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Detection of Above-Ground Physiological Indices of an Apple Rootstock Superior Line 12-2 with Improved Apple Replant Disease (ARD) Resistance

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Abstract: (1) Background: The cultivation of resistant rootstocks is an effective way to prevent ARD. (2) Methods: 12-2 (self-named), T337, and M26 were planted in replanted and sterilized soil. The aboveground physiological indices were determined. (3) Results: The plant heights and the stem thicknesses of T337 and M26 were significantly affected by ARD. Relative chlorophyll content (June–October), P_n (August–September), and G_s (August) of T337 and relative chlorophyll content (June–July, September), P_n (September–October), and C_i (September) of M26 were significantly affected by ARD. ARD had a significant effect on F_v/F_m (June), qP (June–July), and NPQ of T337 (June–October, except August) and F_v/F_m (June) and NPQ (June-October, except July) of M26. Additionally, ARD affected Rfd of M26 and T337 during August. SOD (August and October), POD (August–September), and CAT (July-August, October) activities and MDA (September–October) content of T338 as well as SOD (July–October), POD (June–October), and CAT (July-October) activities and MDA (July, September–October) content of M26 were significantly affected by ARD. ARD significantly reduced nitrogen (October), phosphorus (September–October), and zinc (July) contents of M26 and potassium (June) content of T337. The above physiological indices were not affected by ARD in 12-2. (4) Conclusions: 12-2 could be useful as an important rootstock to relieve ARD due to strong resistance.

Keywords: cultivation of resistant rootstocks; ARD; above-ground physiological indices

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1. Introduction

Apple replant disease (ARD), also known as soil sickness [1,2], refers to the phenomenon that, when the same or closely related crops are continuously planted on the same piece of land, yield decreases, quality deteriorates, and growth status is lessened under normal management [3]. Apple is a worldwide fruit. Many countries list apples as a major consumer product because apples have strong ecological adaptability, high nutritional value, good storability, and a long supply cycle [4]. Limited by land resources, the problem of replanting old orchards is becoming more and more common, and major apple-producing areas in the world are facing ARD [5]. About 50% of the apple orchards in the UK, New Zealand, and Poland have ARD [6]. ARD destroys the diversity of microorganisms in the agroecosystem [7], leading to poor growth of new roots [2,8], slow growth, short plants, reduced resistance, disease, and death of the entire plant, causing a severe economic crisis [9,10]. How to effectively improve the effect of ARD on apple is a problem that needs to be solved.

Many methods are available to improve ARD, such as good crop rotation practices, intercropping, soil disinfection, and the use of beneficial bacterial fertilizer [11], applications of organic amendments [2], and establishing living mulches [8]. Studies showed the decrease of beneficial microorganisms in re-planted soil, the high accumulation of harmful

microorganisms, and the imbalance of the microbial community structure were important factors causing ARD [12]. High-temperature sterilization of replanted soil is an economical, effective, and widely used agricultural practice to control ARD [13]. Although there are many ways to improve ARD, none effectively improve ARD for a long time, and some methods are too costly and cannot be effectively promoted for production.

In the mid-1990s, researchers began to pay attention to the use of resistant rootstocks to prevent ARD [14]. Resistant rootstocks effectively control pests and diseases in replanted soil, alleviate the problem of ARD caused by some pathogenic bacteria [15], strengthen plant resistance, and increase fruit yield and quality [16]. Apple production requires excellent rootstocks resistant to autotoxicity as well as disease and insect pests. Promoting resistant rootstocks is conducive to increasing fruit yield and increasing farmers' income. However, many things can cause ARD, and there are large differences that directly lead to resistance. The selection of a resistant rootstock has become more difficult.

Many studies have been performed on resistant rootstocks. Leinfelder et al. [17] reported the growth of four rootstocks (M26, M7, CG6210, and G30) after being planted in replanted soil for 4 years. The growth of G30 and CG6210 plants increased significantly, and the average life span of the CG6210 root system was five times that of M7. Rumberger et al. [18] selected three CG rootstocks (CG16, CG30, and CG210) and M7 and M26 conventional rootstocks to graft the Royal Empire variety. The results of three consecutive years showed that CG210 and CG30 rootstocks were more resistant to ARD. Mazzola et al. [19] reported that the infection rate of the Geneva rootstock series Rhizopus rot was significantly lower than that of M26, MM111, and MM166 in the Washington State replanting garden. Nevertheless, these rootstocks have not been promoted. The T337 and the M26 dwarf rootstocks are still used as the main apple rootstocks in production, and apples with the T337 rootstock have the advantages of early fruiting and a large yield [20]. Apples with M26 as the rootstock have the advantages of higher graft compatibility and strong healing ability [21], but both rootstocks have a short life span, shallow root systems, and poor resistance to ARD [21].

Through the patented technology of in situ breeding [22] (Figure 1a,b), we selected a new line of apple rootstock with a better ARD resistance called 12-2 (a new line of *Malus spectabilis* that had not been identified in 2010. The new line has red stems and new purple-red leaves in 30 d and 3-year-old plants. We initially selected more than 30 ARD-resistant high-quality lines and planted them all in replanted soil with 20-year-old Fuji/*Malus* × *robusta* (CarriŠre) Rehder apples in 2010. On November 2014, only 12-2 and the other superior lines survived, and the trees have been surviving vigorously until now [23,24]. Thus, we preliminarily judged that 12-2 might be resistant to ARD, and we conducted this test to verify this conclusion. Studies showed that seedlings are more likely to be infected with ARD, and that the growth of rootstock infected with ARD during the seedling period will be affected [25]. Thus, we used 1-year-old 12-2, T337, and M26 as test materials and planted them in replanted soil and sterilized soil. We compared the aboveground traits in the sterilized soil to test the resistance of 12-2 to ARD and provide important test materials for resistance breeding of apple rootstock, which is of great significance to fundamentally solve ARD.

2. Materials and Methods

2.1. Experimental Materials and Treatments

The experiment was conducted at the National Key Rootstock Breeding Base of Shandong Agricultural University, Tai'an City, Shandong Province from March 2015 to October 2016. The 12-2 self-selected tolerant rootstock used breeding patented technology, and the T337 and the M26 tissue culture rootstocks were purchased from Shandong Horticultural Techniques & Services Co. Ltd. (Tai'an, Shandong, China). The three tissue-cultured rootstocks were subcultured under the same conditions for 8 months beginning in early March 2015 (1 L of MS medium contained sucrose 30 g and agar 7.5 g, 6-BA 0.6 mg, and IBA 0.2 mg with pH of 5.8). Five bud bushes were inoculated in each bottle of induction

medium. The temperature of the tissue culture room was 25 ± 2 °C with 16 h·d^{-1}. Light intensity was 1000 lx. In early January 2016, three tissue culture rootstocks subcultured multiple times were inoculated in rooting medium (1 L of 1/2 MS medium contained sucrose 20 g, agar 7.5 g, 6-BA 0.2 mg, and IBA 1.0 mg with pH of 5.8). Five buds were inoculated per bottle of induction medium. The temperature of the tissue culture room was 25 ± 2 °C with 16 h·d^{-1}. Light intensity was 1000 lx. Rooted rootstocks with similar growth from each superior line were selected in early March 2016 and transplanted into a sterile substrate after tempering. At the end of March 2016, each of the three kinds of 60 tissue culture rootstocks with similar growth were transplanted into rootstock pots containing 10 kg of soil, 25 cm stem diameter, and 30 cm depth. Among them, 30 plants were planted in replanted soil, and the others were planted in sterilized soil. Three plants were planted in each rootstock pot. The experimental replanted soil was taken from a 20-year-old apple orchard at Xuanjiazhuang, Daolang District, Tai'an City, Shandong Province, China. Referring to the method of Li et al. [13], half of the replanted soil was autoclaved at 120 °C for 20 min (Zealway Instrument Inc., Xiamen, Fujian, China) and treated as sterilized soil.

2.2. Determining Plant Height and Stem Thickness

The height of the young trees was measured with a ruler starting from the grafting interface, and stem thickness was measured 1 cm above the grafting interface with Vernier calipers. Plant height and stem thickness were measured on 6 June, 6 July, 6 August, 6 September, and 6 October. Each treatment was repeated five times.

2.3. Determining Relative Chlorophyll Content

Fully expanded 5th–7th mature uninjured leaves were removed (from bottom to top), and chlorophyll content was measured with the SPAD-502 portable chlorophyll meter (Beijing Harvesting Science and Technology Co., Ltd., Beijing, China). The relative chlorophyll content was measured on 8 June, 8 July, 8 August, 8 September, and 8 October. Each treatment was repeated five times.

2.4. Determining the Leaf Photosynthetic Parameters

The same fully expanded 5th–7th mature uninjured leaves used to determine relative chlorophyll content were selected, and used in the CIRAS-2 portable photosynthesis measurement system (PP-Systems Hansha Scientific Instruments, Beijing, China) to determine leaf net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), stomatal conductance (G_s), and transpiration rate (T_r). Five replicates were run for each treatment. The measuring times of the leaf photosynthetic parameters were 9 June, 9 July, 9 August, 9 September, and 9 October. Each treatment was repeated five times.

2.5. Determining the Leaf Fluorescence Parameters

The same fully expanded 5th–7th mature uninjured leaves were selected and used with the German WALZ Junior-PAM portable fluorometer (Zealquest Scientific and Technology Co., Ltd., Shanghai, China) to measure the chlorophyll fluorescence parameters. The main fluorescence parameters were: PSII original light energy conversion efficiency (F_v/F_m), PSII actual photochemical efficiency (Φ_{PSII}), non-photochemical quenching coefficient (NPQ), photochemical quenching coefficient (qP), and electron transfer rate (ETR). The measuring times of the leaf photosynthetic parameters were 9 June, 9 July, 9 August, 9 September, and 9 October. Leaf fluorescence was measured and imaged twice on 10 August using the chlorophyll fluorescence imaging system (FlourCam fluorescence imaging system, Czech PSI Co., Shutter = 2, super = 30, Act2 = 50, sensitivity = 80) after the leaves were dark-adapted for 30 min. The instant change in the chlorophyll fluorescence emissions was captured by a top CCD camera. Each treatment was repeated five times.

2.6. Determining Leaf Antioxidant Enzyme Activities and Malondialdehyde (MDA) Content

Mature 5th–7th leaves of the middle branches of the plant (from bottom to top) were selected to determine enzyme activities. The method to determine superoxide dismutase (SOD) activity was referred from Sun et al. [26]. The amount of enzyme required to inhibit 50% of the photochemical reduction of nitrogen blue tetrazolium was one unit of enzyme activity and was expressed as $U \cdot g^{-1}$ FW^{-1}. Peroxidase (POD) activity was measured by the guaiacol method as described by Omran [27]. The change in absorbance was measured at 470 nm. The amount of enzyme that caused an absorbance change of 0.01 at 470 nm/min was one enzyme activity unit and was expressed as $U \cdot g^{-1}$ FW^{-1} min^{-1}. Catalase (CAT) activity was measured according to the method of Singh et al. [28]. The change in absorbance at 240 nm was measured, and the amount of enzyme that reduced the absorbance at 240 nm by 0.1/min was taken as one enzyme activity unit and expressed as $U \cdot G^{-1}$ FW^{-1} min^{-1}. MDA content was determined by the thiobarbituric acid (TBA) method [29]. In total, 1 mL each of the supernatant and water were added to a test tube, and 2 mL of 0.67% TBA was added. The mixture was placed in a boiling water bath for 15 min and quickly placed in ice water to cool. The absorbance of the supernatant was measured at 600, 532, and 450 nm. MDA ($\mu mol \cdot g^{-1}$ FW^{-1}) = 0.1548 ($A_{532} - A_{600}$) − $0.01344A_{450}$. Each treatment was repeated five times, and the average value was used. The measuring times were 11 June, 11 July, 11 August, 11 September, and 11 October. Each treatment was repeated five times.

2.7. Determining Mineral Nutrient Element Contents in Leaves

Mature 5th–7th leaves of the middle branches of the plant (from bottom to top) were selected. The leaves were rinsed with deionized water and placed in an oven at 105 °C for 15 min and then dried at 80 °C. The dried, ground, and sieved leaves were digested with H_2SO_4-H_2O. Total nitrogen was determined with the multi N/C3100 (Analytik Jena AG, Beijing, China). Total phosphorus was determined by platinum blue colorimetry. Total potassium was determined by flame photometry, and atomic absorption spectrophotometry was used to determine the contents of total calcium, magnesium, iron, copper, and zinc in the leaves [30,31]. The measuring times were 12 June, 12 July, 12 August, 12 September, and 12 October. Each treatment was repeated five times.

2.8. Data Analysis

Analysis of variance was performed using DPS7.05. Mean difference comparison among different treatments was performed by t-test and Duncan's multiple range test (DMRT) at a 0.05 probability level, unless otherwise noted.

3. Results

3.1. Plant Height and Stem Thickness Analysis

ARD had a significant effect on T337 and M26 plant height (Table 1) and stem diameters (Table 2) from June to October but no significant effect on 12-2. From June to October, compared with their respective controls in the replanted soil, the plant heights of T337 in the sterilized soil increased by 20.59%, 29.96%, 46.00%, 43.23%, and 41.43%, respectively, and the stem diameters increased by 17.92%, 18.31%, 23.10%, 13.65%, and 14.44%, respectively. The plant heights of M26 in the sterilized soil increased by 30.91%, 40.35%, 42.47%, 36.60%, and 35.01%, respectively, and the stem diameters increased by 20.00%, 32.59%, 22.54%, 20.09%, and 17.49%, respectively.

Table 1. The height (cm) of the plants with different rootstock types planted in sterilized and replanted soil.

Treatment		June	July	August	September	October
T337	Replanted soil	43.2 ± 1.0	47.3 ± 1.9	47.2 ± 2.6	54.1 ± 1.5	56.3 ± 2.4
	Sterilized soil	52.1 ± 0.7 *	61.4 ± 0.5 *	68.9 ± 3.1 *	77.5 ± 2.1 **	79.7 ± 1.2 *
M26	Replanted soil	43.0 ± 1.7	54.7 ± 3.0	55.0 ± 3.9	63.2 ± 0.9	67.3 ± 2.9
	Sterilized soil	56.3 ± 0.9 *	76.7 ± 2.2 *	78.4 ± 2.5 **	86.3 ± 1.9 **	90.9 ± 1.3 *
12-2	Replanted soil	79.4 ± 2.0	94.8 ± 1.3	94.7 ± 2.7	96.3 ± 2.1	99.7 ± 1.2
	Sterilized soil	79.7 ± 1.5	90.3 ± 2.7	99.1 ± 1.4	99.3 ± 2.4	97.5 ± 1.3

Note: A *t*-test was used to determine the significance of the difference between the two assays. The data in the table are expressed as mean ± SE. * $p < 0.05$; ** $p < 0.01$. The same below.

Table 2. The stem thickness (mm) of the plants with different rootstock types planted in sterilized and replanted soil.

Treatment		June	July	August	September	October
T337	Replanted soil	4.8 ± 0.2	5.0 ± 0.1	6.6 ± 0.1	7.2 ± 0.2	7.2 ± 0.0
	Sterilized soil	5.7 ± 0.0 *	5.9 ± 0. 1 **	8.1 ± 0.2 **	8.2 ± 0.1 **	8.2 ± 0.1 *
M26	Replanted soil	4.1 ± 0.1	4.5 ± 0.2	5.9 ± 0.1	6.4 ± 0.1	6.9 ± 0.1
	Sterilized soil	4.9 ± 0.1 *	5.9 ± 0.1 **	7.2 ± 0.1 *	7.7 ± 0.1 *	8.1 ± 0.1 *
12-2	Replanted soil	5.9 ± 0.1	6.4 ± 0.3	8.0 ± 0.1	8.2 ± 0.1	8.3 ± 0.1
	Sterilized soil	5.9 ± 0.2	6.4 ± 0.2	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.1

Note: A *t*-test was used to determine the significance of the difference between the two assays. The data in the table are expressed as mean ± SE. * $p < 0.05$; ** $p < 0.01$.

3.2. Analysis of Relative Chlorophyll Content in Plant Leaves

The relative chlorophyll content of T337 and M26 was affected by ARD (Figure 1c–e). The relative chlorophyll content of T337 in replanted soil was significantly lower than that in sterilized soil from June to October. The relative chlorophyll content of M26 in replanted soil was significantly lower than that in sterilized soil in June, July, and September. The relative chlorophyll content of 12-2 was not different between replanted and sterilized soil. The relative chlorophyll content of 12-2 was less affected by ARD from June to October.

3.3. Analysis of Plant Leaf Photosynthetic Parameters

No significant differences in P_n, C_i, G_s, or T_r were observed in T337, M26, or 12-2 from June and July compared with the respective controls (Figure 2). The P_n and the G_s of T337 in replanted soil were significantly lower than those in sterilized soil, but no significant differences were observed in the photosynthetic parameters of M26 and 12-2 in replanted or sterilized soil during August. During Sepetmber, the P_n value of T337 and the P_n and the C_i values of M26 in replanted soil were significantly lower in replanted soil than those in sterilized soil, respectively. The 12-2 photosynthetic parameters were not significantly different between replanted and sterilized soil. No significant differences were observed in the photosynthetic parameters of the other treatments during October, except that M26 P_n was significantly lower in replanted soil than in sterilized soil.

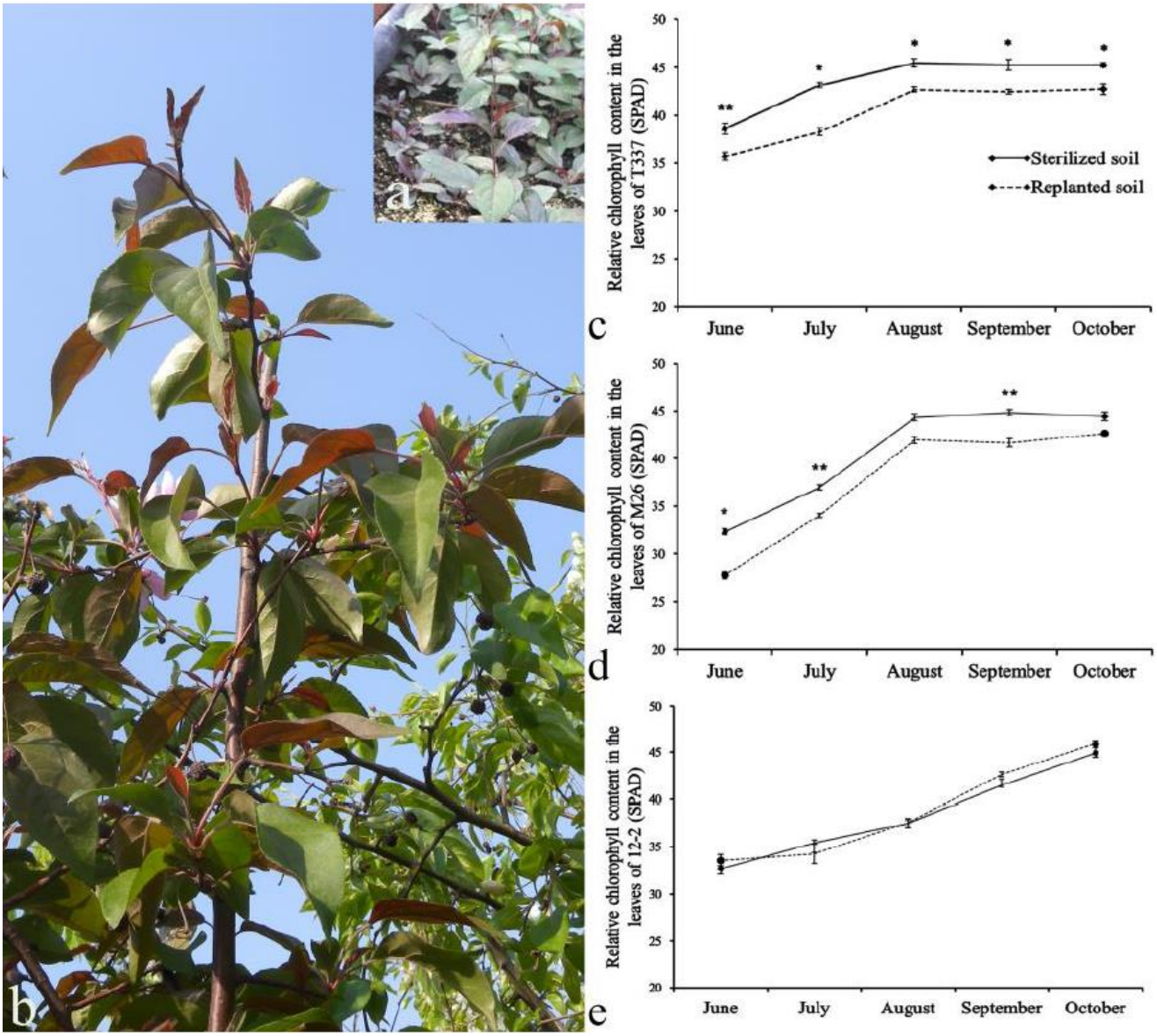

Figure 1. Growth of 12-2 and the relative chlorophyll content of the plants with different rootstock types planted in sterilized and replanted soil. (**a**): 30 d after transplanting the 12-2 plugs; (**b**): 3-year-old 12-2 plant; (**c**–**e**): relative chlorophyll content in the leaves of the three rootstocks. * $p < 0.05$; ** $p < 0.01$. Solid line represents the treatments in sterilized soil; dotted line represents the treatments in replanted soil. The same below.

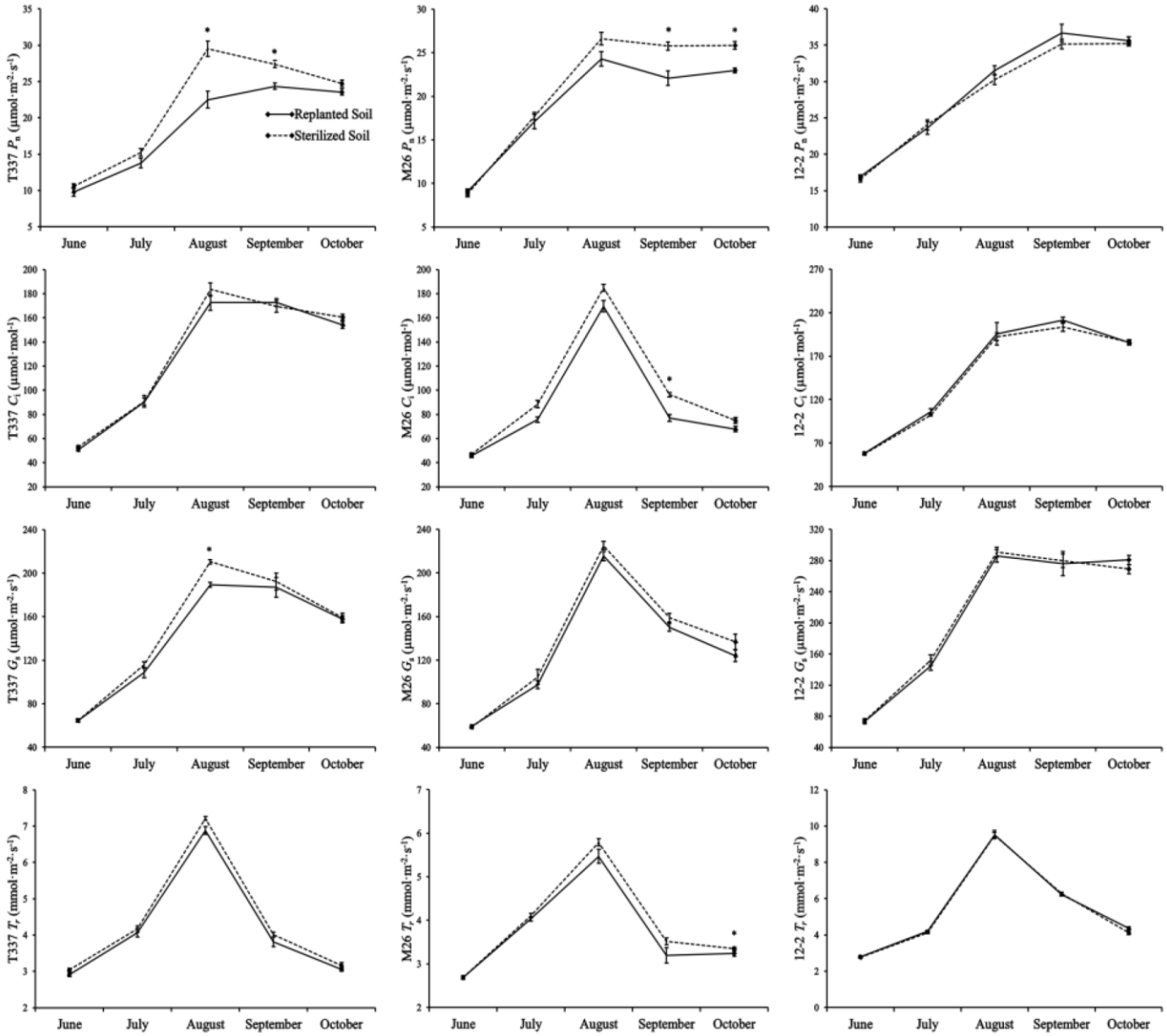

Figure 2. Photosynthesis in the leaves of plants with different rootstock types planted in sterilized and replanted soil.

3.4. Analysis of Fluorescence Parameters and Fluorescence Imaging of Plant Leaves

3.4.1. Analysis of Fluorescence Parameters

The T337 qP (Figure 3) value was very significantly lower in replanted soil than in sterilized soil in June and July, and no significant differences were detected in the other treatments. ARD had a significant effect on T337 and M26 NPQ values. The T337 NPQ value in replanted soil was significantly higher than that in sterilized soil from June to October, except August, and the M26 NPQ value in replanted soil was significantly higher than that in sterilized soil from June to October, except July. The T337 and the M26 F_v/F_m values were significantly higher in replanted soil than those of sterilized soil in June, respectively, with no significant differences in the other treatments. No significant differences in T337, M26, or 12-2 Φ_{PSII} and ETR (Figure 4a) values were observed in replanted or sterilized soil from June to October.

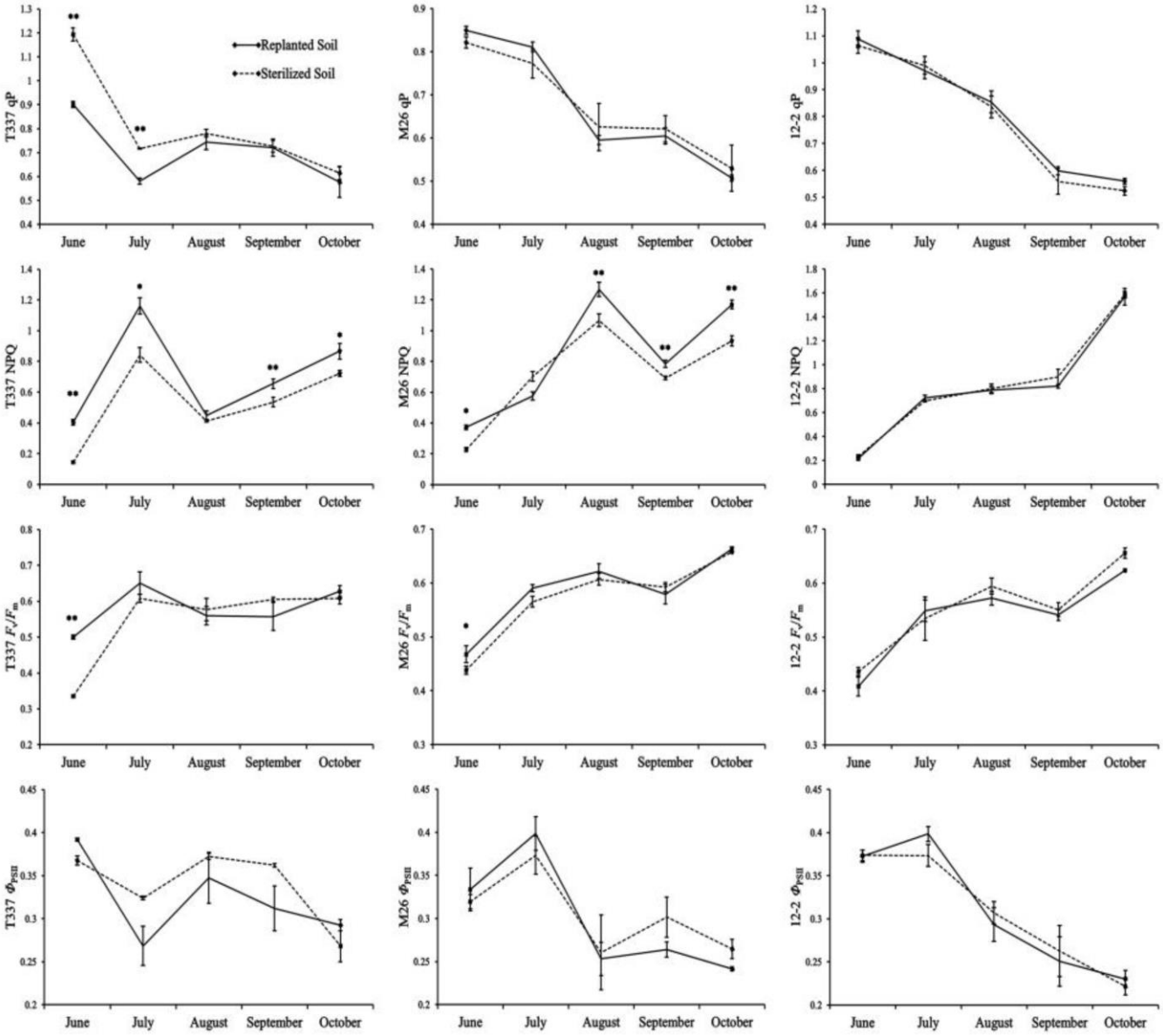

Figure 3. Fluorescence (qP, NPQ, F_v/F_m, and Φ_{PSII}) in the leaves of plants with different rootstock types planted in sterilized and replanted soil. Effects of replanted soil on fluorescence of leaves from the different rootstock treatments.

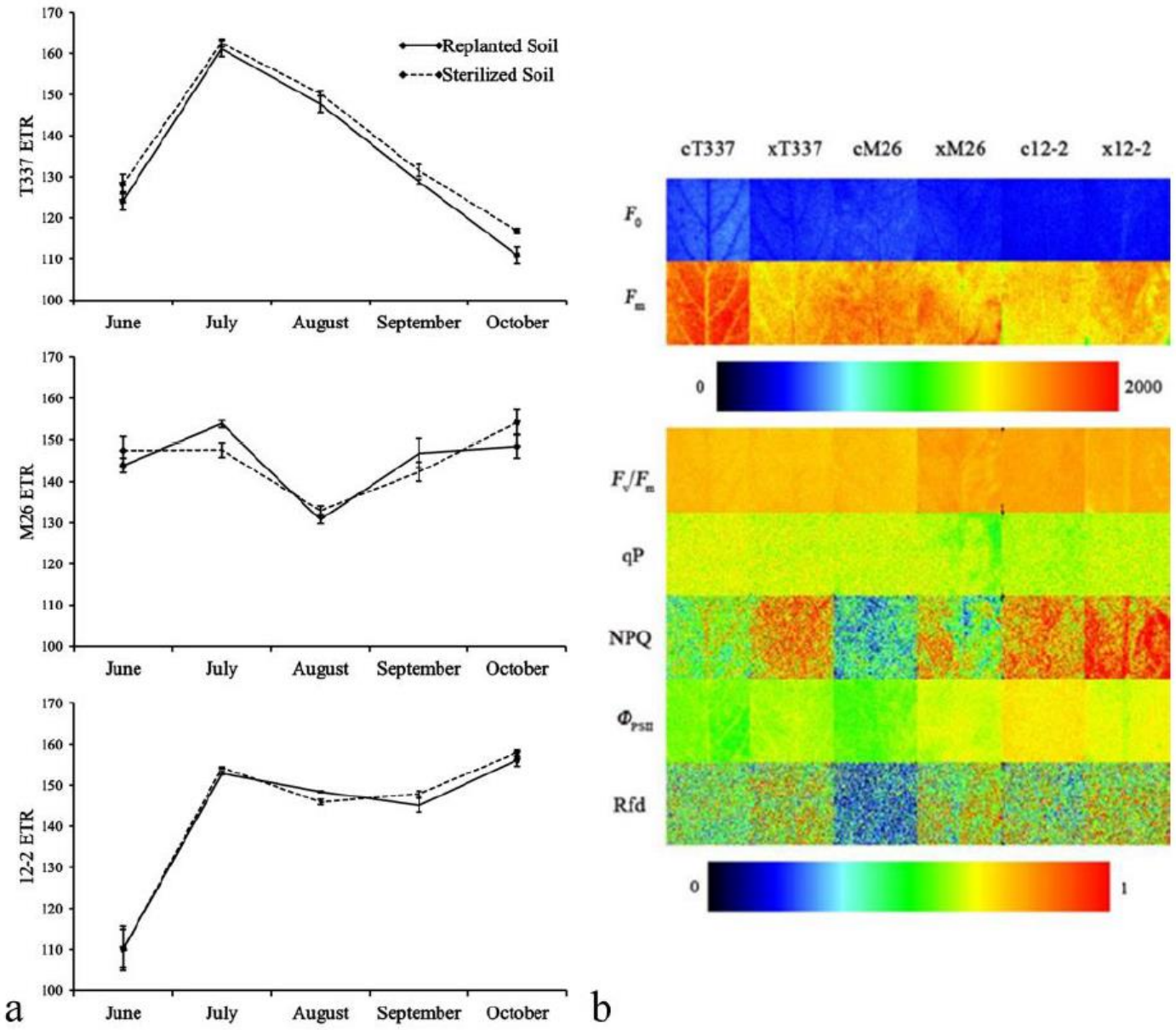

Figure 4. Fluorescence (ETR) (**a**) and the chlorophyll fluorescence imaging system (**b**) in the leaves of the plants with different rootstock types planted in sterilized and replanted soil. cT337, T337 planted in replanted soil; xT337, T337 planted in sterilized soil; cM26, M26 planted in replanted soil; xM26, M26 planted in sterilized soil; c12-2, 12-2 planted in replanted soil; x12-2, 12-2 planted in sterilized soil. F_0 and F_m scale is 0–2000, and F_v/F_m, qP, NPQ, Φ_{PSII}, and ETR scale is 0–1.

3.4.2. Analysis of Fluorescence Imaging

The second fluorescence measurements of T337, M26, and 12-2 in August (Table 3) showed that F_v/F_m, qP, Φ_{PSII}, and ETR values were not significantly different from the first measurements, and the initial fluorescence for dark adaptation (F_0) and maximum fluorescence (F_m) indicated that ARD did not have significant effects on T337, M26, or 12-2. Figure 4b showed that ARD had an effect on NPQ of M26 and rapid fluorescence quenching (Rfd) of M26 and T337, and the effect of Rfd on M26 was higher than that on T337. No effect on Rfd was detected in 12-2.

Table 3. Fluorescence in the leaves from plants with different rootstock types planted in sterilized and replanted soil on 10 August.

Treatment		F_0	F_m	F_v/F_m	qP	NPQ	Φ_{PSII}	ETR
T337	Replanted soil	383.0 ± 8.4	1537.1 ± 32.7	0.8 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	1.1 ± 0.0	0.8 ± 0.0
	Sterilized soil	375.4 ± 13.7	1461.4 ± 22.0	0.7 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	0.9 ± 0.1	0.8 ± 0.0
M26	Replanted soil	369.5 ± 16.1	1455.7 ± 75.9	0.8 ± 0.0	1.0 ± 0.0	0.3 ± 0.0 **	0.8 ± 0.1	0.8 ± 0.0
	Sterilized soil	338.3 ± 6.0	1497.2 ± 42.9	0.8 ± 0.0	1.0 ± 0.0	0.2 ± 0.0	0.9 ± 0.1	0.8 ± 0.0
12-2	Replanted soil	284.5 ± 10.4	1271.8 ± 60.6	0.8 ± 0.0	0.9 ± 0.0	0.5 ± 0.1	1.1 ± 0.1	0.8 ± 0.0
	Sterilized soil	300.7 ± 11.0	1324.4 ± 45.1	0.8 ± 0.0	1.0 ± 0.0	0.5 ± 0.0	1.1 ± 0.1	0.8 ± 0.0

Note: A *t*-test was used to determine the significance of the difference between the two assays. The data in the table are expressed as mean ± SE. ** $p < 0.01$.

3.5. Analysis of Antioxidant Enzyme Activities and MDA Content in Leaves

Analysis of the content of antioxidant enzymes and MDA in leaves (Figure 5) showed that ARD affected T337 and M26. T337 SOD activity was significantly higher in replanted soil than that in sterilized soil in August and Ocotber. M26 SOD activity was significantly higher in replanted soil than that in sterilized soil from July to September, whereas SOD activity in October was significantly lower in the continuous soil treatment than in sterilized soil. The T337 POD activity was significantly higher in replanted soil than in sterilized soil during August and September, and M26 POD activity was significantly higher in replanted soil than in sterilized soil during June and July. M26 POD activity was significantly lower in replanted soil than in sterilized soil from August to October. T337 CAT activity was significantly lower in replanted soil than in sterilized soil in July, August, and October. M26 CAT activity was significantly higher in replanted soil than in sterilized soil during July. M26 CAT activity was significantly lower in replanted soil than in sterilized soil from August to October. T337 MDA content was significantly lower in replanted soil than in sterilized soil during August, but the opposite trend was observed during September and October. M26 MDA content was significantly lower in replanted soil than in sterilized soil during July. The M26 MDA content was significantly higher in replanted soil than in sterilized soil during September and October. The antioxidant enzyme activities and the MDA content in the leaves of 12-2 were not significantly affected by ARD.

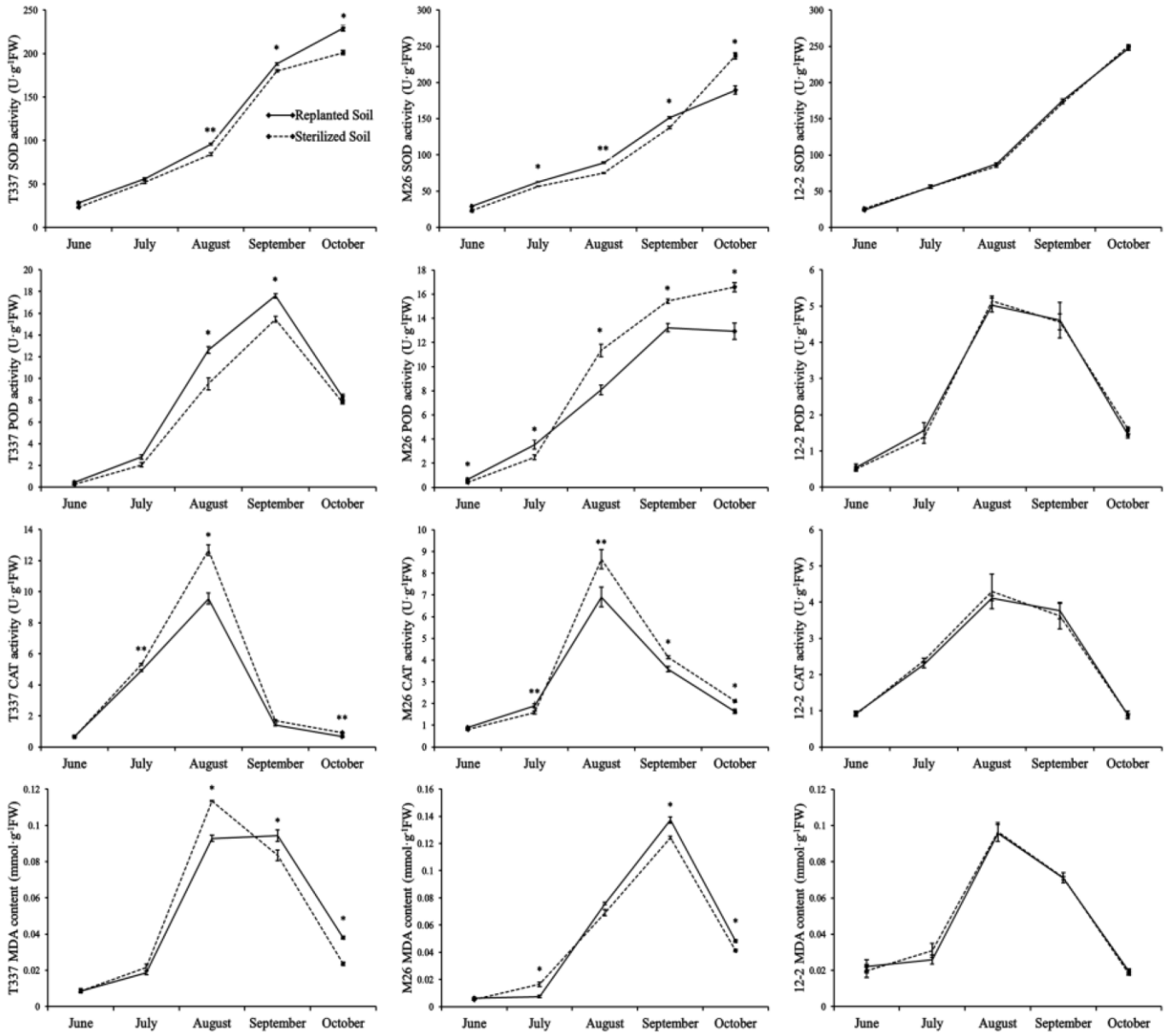

Figure 5. The antioxidant enzyme activities and the malondialdehyde content in leaves of plants with different rootstock types planted in sterilized and replanted soil.

3.6. Analysis of Mineral Elements in the Leaves

The effects of ARD on the mineral elements differed (Figure 6). Nitrogen content in M26 was significantly lower in replanted soil than in sterilized soil during October, but no other significant differences were detected in the other treatments from June to October. T337 phosphorus content was significantly higher in replanted soil than in sterilized soil from August to October, and M26 phosphorus content was significantly lower in replanted soil than in sterilized soil during September and October. T337 potassium content was significantly lower in replanted soil than in sterilized soil during June, significantly higher in replanted soil than in sterilized soil from September to October, and there were no significant differences in potassium contents of M26 and 12-2. The zinc content of M26 was significantly lower in replanted soil than in sterilized soil during July, but no other significant differences were detected in the other treatments from June to October. ARD had no effect on calcium, magnesium, iron, or copper contents (Figure 7) in any of the treatments.

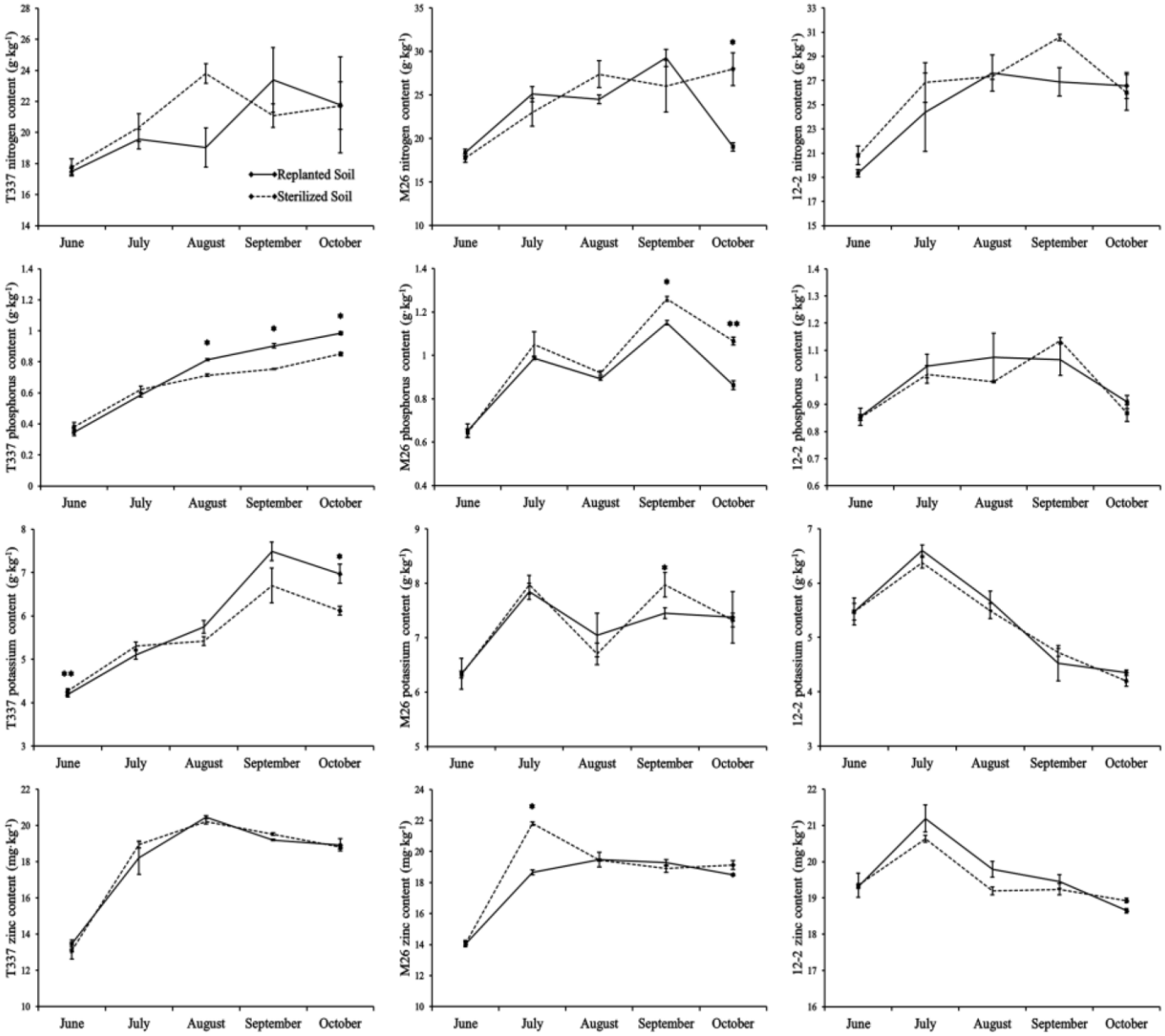

Figure 6. The mineral element content (nitrogen, phosphorus, potassium, and zinc) in the leaves of plants with different rootstock types planted in sterilized and replanted soil.

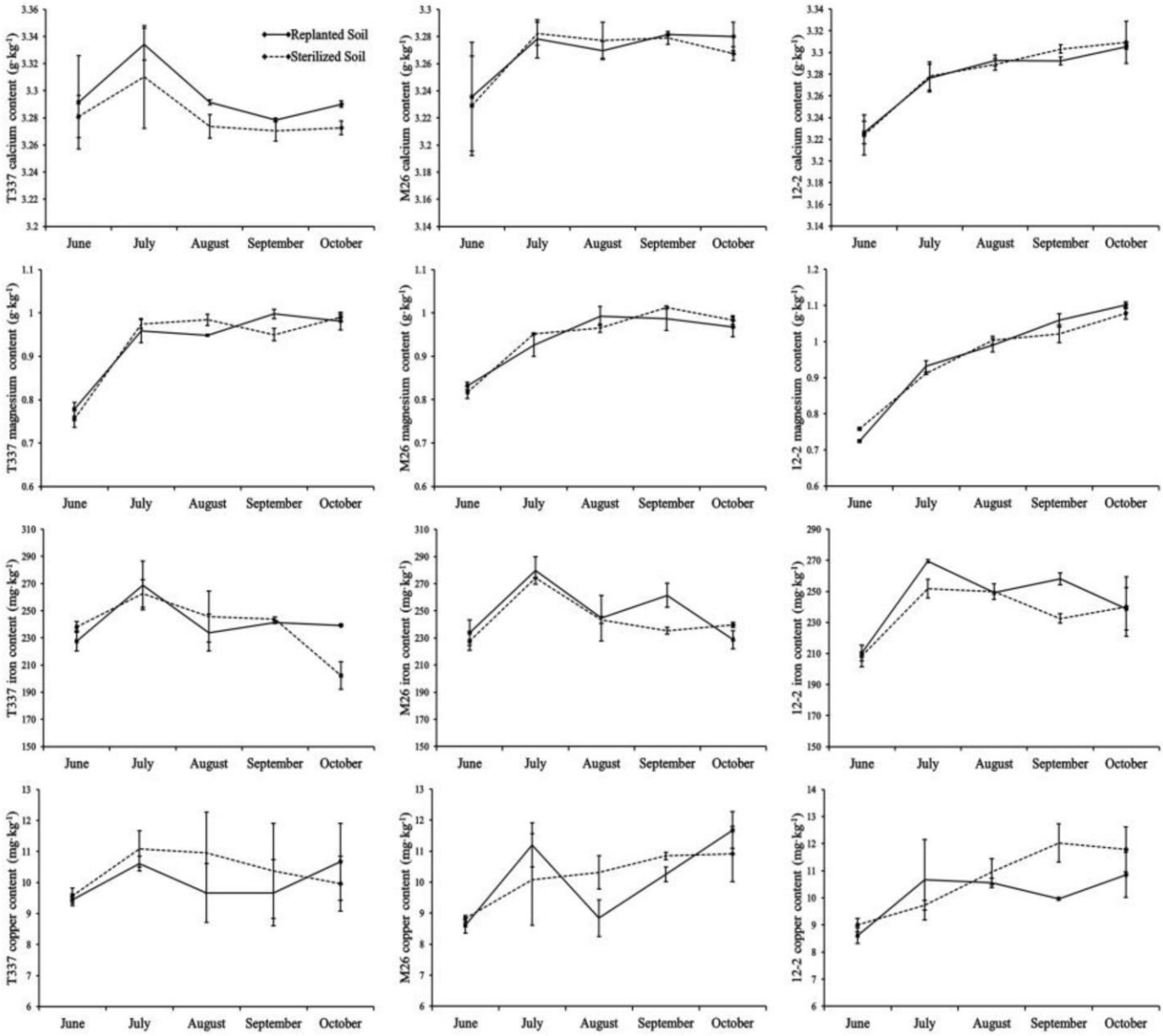

Figure 7. The mineral element content (calcium, magnesium, iron, and copper) in the leaves of plants with different rootstock types planted in sterilized and replanted soil.

4. Discussion

4.1. Effect of ARD on Plant Height, Stem Diameter

Using resistant rootstock to prevent and control ARD has attracted attention since the mid-1990s [14]. Now, breeding resistant rootstocks is considered one of the most economical, effective, and feasible means to overcome ARD [21]. Plant height, stem diameter, and relative leaf chlorophyll content are the most direct manifestations of plant growth status [32]. Studies showed that reduced plant growth (or biomass) is one of the main phenotypic parameters of ARD infection [25,33–36]. In this experiment, the plant heights and the stem thicknesses of T337 and M26 were significantly affected by ARD, whereas 12-2 was not affected. These results follow those of Guo et al. [37].

4.2. Effect of ARD on Relative Leaf Chlorophyll Content

Chlorophyll absorbs light energy, which is necessary for photosynthesis. A decrease in chlorophyll content will inevitably cause a decline in the photosynthetic rate and the

accumulation of photosynthetic products, which ultimately affects the health of growing plants [38]. The study of Botyanszka et al. [39] showed that chlorophyll content could be used as a technical indicator to easily identify rootstock resistance; the poorer the rootstock resistance is, the more significant is the impact on chlorophyll content. This was also the result of the test in which T337 (June–October) and M26 (June, July, and September) were significantly affected, but 12-2 was not affected.

4.3. Effect of ARD on Plant Leaf Photosynthetic Parameters

Chlorophyll is the main photosynthetic pigment in plants, and photosynthesis uses light energy for material production. Therefore, chlorophyll content is directly related to the strength of photosynthesis [40]. Studies showed that C_i increases most of the time when G_s decreases, as carboxylation efficiency is reduced [41]. In this experiment, the T337 G_s (August) and the M26 C_i (September) were suppressed, causing a decrease in P_n, indicating that T337 and M26 suffered mainly from inhibited stomata [42]. The decreases of T337 P_n in September and M26 P_n in October may have been the result of ARD destroying the inner thylakoid membrane structure of T337 and M26, causing membrane peroxidation and a decrease in chlorophyll content and ultimately resulting in a lower net photosynthetic rate [43]. The 12-2 photosynthetic parameters were not significantly different between replanted and sterilized soil, indicating that ARD affected photosynthesis of T337 and M26 but not of 12-2.

4.4. Effect of ARD on Fluorescence Parameters and Fluorescence Imaging of Plant Leaves

Chlorophyll fluorescence can be utilized as a photosynthetic probe. A series of important regulatory processes within the photosynthetic apparatus can be understood through fluorescence parameter analysis. The greater the maximum PSII photochemical efficiency of F_v/F_m is, the higher is the original PSII light energy conversion efficiency. F_v/F_m decreases when a plant is under stress [44]. The change in the NPQ value reflects non-photochemical dissipated energy, including heat dissipation energy from the thylakoid membranes, which is a self-protection mechanism for the plant photosynthetic machinery [45]. The results of the present experiment show that T337 and M26 F_v/F_m values were significantly higher in replanted soil than those in sterilized soil during June, which disagreed with the results of Wright et al. [44]. However, replanted soil had a significant effect on the NPQ values of T337 (June–October, except August) and M26 (June–October, except July) but had no significant effect on 12-2. The reasons might be that T337 and M26 maintained photosynthetic activity by activating an acclimation mechanism under the replanted condition. Energy dissipation capacity may have increased, which was detected by an increase in NPQ, but the PSII F_v/F_m value did not change. When stress exceeded capacity, permanent photoinhibition occurred, which was detected by a decrease in the F_v/F_m value [46]. When the stress was strong enough, the PSII Φ_{PSII} and the qP decreased, indicating that the ETR was inhibited [47]. In the present experiment, no significant changes in Φ_{PSII}, ETR, or qP (except T337 in replanted soil in June and July) of T337, M26, or 12-2 after ARD were detected. The reason was that T337, M26, and 12-2 were all subjected to ARD, but the degree of stress did not exceed the ability of T337, M26, and 12-2 to adapt [48]. The ability of PSII to receive maximum light quanta can be determined by combining F_0 and F_m. If the F_0 value is low and the F_m value is high, the range of receiving light quanta is wider, and more light quanta enter the photochemical reaction pathway [49]. In this study, the results of F_0 and F_m of T337, M26, and 12-2 showed that ARD did not change the photons in their photochemical reaction pathways. Rfd is an empirical parameter that measures plant health [50]. In this experiment, ARD affected the health of M26 and T337 to a certain extent but did not affect the health of 12-2.

4.5. Effect of ARD on Antioxidant Enzyme Activities and MDA Content in Leaves

SOD, POD, and CAT activities are the core of the plant's cross-protection mechanism [51]. Our results show that SOD, POD, and CAT activities in leaves of different apple

rootstocks were significantly affected by ARD, and the trends were the same basically. The antioxidant enzyme activities of T337 and M26 (T337 SOD in August and October, POD in August and September, CAT in July, August, and October; M26 SOD in July–September, POD in June and July, and CAT in July) were significantly higher in replanted soil than in sterilized soil, indicating that, under ARD, T337 and M26 produced reactive oxygen species, which enhance SOD activity to convert the disproportionately activated oxygen into H_2O_2 and O_2 and reduce damage to the plant [52,53], However, the H_2O_2 produced by the disproportionation form OH^- after accumulation in the plant, which causes membrane lipid peroxidation. There are no enzymes to remove OH^- specifically, thus the plant is easily damaged under stress [54]. CAT and POD remove H_2O_2. CAT has a higher efficiency but a weaker affinity for H_2O_2, while POD has a higher affinity for H_2O_2 but also degrades chlorophyll, causing membrane lipid peroxidation, thus POD has a dual role [53]. However, in the next few months, M26 antioxidant enzyme activity (SOD in October, POD during August–October, and CAT during August–October) was significantly lower in replanted soil than in sterilized soil. The possible reasons were that the stress exceeded a certain threshold and M26 could not remove the oxygen free radicals in time, which eventually led to reduced enzyme activities and oxidative damage [55]. MDA is a product of membrane lipid peroxidation and a marker of cell membrane destruction [56]. MDA reacts with macromolecules, such as proteins and nucleic acids, to denature proteins and nucleic acids and affect the structure and the function of cells, ultimately leading to cell death [57]. The results of this experiment showed that MDA contents of T337 (August) and M26 (July) were significantly lower in replanted soil after several months than in sterilized soil. The reasons might be that T337 and M26 adapted to the ARD [58]. After a few months, the MDA content of T337 (September and October) and M26 (September and October) was significantly higher in replanted soil than in sterilized soil. ARD destroyed the cell structure of T337 and M26, resulting in increased MDA content [59]. No significant differences were observed between SOD, POD, CAT activities and MDA content of 12-2 in replanted or sterilized soil, which further confirmed that 12-2 has a stronger tolerance to ARD than T337 and M26.

4.6. Effect of ARD on Mineral Elements in the Leaves

Mineral nutrient elements play an important role in the material composition and the metabolic processes of crops, and plant leaves are a sensitive organ that reflects mineral content, and their nutrient content is directly related to the nutrient level of the tree [60]. Nitrogen is a main component of protein and plays an important role in leaf growth [61]. A phosphorus deficiency could cause plants to be short, dark green in color, and dull [62]. Potassium-deficient plants have a weakened resistance to stress, making them vulnerable to disease [63]. Zinc changes the ratio of organic nitrogen to inorganic nitrogen in plants, promotes healthy growth of branches and leaves, and participates in chlorophyll synthesis and the formation of carbohydrates [64]. Zinc also contributes to the synthesis of auxin, activates enzymes, and plays important roles in physiology and biochemistry [65]. Calcium is necessary for plant growth and development, as it maintains the stability of cell walls and membranes [66]. Magnesium is part of the chlorophyll structure and plays an important role in the development and the functioning of chloroplasts. Magnesium is also an activator of many enzymes [67]. Iron is a component of many important enzymes in plants and participates in the formation of chlorophyllin [68]. Copper is an essential element for plant growth [64]. Previous studies showed that ARD reduces the mineral nutrient content in plant leaves [69]. In this experiment, the effect of ARD on plant mineral elements was not as significant as that on the growth indicators. The reason may be that different rootstock varieties have different abilities to process nutrients, and differences were observed in the ability of the aboveground tissues to obtain elements from underground, even under the same rootstock conditions [70]. Additionally, many factors, such as soil texture, fertilization status, and cultivation management, affect the mineral element content of apple leaves [65].

Therefore, the mechanism of ARD on absorption, operation, and utilization of nutrient elements in plants remains unclear, and further research is needed.

5. Conclusions

The 12-2 rootstock had better resistance to ARD than the control. Plant height, stem thick, relative leaf chlorophyll content, photosynthesis of leaves, leaf fluorescence parameters, leaf antioxidant enzyme activities, MDA content, and leaf mineral contents in 12-2 were not significantly different in replanted soil compared to sterilized soil. ARD had a greater effect on most of the aboveground physiological indicators of T337 and M26 compared to those of 12-2. The test of the aboveground indicators for 12-2 showed that 12-2 was a more resistant ARD rootstock and could be used as important test material in apple rootstock resistance breeding.

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Abbreviations

ARD: apple replant disease; 12-2: apple rootstock superior line named 12-2; Pn: net photosynthetic rate; Ci: intercellular CO2 concentration; Gs: stomatal conductance; Tr: transpiration Rate; Fv/Fm: PSII original light energy conversion efficiency; ΦPSII: PSII actual photochemical efficiency; NPQ: non-photochemical quenching coefficient; qP: photochemical quenching coefficient; ETR: electron transfer rate; MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase.

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