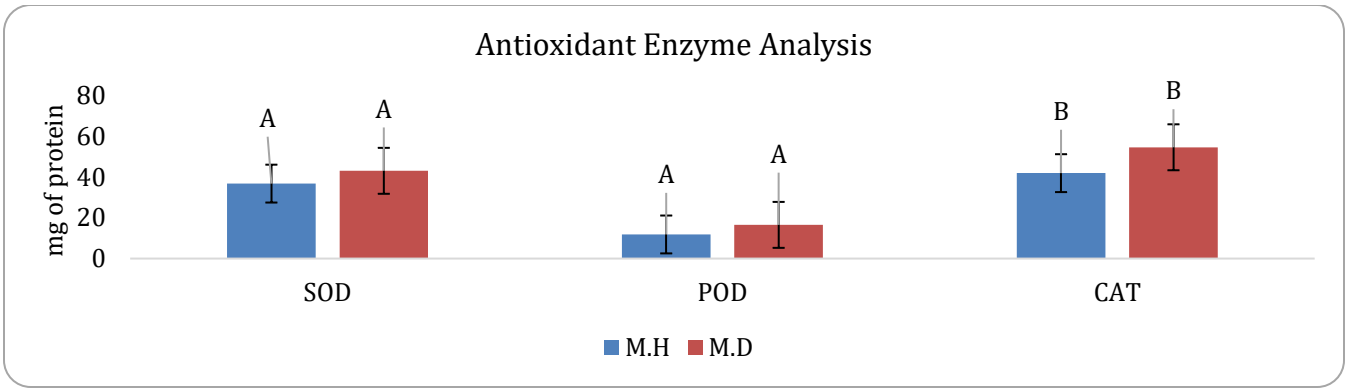

Figure 3.Antioxidant enzymes analysis of Citrus gummosis (mean±SE) in healthy leaves and diseased leaves means with similar letter are significantly different at *P<0.05.*

Table 1. Concentration of mineral elements in healthy and infected leaves

Sr. No.	Minerals	Healthy (mg/kg)	Infected (mg/kg)
1	Fe	116 a	51.4 a
2	Zn	78.4 b	48.2 b
3	Mn	74.16 c	30.6 c
LSD		1.2	1.5

Different letters in a column indicate significantly different values at 5% probability level

DISCUSSIONS

The amount of total soluble phenols is considerably reduced to the incidence of gummosis disease in leaves and bark of citrus plants. Phenolics are the most abundant secondary metabolites in plants that take part in different biosynthesis pathways (Boudet, 2007). Phenols serve as plant protectors by inhibiting the activities of invading pathogens as pesticide agents (Lattanzio *et al*., 2006). The phenols in plants and functions as phytoalexins and phytoanticipins against soil-borne pathogens (Akhtar and Malik, 2000). Plants react to pathogen invasion by building up phytoalexins i.e. hydroxycoumarins and hydroxycinnamate (Karou*et al*., 2005). In diseased plants, the amount of phenolics is significantly reduced that results in lowering the defense response against invading pathogens. In current study, total soluble phenols was reduced in diseased citrus plant samples as compared to healthy ones. As the phenols occupy a key position in plant defense responses, these can also be stimulated by the application of salicylic acid (Tsuda *et al*., 2008). Phenolics stabilizes lipid compounds against peroxidation of many oxidizing enzymes (Cos *et al*., 1998). The host plants activated diversified defense to fight against invading fungi, that are stimulated by pathogen invasion. These reactions consist of stimulation of phenolics, phytoalexins, and flavonoids (Doehlemann *et al*. 2008). Citrus plants have phenolic compounds that tolerates the devastating effect of hydrogen peroxide, hydroxyl free radical and singlet oxygen caused by various stresses. (Lin *et al*., 2014). It is evident that phenols have significant role in plants to cope with stresses because it manages the harmful metabolites produced as a result of pathogenic attack. The plants with low quantity of phenols remained unsuccessful in stimulating defense system against the biotic stresses. These facts are in contrast with Lee *et al*., (2003) who found more phenols in CTV infected plants as compared healthy citrus samples. This may be due to the genetic resistance of the cultivars under attack that produced more phenols to cope with harmful radicals.

In current study, the amount of total soluble sugars decreased as a result of gummosis infection. In the earlier experiments, the effect of citrus greening disease on total soluble sugars in citrus was described and it was found that the quantity of sugars severely reduced in roots, leaves, bark and fruits of the affected plants (Zheng*et al*., 2018). Fan *et al*. (2010) analyzed the citrus juices both from healthy and greening affected oranges and showed lower amount of total soluble sugars in diseased samples due to disruption in carbohydrate metabolism. There was an increase in invertase enzyme in the cell wall of greening infected citrus leaves that lead to lowering the sugar contents (Slisz *et al*., 2012). Poiroux-Gonord *et al*. (2013) described the enhanced sucrose substances in orange juice that was extracted from resistant cultivars.

The stimulation of antioxidant enzymes is an important factor of the defense system against biotic and abiotic stresses against reactive oxygen species. The results of present research implies that the alters the antioxidants activity of many citrus cultivars from Sargodha region are linked with resistance status. In previous experiments, superoxide dismutase was increased in gummosis infected cultivars due to resistance. These results are in agreement with the research of Ashry and Mohamed, (2011) who also determined more peroxidase in diseased citrus as compared to healthy ones. Baker and Orlandi (2005) stated that CTV infection causes huge alterations in antioxidants in citrus plants. The antioxidants are significant biochemical markers to map out the level of disease resistance (Mittler*et al*., 2004). Garcia-Limones *et al*., (2002) described that increased in SOD activity of citrus can be associated with tolerance to CTV diseases.

The appropriation of iron (Fe) may stimulates the defense related genes of plants against microbial invasion (Dellagi *et al.* 2005). The expressions of apoplastic Fe protein is associated with the attack of *Pectobacteriumcarotovorum* subsp. *carotovorum* (Pcc) (Hsiao *et al*., 2017). Zinc (Zn) occupies a vital position in growth, development, and defense of plants. It affects host pathogen interaction and act as a catalyst for many defense related enzymes (Hambidge *et al*., 2000). Many studies conferred that diseased plants were deficient in Zn because strengthens the structural and biochemical defenses of the plant (Machado *et al*., 2018). Zinc activates many metalloenzymes and impact the plant-microbe interactions (Fones and Preston, 2012). Zn may interrupt the production of harmful radicals and thus resumes the plant growth (Cakmak, 2000). Zn also stimulates antioxidant production against the damaging effects of oxygen free radicals and triggers SAR mechanism (Feigl *et al.,* 2015). Helfenstein *et al*. (2015) conducted extensive studies on the effect of Zn on plant defense and described that Zn-deficient plants are more susceptible to diseases. Manganese (Mn) takes part in the production of phenolic compounds and other plant defense mechanisms (Fernando *et al.,* 2009)

CONCLUSIONS

In the present study, it was found that the healthy leaves of plants showed higher content than the infected leaves. It means, the pathogen of citrus gummosis reduces the amount of minerals that reduce the quality of fruits. Therefore, to manage the citrus gummosis disease adopt the alternative approaches like certified varieties, bio control and use of fungicides.

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