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DECIPHERING THE ROLE OF ARBUSCULAR MYCORRHIZAL INOCULATION IN ALLEVIATING FLOODING STRESS IN DIPLOID AND TETRAPLOID VOLKAMER LEMON ROOTSTOCKS

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Abstract

Flooding stress is an important environmental constraint that greatly affects the growth and productivity of plants, particularly in citrus orchards. The rootstock genotypes and the symbiotic associations with beneficial soil bacteria, such as arbuscular mycorrhizal fungus (AMF), play a crucial role in determining how plants react to abiotic stimuli. However, there is limited knowledge regarding the specific responses of diploid and tetraploid citrus rootstocks to flooding stress and AMF inoculation. This study investigates the physical, physiological, and biochemical responses of diploid and tetraploid volkamer lemon citrus rootstocks to flooding stress. It specifically focuses on the impact of the presence or absence of arbuscular mycorrhizal fungus (AMF) on these responses. The findings suggest that the application of arbuscular mycorrhizal fungi (AMF) enhances the resilience of both diploid and tetraploid plants to flooding stress, with tetraploid plants exhibiting superior performance. Differences in physical attributes, gas exchange, and chlorophyll fluorescence parameters, the impact of antioxidant enzymes and reactive oxygen species, amount of soluble proteins, and profiles of phenolic components have been observed between diploid and tetraploid citrus rootstocks. HPLC analysis of phenolic compounds of leaf samples revealed 10 phenolic acids and one flavonoid i.e., quercitrin. However, the concentrations of quercetin and gallic acid significantly differed between AMF-inoculated tetraploid (4x) and AMF-inoculated diploid (2x) volkamer lemon citrus rootstocks under severe flooding stress. Overall, results depicted that tetraploid citrus rootstocks are more resistant than diploid rootstocks under flooding stress, and incubation of AM fungi further increases their efficiency of resistance against flooding conditions.

Key words: Volkamer lemon, Polyploidy, Flooding stress, Gas exchange, Antioxidant enzymes, Flavonoid, Phenolic acids.

Introduction

Waterlogging is an environmental factor that can harm the growth and development of plants. It is a major stressor for plants in agricultural, horticultural, and natural systems (Tuheteru and Wu, 2017; Parent *et al*., 2008). When the soil becomes waterlogged, it lacks oxygen and the plants cannot absorb water and nutrients properly, which affects sugar mobilization and photosynthesis (Grassini *et al*., 2007; Sairam *et al*., 2009a). This leads to an increase in reactive oxygen species (ROS) within the plant's system, which results in oxidative stress (Sairam *et al*. 2009b). This stress can damage the plant's lipids, proteins, and nucleic acids (Hossain *et al*., 2009). However, plants possess antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase, to protect themselves from this damage (Gill and Tuteja, 2010).

Arbuscular mycorrhizal (AM) fungi belong to the Glomeromycota phylum and have the potential to establish symbiotic relationships with up to 90% of terrestrial plants, including cereals, vegetables, and fruit trees (Zou *et al*., 2021). AM fungi form a beneficial association with plants by providing improved uptake of nutrients, water, and protection against stress (Hardoim *et al*., 2015; Cheng *et al*., 2022; Rillig *et al*., 2019; Emmanuel and Babalola, 2020; Dong *et al*., 2022). This mutually beneficial relationship involves the host plant providing photosynthates, which the AM fungi can use to grow. In return, the extraradical AM hyphae extend the root's absorption area and provide water and nutrients to the host (He *et al*., 2017).

In addition to this, the AM hyphae secrete a water-insoluble protein (glomalin) known as glomalin-related soil protein (GRSP) in soil. The GRSP is largely responsible for stabilizing soil aggregates (Wright & Upadhyaya, 1998) and contributes to soil organic carbon pools, benefiting plant growth (He *et al*., 2020; Meng *et al*., 1998). Furthermore, AM symbiosis can generate changes in plant secondary metabolism, bolstering the synthesis of phytochemicals, increasing nutrient uptake, and improving plant stress tolerance (Raklami *et al*., 2019). Therefore, AM fungi offer numerous advantages to host plants and enable the implementation of sustainable agricultural and environmental practices (Ali *et al*., 2019).

Citrus plants rely heavily on AM fungal colonization due to their few root hairs, and due to poor soil texture, environmental factors, and genetic factors, in the field, yet the colonization rate is quite low, below 20% (Wang *et al*., 2019), limiting the high yield and quality of citrus. Studies in the past decades have explored the effects of AM fungal inoculation on nutrient uptake (Syvertsen & Graham,

1999), photosynthesis (Levy & Krikun, 1980), phytohormone balance (Wu *et al*., 2008), rhizosphere dynamics, soil fertility (Wu *et al*., 2013) and fruit quality (Cheng *et al*., 2022) in citrus plants, particularly in response to abiotic stress. It has been observed that about 20% of the host's photosynthates are obtained by mycorrhizal symbionts in citrus plants growing in High-Phosphorus soil, which, in turn, improves their water and nutrient uptake (Peng *et al*., 1993). Moreover, AM fungi have been found to increase the host plant's resistance to various abiotic stresses (Wu *et al*., 2013), such as salinity, drought, high temperatures, and heavy metal toxicity (Miransari 2010; Wu & Zou, 2011). Additionally, some reports have highlighted the ability of AM fungi to enhance the tolerance of citrus plants to waterlogged soil (Miller & Sharitz, 2000; Neto *et al*., 2006; Fougnies *et al*., 2007).

The use of citrus diploid and tetraploid rootstocks has become increasingly popular, since they can provide many economic advantages to growers, including increased fruit quality and yield, earlier fruiting, better disease resistance, uniform cropping, and smaller, more manageable trees. Although any variety can be used as a rootstock, some are more suitable for certain conditions than others (Davies & Albrigo, 1994; Lawrence & Bridges, 1974). The difference between diploids and their corresponding tetraploid rootstocks in anatomy, morphology, and physiological and gene expression changes have been shown to lead to better adaptive strength to various environmental stresses (Tan *et al*., 2015). Studies have discovered that polyploid, particularly tetraploid, plants have better tolerance to stress than their diploid counterparts due to the morphological and physiological variations between the two (Allario *et al*., 2013; Oustric *et al*., 2019).

Citrus plants are often exposed to heavy rain and subsequent periods of soil waterlogging which severely restrict tree growth and morphological, physiological, and biochemical changes occurring in the plant. However, limited efforts have been made in the past to expand our understanding of the citrus plant's responses to waterlogged conditions through mycorrhizal inoculation. The goal of this study was to investigate the impact of mycorrhizal inoculation in a new rootstock variety, Volkamer lemon at diploid and tetraploid ploidy levels to improve tolerance to flooding conditions as a result enhance the growth and yield of citrus plants in Pakistan. The present study compared physiological, biochemical, and phenolic acid changes that occurred after the AM fungi inoculation in the diploid and tetraploid Volkamer lemon rootstocks at control and flooding conditions. It was hypothesized that AM fungi-inoculated tetraploid Volkamer lemon rootstock would show greater resilience to flooding than AM fungi-inoculated diploid rootstock. To verify this, AM fungi-inoculated tetraploid and diploid Volkamer lemon citrus rootstocks, known for their varying tolerance to flooding, were subjected to continuous stress to provide data-supported evidence for the applicability of AM fungi inoculation in citriculture.

Material and Methods

Plant materials: Seeds of diploid and tetraploid volkamer lemon rootstocks were obtained from the INRA-CIRAD Citrus Germplasm Collection of San Giuliano, Corsica, France. These seeds were sown in plastic containers in the citrus nursery area of the Department of Horticulture, Bahauddin Zakariya University Multan, Pakistan. After 6 months, healthy and vigorous seedlings were transplanted in earthen pots (Ø30 cm) and 1-year-old uniform-sized plants were used for the present study.

Experimental conditions and layout: The 16 plants of each diploid and tetraploid volkamer lemon rootstock of uniform size were selected for this experiment. From each 16 diploid and tetraploid rootstocks, eight plants were inoculated with mycorrhizal fungi while the remaining eight plants were non-inoculated. All the plants were treated equally with full-strength Hoagland nutrient solution until the inoculation of mycorrhiza. After the inoculation of mycorrhiza, the plants with mycorrhiza were treated with full-strength Hoagland nutrients solution except phosphorus, while the remaining plants were treated with full-strength Hoagland nutrients solution with all nutrients present in it. The temperature during day and night were around 28°C ± 3 and 14°C ± 3 respectively, while the relative humidity varied from 50 to 80%. After 60 days of inoculation plants were divided into two groups: (1) Mycorrhizal inoculation and flooding stress (T1) in which the plants were inoculated and subjected to flooding stress and (2) Non-mycorrhizal and flooding stress (T2) in which the plants were not inoculated and subjected to flooding stress. The flood condition was obtained by keeping the pots in plastic containers full of tap water. All the plants used for flooding were marked on stems with a permanent marker 3cm above the soil level to maintain the water level up to the mark. When needed, more water was added. An opaque plastic sheet was used to cover the surface of containers to avoid algae proliferation. The flooding condition was maintained until plants showed stress symptoms. The experimental layout was arranged according to a completely randomized design with a factorial arrangement. There were four replications, and two plants were in each replication.

Physiological parameters and phenotypic response: The physiological response of both diploid and tetraploid volkamer lemon rootstocks was evaluated by measuring different physiological parameters throughout the experiment. Four leaves were tagged on each plant to measure different physiological parameters at a four-day interval, in case of leaf fall the adjacent leaf was selected for observation. The different physiological parameters include transpiration rate (*E*), stomatal conductance (gs), and photosynthetic rate (*Pn*) measured using an Infrared Gas Analyser (LCi-SD, ADC Bioscientific Ltd., the United Kingdom), non-photochemical quenching (NPQ), quantum yield at day-time (Fv′/Fm′), quantum yield at night-time (Fv/Fm) were measured with chlorophyll fluorometer (FluorPen FP-100. Czech Republic) and leaf greenness (SPAD) was measured with SPAD meter.

Collection of plant materials for biochemical assay and phenolic acids measurement: Leaves and roots of each diploid and tetraploid inoculated plants were collected after

28 days. Similarly for Non-inoculated plants leaves and roots were collected after 16 days as plants showed stress symptoms. For antioxidant enzymes measurement leaves and roots from each experimental unit were crushed in liquid nitrogen to stop enzymatic activity and stored immediately at -80°C for further analysis. For phenolic acids measurements, plant roots were shade-dried and ground into a fine powder using a West Point coffee grinder (WF 9222). The fine powder was sieved with a 0.25 mm sieve and stored in opaque, screw-capped containers at room temperature for later use.

Enzymatic assay: To measure superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), 300 mg of each sample was taken and homogenized in 3 mL of sodium phosphate buffer (pH 7.8) with a chilled mortar and pestle. The mixture was centrifuged at 15000 rpm at 4°C for 5 min, and the supernatant was used for further analyses.

Superoxide dismutase (SOD, EC: 1.15.1.1) activity was determined using the methodology outlined in Giannopolitis and Ries (1977). The reaction mixture contained 0.5 mL ethylenediaminetetraacetic acid (75 mM), 1 mL nitroblue tetrazolium (50 mM), 0.95 mL sodium phosphate buffer (50 mM, pH 7.8), 1 mL riboflavin (1.3 µM), 0.5 mL methionine (13 mM), and 0.05 mL enzyme extract. Absorbance was noted at a wavelength of 560 nm using a spectrophotometer. Catalase (CAT, EC: 1.11.1.6) activity was estimated as in Chance & Maehly (1955), and a mixture containing 0.9 mL hydrogen peroxide (5.9 mM), 2 mL sodium phosphate buffer (50 mM, pH 7.8), and 0.1 mL extracted enzyme was used. Peroxidase (POD, EC: 1.11.1.7) activity was estimated as outlined in Chance & Maehly (1955), and the reaction solution contained 0.4 mL guaiacol (20 mM), 2 mL of 7.8 pH sodium phosphate buffer (50 mM), 0.5 mL hydrogen peroxide (40 mM), and 0.1 mL enzyme extract. The absorbances measured for catalase and peroxidase were at wavelengths of 240 nm and 470 nm, respectively.

Estimation of hydrogen peroxide and total soluble proteins: Stored samples of 300 mg from each experimental unit were homogenized in 3 mL of trichloroacetic acid (0.1%) before being centrifuged at 12,000 rpm for 15 min. The hydrogen peroxide (H_2O_2) was estimated by the method of Velikova *et al*., (2000). The solution had 1 M potassium iodide, 10 mM potassium phosphate buffer, and an extracted sample. The absorbance was noted at 390 nm.

Total soluble proteins (TSP) were extracted (phosphate buffer saline (PBS) with pH 7.2), according to the methodology in Sambrook and Russell (2001). Each 0.5 g leaf and root samples were taken and homogenized in 1 mL phosphate saline buffer (pH 7.2). The phosphate saline buffer contained 2.7 mM potassium chloride, 2 mM potassium dihydrogen phosphate, 10 mM disodium hydrogen phosphate, and 1.37 mM sodium chloride. The homogenized samples were then centrifuged at 10,000 rpm for 5 min. Total soluble proteins were determined using the method detailed in Bradford (1976). Briefly, 0.2 mL extracted plant material was added to a reaction mixture containing 0.78 mL deionized water and 0.02 mL Coomassie blue dye. The absorbance of the reaction solution was read at a wavelength of 595 nm. To calculate total soluble proteins, a standard graph was generated, with changing concentrations of Bradford protein and absorbances.

Preparation of plant extracts for phenolic acids measurements: Samples prepared for phenolic acids were macerated in methanol solvent for 15 days. The resulting soluble fractions in methanol were filtered and the filtrate was then concentrated at 400°C using a rotary evaporator to give a crude extract. This crude extract was then dissolved in 150 mL of methanol. The concentrated methanolic extract was transferred to sample vials and stored at -4°C for further analysis.

Analysis of phenolic compounds: The hydrolysis of extracts was conducted as previously described (Pak-Dek *et al*., 2011). Briefly, 50 mg of extract was dissolved in 24 mL of methanol and homogenized. It was then mixed with 16 mL of distilled water and 10 mL of 6M HCl and thermostat for two hours at 95°C. The solution was filtered using a 0.45 µm nylon membrane filter (Biotech, Germany) before it was analyzed using high-performance liquid chromatography (HPLC). The chromatographic separation was carried out on a Shimadzu LC-10A (Japan) with a Shim-Pack CLC-ODS (C118) 25cm X 4.6 mm, 5 µm column. The mobile phase gradient used was A (H_2O: Acetic acid-94:6, pH = 2.27), B (acetonitrile 100%), with 15% solvent B (0-15 min), 45% solvent B (15-30 min) and 100% solvent B (35-45 min) with a flow rate of 1 mL/min. The UV-visible detector (λ max 280 nm) was used to separate the phenolic compounds. The peaks were identified by comparing the retention time and UV-visible spectra to those of standards. Quantification of the compounds was done through external calibration.

Identification of AMF by staining technique: First, the fine roots from the plant's rhizosphere were collected. The root samples were washed under running water. Root samples were taken for the staining process, and the sample was stored in 50:50 of ethanol and water at 4°C. The 10% KOH was prepared by dissolving 4.7g of KOH in 36ml distilled water and samples were kept in this solution. After one hour the samples were poured on the sieve and washed with distilled water. The samples were kept in 1% HCl solution for 1 hour at room temperature to get the root neutralized. After that, the samples were kept in staining solution for 1 hour at 90ºC for staining. The staining solution was prepared by dissolving lactic acid, glycerol, and water by 1:1:1 respectively with 0.05% trypan blue and methylene blue at 0.05%. The samples were sieved and washed with water. After that the samples were stored in water at 4ºc room temperature for 24 hours to get de-stain and then slides of fine roots were made for observation.

Statistical analysis

The data was statistically analyzed using the One-Way Analysis of Variance (ANOVA) technique. The Statistix 8.1 software was used for statistical analysis. The means were separated by the LSD (Least Significance Difference) test.

Fig. 1. The phenotypes of AMF inoculated and non-inoculated diploid and tetraploid volkamer lemon rootstocks under flooding stress. (A) AMF inoculated diploid; (B) AMF inoculated tetraploid; (C) Non-inoculated diploid and (D) Non-inoculated tetraploid under flooding stress.

Results

Phenotypical symptoms: The results of this study showed that AM fungi inoculated diploid and tetraploid volkamer lemon citrus rootstocks responded differently). The volkamer lemon plants inoculated with AMF performed better as compared to non-inoculated plants under flood conditions. As far as diploid and tetraploid rootstocks are concerned, the tetraploid plants performed better than diploid plants. The non-inoculated diploid and tetraploid plants under flood conditions (T2) showed the rolling and wilting of leaves after eight days of stress. Between diploid and tetraploid plants, the diploid plants showed early symptoms compared to tetraploids. Moreover, inoculated diploid and tetraploid plants under flood conditions (T1) started exhibiting these symptoms after the 18th day (Fig. 1).

Leaf gas exchange and chlorophyll fluorescence: Plants that were inoculated with AMF and subjected to flooding stress were able to survive for 28 days. However, non-inoculated plants under flooding stress could only survive for 16 days. The flooding stress and AMF inoculation significantly affected various physiological parameters such as transpiration rate (*E*), stomatal conductance (*gs*), and photosynthesis rate (*Pn*). Notably, these effects varied between diploid and tetraploid volkamer lemon rootstocks (Fig. 2A, B, and C).

Under flooding stress, non-inoculated diploid and tetraploid plants demonstrated the lowest levels of transpiration rate, photosynthesis rate, and stomatal conductance on the 16th day. Diploid plants were particularly more vulnerable compared to their tetraploid counterparts. Conversely, AMF inoculation markedly improved gas exchange parameters in both diploid and tetraploid plants under flood conditions, with tetraploid plants exhibiting a more pronounced improvement. In addition, the changes in leaf gas exchange parameters were more rapid in diploid plants compared to tetraploid plants under flooding stress in both inoculated and non-inoculated conditions (Fig. 2A, B, and C).

A significant increase in NPQ was observed in both diploid and tetraploid plants when exposed to flooding stress, with diploid plants displaying higher NPQ values than tetraploid plants, regardless of inoculation (Fig. 2D). During flooding stress, both diploid and tetraploid plants experienced a significant decrease in the maximum quantum yield of PSII, in both light-adopted (Fv′/Fm′) and dark-adopted (Fv/Fm) leaves, regardless of inoculation. Figure 2 E and F showed varying trends in the maximum quantum yield of PSII between diploid and tetraploid plants in response to flooding stress. Non-inoculated diploid plants experienced a significant decrease in the maximum quantum yield of PSII compared to tetraploid plants under flooding stress. However, AMF inoculation improved the values of quantum yield in both diploid and tetraploid plants under flooding stress.

Under flooding stress, the maximum decrease in leaf greenness (SPAD) content was observed in non-inoculated diploid plants (Fig. 2G). However, AMF inoculation alleviated the leaf greenness under the flooding stress in both diploid and tetraploid plants.

Antioxidant enzymatic activity: The diploid and tetraploid plants, both inoculated and non-inoculated, showed increased SOD activity in response to flooding stress in their leaves and roots (Fig. 3A). In non-inoculated diploid plants, there was a rapid increase in SOD activity in both leaves and roots compared to inoculated diploid plants under flooding stress. SOD levels were significantly higher in non-inoculated plants compared to inoculated plants under flooding stress. Tetraploid plants also exhibited increased SOD activity in response to flooding stress, although the increase was less compared to diploid plants. The AMF inoculation led to a decrease in SOD activity under flooding stress in tetraploid plants in both roots and leaves.

Furthermore, both inoculated and non-inoculated diploid and tetraploid plants displayed differential POD activity in their leaves and roots in response to flooding stress (Fig. 3B). Higher POD activity was observed in the roots of both diploid and tetraploid plants compared to the leaves. The maximum POD activity was observed in non-inoculated diploid plants under flooding stress. The AMF inoculation lowered the POD activity in both the roots and leaves of both diploid and tetraploid plants.

Additionally, the catalase activity was higher in leaves compared to roots in both diploid and tetraploid plants (Fig. 3C). Non-inoculated diploid and tetraploid plants exhibited high CAT activity compared to inoculated plants in both roots and leaves under flooding stress. In comparison to diploid plants, the CAT activity was lower in tetraploid plants in both inoculated and non-inoculated plants under flooding stress.

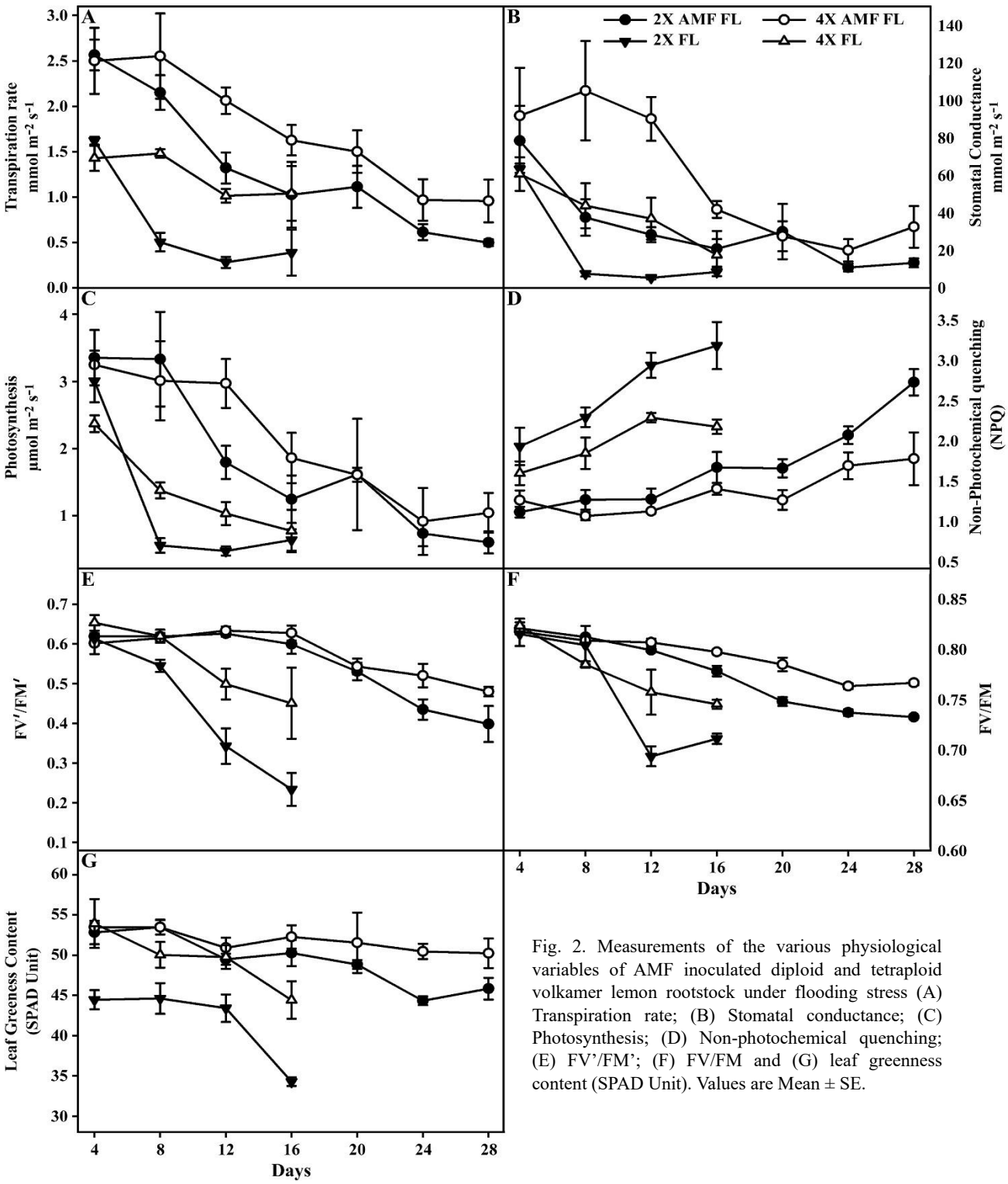

Fig. 2. Measurements of the various physiological variables of AMF inoculated diploid and tetraploid volkamer lemon rootstock under flooding stress (A) Transpiration rate; (B) Stomatal conductance; (C) Photosynthesis; (D) Non-photochemical quenching; (E) FV'/FM'; (F) FV/FM and (G) leaf greenness content (SPAD Unit). Values are Mean ± SE.

Oxidative status: The study aimed to investigate the oxidative damages caused by H_2O_2 content in leaves and roots of diploid and tetraploid plants under flooding stress (Fig. 4A and B). Both inoculated and non-inoculated plants were compared. The results showed that H_2O_2 content increased in both diploid and tetraploid plants during flooding stress. Among the conditions, the non-inoculated diploid leaves had the highest H_2O_2 content under flooding stress.

Total soluble protein: The study showed that both diploid and tetraploid plants, whether inoculated or not, had higher levels of soluble protein in their leaves and roots as a response to flooding stress (Figs. 5A and B). Non-inoculated diploid plants showed a faster increase in soluble protein in their leaves and roots compared to inoculated diploid plant s under the same flooding stress. Furthermore, the protein levels were significantly higher in non-inoculated plants than in the inoculated ones. While tetraploid plants also showed an increase in protein content under flooding stress, the increase was less compared to the diploid plants. Additionally, the inoculation of arbuscular mycorrhizal fungi decreased the soluble protein level in tetraploid plants in both roots and leaves.

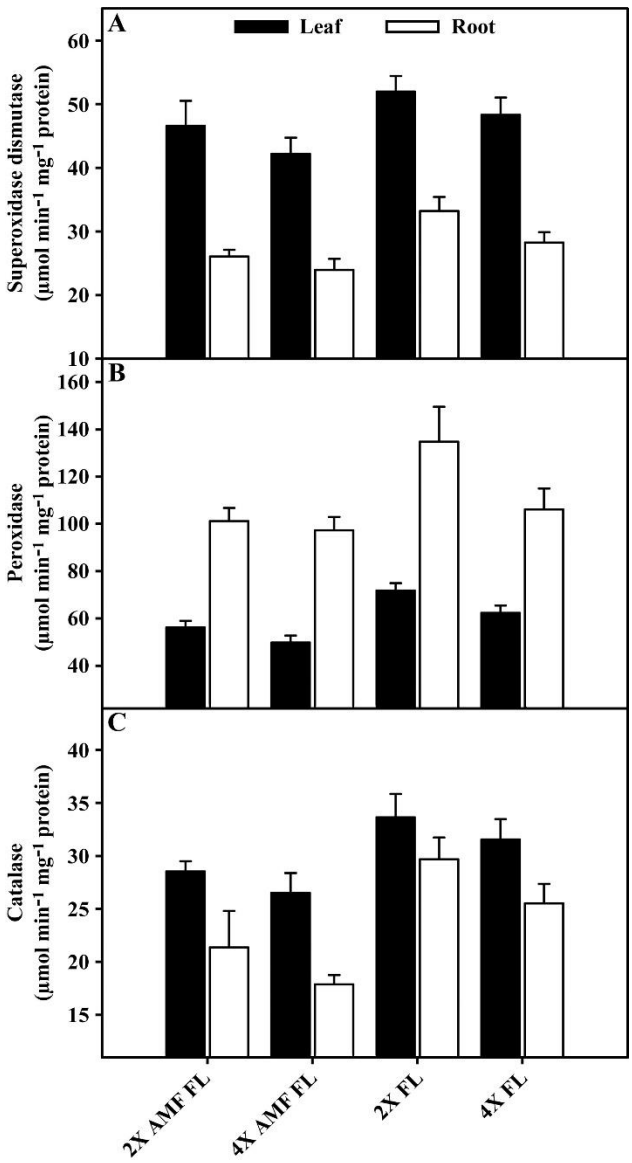

Fig. 3. Measurements of the various antioxidative enzymes activity of AMF inoculated diploid and tetraploid volkamer lemon rootstock under flooding stress (A) Superoxidase dismutase; (B) Peroxidase; (C) Catalase. Values are Mean ± SE.

Phenolic compounds: HPLC-DAD analysis was utilized to quantitatively determine the phenolic compounds present in both AMF-inoculated and non-inoculated diploid (2x) and tetraploid (4x) volkamer lemon citrus plant roots under flooding stress. Eleven constituents were identified in the roots, as shown in (Table 1) including ten phenolic acids (Gallic acid, Chlorogenic acid, p-coumaric acid, Benzoic acid, Syringic acid, Ferulic acid, Vanillic acid, M-coumeric acid, Cinamic acid, and sinapinic acid) and one flavonoid (quercitrin). The chromatogram of the measured samples is shown in Fig. 6.

The concentration of flavonoid (quercitrin) was found to be significantly different ($p \leq 0.05$) between inoculated diploid and tetraploid (4x) and non-inoculated diploid (2x) and tetraploid plants under severe flooding stress in roots. The quercetin under flooding stress, in inoculated diploid and tetraploid roots was 3.41 µg/mg and 4.56 µg/mg, respectively. Notably, non-inoculated diploid and tetraploid plants showed significant differences in quercitrin concentration under flooding stress in roots. The non-inoculated tetraploid plants showed 5.89 µg/mg quercitrin in roots and 0.64 µg/mg in diploid plants under flooding stress.

The levels of various phenolic acids in the roots of different plants were measured under flooding stress conditions. The results showed a significant difference ($p \leq 0.05$) in the levels of these acids between inoculated and non-inoculated diploid and tetraploid plants. The gallic acid was not detected in inoculated diploid plants under flooding stress, but inoculated tetraploid plants had 0.61 µg/mg of this acid in their roots. Under flooding stress, non-inoculated diploid plants had 1.51 µg/mg of gallic acid, while non-inoculated tetraploid plants had 0.76 µg/mg. Chlorogenic acid was present in tetraploid and under flooding stress, inoculated tetraploid plants had 5.38 µg/mg of chlorogenic acid in their roots, while inoculated diploid plants had none. P-coumaric acid was found under waterlogging conditions, inoculated tetraploid plants had 0.65 µg/mg of this acid in their roots, while inoculated diploid plants had only 0.18 µg/mg. Similarly, under waterlogging conditions, non-inoculated tetraploid plants had 1.49 µg/mg of p-coumaric acid in their roots, while non-inoculated diploid plants had 1.12 µg/mg. The concentration of benzoic acid in inoculated tetraploid plants was 3.16 µg/mg in their roots, while inoculated diploid plants had none under flooding stress. Similarly, under flooding stress, non-inoculated diploid plants had 7.79 µg/mg of benzoic acid in their roots, while inoculated tetraploid plants had none.

The concentration of ferulic acid in inoculated diploid plants was recorded at 2.85 µg/mg under flooding stress, and it was not present in inoculated tetraploid plants. Similarly, ferulic acid was not found in both diploid and tetraploid in non-inoculated plants under flooding stress. In inoculated diploid plants, 0.45 µg/mg of vanillic acid concentration was recorded, and it was absent in inoculated tetraploid plants under flooding stress. However, when non-inoculated plants were subjected to flooding stress, a significant difference ($p \leq 0.05$) in vanillic acid concentration was observed. Under flooding stress, non-inoculated diploid plants showed 5.14 µg/mg, and non-inoculated tetraploid plants showed 11.55 µg/mg of vanillic acid in their roots. M-Coumeric acid was recorded at 0.39 µg/mg in inoculated tetraploid plants and was absent in inoculated diploid plants under flooding stress. However, in non-inoculated tetraploid plants, 2.54 µg/mg was observed, and it was not found in non-inoculated diploid plants under flooding stress.

Under flooding stress, the concentration of cinamic acid was not found in inoculated tetraploid plants, while in inoculated diploid plants, the concentration of cinamic acid was recorded at 26.56 µg/mg. However, in non-inoculated diploid plants, 6.83 µg/mg of cinamic acid was recorded, and it was not found in non-inoculated tetraploid plants under flooding stress. The concentration of sinapinic acid in inoculated diploid plants was observed at 0.62 µg/mg and was not found in inoculated tetraploid plants under flooding stress. However, when non-inoculated plants were subjected to flooding stress, a significant difference ($p \leq 0.05$) in sinapinic acid concentration was observed. In non-inoculated tetraploid plants, 2.92 µg/mg of sinapinic acid was recorded, and in non-inoculated diploid plants, 0.98 µg/mg was recorded under flooding stress. The syringic acid rosmarinic acid and caffeic acid were absent in both diploid and tetraploid plants, in inoculated and non-inoculated plants under flooding stress.

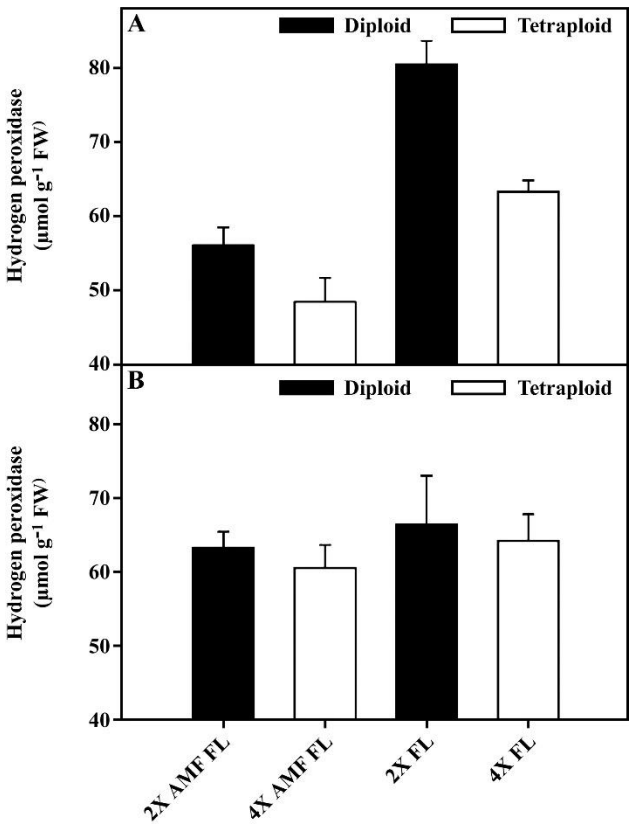

Fig. 4. Measurements of oxidative status of AMF inoculated diploid and tetraploid volkamer lemon rootstock under flooding stress (A) H_2O_2 in leaves and (B) H_2O_2 in roots. Values are Mean ± SE.

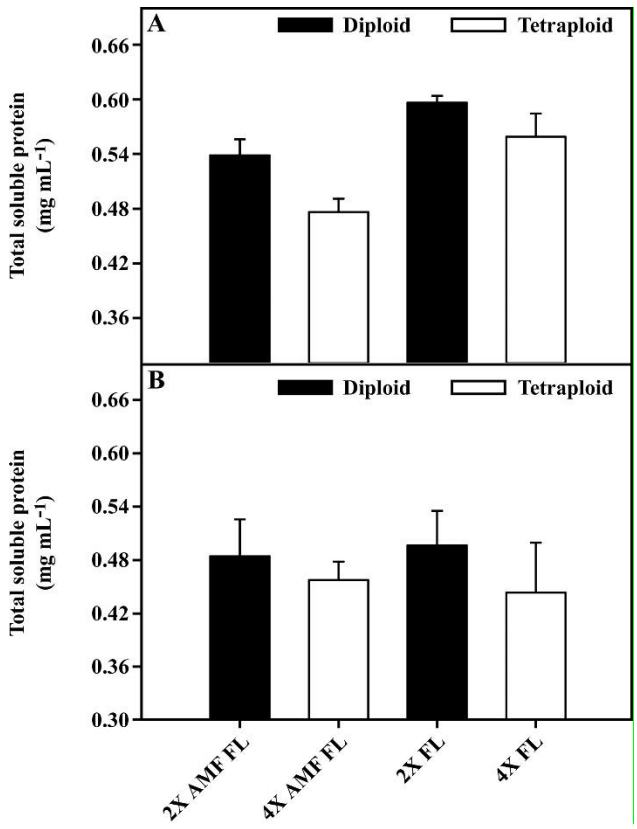

Fig. 5. Measurements of total soluble protein of AMF inoculated diploid and tetraploid volkamer lemon rootstock under flooding stress (A) Total soluble protein in leaves and (B) Total soluble protein in roots. Values are Mean ± SE.

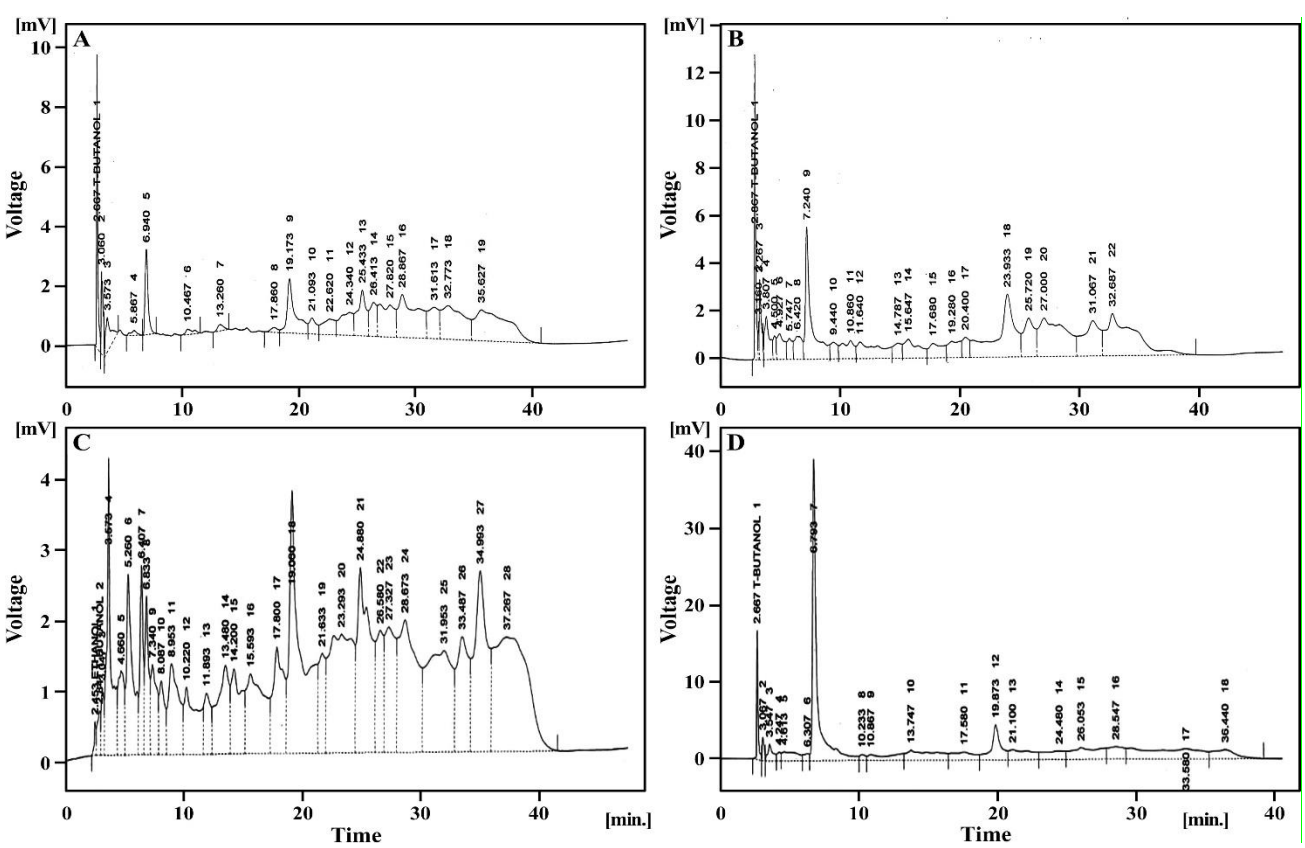

Fig. 6. Chromatogram of the measured samples (A) AMF inoculated diploid (B) AMF inoculated tetraploid (C) Non-inoculated diploid (D) Non-inoculated tetraploid.

Table 1. The phenolic constituents in inoculated and non-inoculated diploid and tetraploid volkamer lemon rootstock root samples were obtained by HPLC-DAD analysis. All chemical compounds were measured in (µg/mg). (-) = Not detected. Data are shown as the mean of triplicate determinations.

Name of compounds	Diploid (2x)		Tetraploid (4x)	
	AMF inoculated	Non-inoculated	AMF inoculated	Non-inoculated
Flavonoids				
Quercetin	**3.41**	**0.64**	**4.56**	**5.89**
Phenolic compounds				
Gallic acid	-	1.51	0.61	0.76
Chlorogenic acid	-	9.11	5.38	-
p-Coumaric acid	0.18	1.12	0.65	1.49
Benzoic acid	-	7.79	3.16	-
Ferulic acid	2.85	-	-	-
Vanillic acid	0.45	5.14	-	11.55
M-Coumeric acid	-	-	0.39	2.54
Cinamic acid	26.56	6.83	-	-
Sinapinic acid	0.62	0.98	-	2.92
Rosmarinic acid	-	-	-	-
Caffic acid	-	-	-	-
Syringic acid	-	-	-	-

Discussion

Plants undergo various abiotic stressors, which trigger specific morphological, physiological, and molecular reactions to adapt and effectively cope with new challenges that are different from each stressor (Mittler & Blumwald, 2010; Suzuki *et al*., 2014; Liu *et al*., 2015; Zhang *et al*., 2015). Environmental problems, both in general for plants and specifically for citrus, result in oxidative stress (Halliwell & Gutteridge, 1989; Arbona *et al*., 2008). Flooding has a harmful impact by damaging the photosynthetic machinery, which can lead to excessive generation of reactive oxygen species (ROS). Furthermore, the alleviation of stress can potentially intensify oxidative stress as a result of the return to normal levels of oxygen following a period of oxygen deprivation (Yordanova *et al*., 2004). Citrus plants frequently experience intense rainfall and subsequent episodes of soil waterlogging, resulting in significant limitations to tree growth and causing morphological, physiological, and biochemical alterations in the plant. Previous research has made only minimal attempts to enhance our comprehension of how the citrus plant reacts to flooding stress by using mycorrhizal inoculation. Various research studies have emphasized the capacity of arbuscular mycorrhizal fungi to improve plant tolerance in waterlogged soil conditions (Miller & Sharitz, 2000; Neto *et al*., 2006; Fougnies *et al*., 2007). However, there is currently no available data on the impact of AM fungi inoculation on the growth of tetraploid and diploid Volkamer lemon citrus seedlings in a continuous waterlogged environment. The current study evaluated the effects of arbuscular mycorrhizal fungi (AMF) inoculation on the photosynthetic activity, oxidative damage, and antioxidant enzyme capacity of diploid and tetraploid volkamer lemon plants under flooding stress. The primary findings of this study confirm the hypothesis that tetraploid Volkamer lemon rootstock infected with arbuscular mycorrhizal (AM) fungus would exhibit higher resistance to flooding compared to diploid rootstock inoculated with AM fungi. Additionally, the results demonstrate that AM fungal inoculation enhances flood tolerance in Volkamer lemon rootstocks.

The study's findings revealed that diploid and tetraploid volkamer lemon rootstock citrus plants, both inoculated and non-inoculated with AMF, had distinct responses to flooding stress. At first, the plants in both genotypes exhibited no indications of distress until the 8th day. Subsequently, the diploid plants that were not inoculated with AM fungi began to experience leaf rolling and withering, whereas the tetraploid plants that were not inoculated with AM fungi only displayed these symptoms after the 18th day. In addition, diploid plants inoculated with AM fungi exhibited a reduction in green pigments, whereas tetraploid plants inoculated with AM fungi displayed milder symptoms, such as leaf chlorosis and rolling, but retained their green pigments. Citrus tetraploid plants exhibit bigger and greener leaves in comparison to their diploid counterparts. They also have shorter and thicker roots, but their overall growth is diminished. Moreover, these trees yield greater fruit. This phenomenon has been documented in research conducted by Ruiz *et al*., (2016). Citrus plants may display a range of symptoms, including leaf wilting, yellowing, and progressive senescence, when they are exposed to waterlogging (Graser & Allen, 1988, Yelenosky *et al*., 1995; Arbona *et al*., 2008). Plants that were inoculated with AMF, especially those that were tetraploid, showed superior performance when subjected to flood conditions in comparison to plants that were not inoculated. Tetraploid plants exhibited a delayed onset of stress symptoms in comparison to diploid plants, suggesting a possibility for higher resilience in tetraploid plants.

The flooding stress led to a significant reduction in various physiological functions, including transpiration rate, stomatal conductance, photosynthetic rate, leaf greenness (SPAD), non-photochemical quenching, quantum yield during the day, and quantum yield during the night (Fig. 2) in both AMF inoculated and non-inoculated diploid and tetraploid citrus plants. AMF-inoculated diploid plants exhibited greater susceptibility compared to tetraploid plants, with a statistically significant difference (p<0.005) detected. Research has

demonstrated that the use of tetraploid rootstocks offers greater stress tolerance or resistance when compared to diploid rootstocks (Saleh *et al*., 2008; Allario *et al*., 2013; Tan *et al*., 2015; Oustric *et al*., 2017).

One of the first things noticed in the diploid volkamer lemon following waterlogging was a significant and quick reduction in the rate at which water is lost by transpiration, which was caused by the closing of stomata. Some species have shown changes in stomatal conductance following flooding that is associated with ABA (Ahmed *et al*., 2006; Jackson *et al*., 1987; Neuman *et al*,.1991; Zhang *et al*,.1994). The sensitivity of the photosynthetic system of non-inoculated diploid plants to various conditions, such as flooding, might lead to oxidative damage. The damage seen may be associated with a reduction in the effectiveness and functioning of the photosynthetic system (Lopez-Climent *et al*., 2008). Stress can affect the functioning of light-harvesting complexes and the transfer of electrons between photosystems in citrus diploid genotypes such as volkamer lemon. This disturbance can cause a rise in the use of non-photochemically active electrons, ultimately leading to the creation of various reactive oxygen species (ROS). AMF inoculation enhanced gas exchange characteristics in both diploid and tetraploid plants under flooding, with tetraploid plants exhibiting a more significant increase. Under flooding stress, diploid plants exhibited faster alterations in leaf gas exchange characteristics compared to tetraploid plants, regardless of their inoculation status.

Superoxide dismutase (SOD) serves as the primary barrier against reactive oxygen species (ROS). It has a vital function in protecting cells from oxidative stress by directly regulating the levels of the superoxide anion ($O2•–$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO•). During water-logging stress, an excessive amount of reactive oxygen species can be produced. These reactive oxygen species can be converted into hydrogen peroxide (H_2O_2) by the activity of superoxide dismutase (SOD). The H_2O_2 is subsequently metabolized by the components of the ascorbate-glutathione cycle. Both diploid and tetraploid plants displayed elevated superoxide dismutase (SOD) activity in response to flooding stress, with diploid plants demonstrating a more rapid escalation. Under flooding stress, tetraploid plants exhibited reduced superoxide dismutase (SOD) activity in comparison to diploid plants. The application of AMF inoculation resulted in a reduction in Superoxide Dismutase (SOD) activity when tetraploid plants were subjected to flooding stress. The prompt activation of superoxide dismutase (SOD) and catalase (CAT) enzymes reported in plants subjected to continual flooding appears to be a proactive and effective response of plants to mitigate the effects of waterlogging stress. The elevated SOD and CAT activity seen in diploid plants, as opposed to tetraploid plants, indicates that these two enzymes play a crucial role in reducing oxidative stress damage caused by waterlogging. Both diploid and tetraploid plants exhibited elevated levels of hydrogen peroxide (H_2O_2) in response to flooding stress. Among the diploid plants, those that were not inoculated had the greatest H_2O_2 content. Both diploid and tetraploid plants exhibited elevated amounts of soluble protein in response to flooding stress, with non-inoculated diploid plants demonstrating a more rapid increase. Tetraploid plants demonstrated a comparatively smaller augmentation in protein content in response to flooding stress when compared to diploid plants.

The phenolic compounds in the roots of volkamer lemon citrus plants, infected with both AM fungus and subjected to severe flooding stress, were quantitatively and qualitatively analyzed by HPLC-DAD analysis. The amounts of phenolic compounds in roots were affected by AMF inoculation under flooding stress. There were substantial changes identified between plants that were infected and those that were not, as well as between diploid and tetraploid plants. Under flooding stress, tetraploid plants consistently displayed reduced levels of phenolic acids relative to diploid plants, regardless of whether they were inoculated or not. Citrus plants consist of limonoids, which are the characteristic triterpenoids found in citrus, as well as phenolic components such as phenolic acids, flavonoids, tocopherols, and carotenoids (Moulehi *et al*., 2012). The primary phenolic acids found in citrus extracts are caffeic, p-coumaric, and ferulic acids (Bocco *et al*., 1998). Phenolic chemicals are phenylpropanoids that are produced in response to stress (Zandalinas *et al*., 2017). Phenolic chemicals, including flavonoids, function as antioxidants by safeguarding plant cells against oxidative harm induced by waterlogging. Arbuscular mycorrhizal (AM) fungi can enhance the ability of plants to tolerate wet environments by improving root aeration and nutrient absorption. Decreased waterlogging stress can lead to a decrease in the formation of stress-induced phenolic compounds, as the plant undergoes less oxidative damage. The findings of our study indicate that the influence of arbuscular mycorrhizal fungus varies across different rootstocks. Phenols in plants are commonly regarded as stress metabolites (Strack *et al*., 2003; Sheppard & Peterson 1976), and their accumulation in plants is influenced by various conditions. Wahid & Ghazanfar (2006) and Wahid & Close (2007) corroborated these findings in several plant species, demonstrating an augmented production of phenolic chemicals, flavonoids, and phenylpropanoids. The elevation in phenylalanine ammonia-lyase (PAL) activity caused by stress might be seen as the initial stage of cellular adaptation to water stress. In summary, the findings indicate that the introduction of AMF can improve the ability of both diploid and tetraploid volkamer lemon rootstock plants to withstand flooding stress. Tetraploid plants tend to exhibit superior performance and potentially experience less oxidative stress than diploid plants. Moreover, the contrasting reaction of diploid and tetraploid plants emphasizes the significance of genetic elements in influencing plant responses to environmental stressors.

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