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Full Length Research Paper

Anatomical studies of stems, roots and leaves of selected citrus rootstock varieties in relation to their vigour

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The present studies were carried out to consolidate information on anatomical features of stem, root and leaves from different vigour groups of citrus rootstock varieties. Troyer citrange (*Citrus sinensis × Poncirus trifoliata*), rough lemon (*Citrus jambhiri*), swingle citrumelo (*Citrus paradisi × P. trifoliata*), sweet lime (*Citrus limettioides*), carrizo citrange (*C. sinensis × Poncirus trifoliata*), sour orange (*Citrus aurantium*) and flying dragon (*P. trifoliata*) were examined to investigate relationships between their internal structure and vigour. It was found that vigorous rootstock, rough lemon (*C. jambhiri*) possessed lower proportions of bark (phloem) in the stems and roots as well as larger vessel elements in the xylem, when compared with least vigorous rootstocks, flying dragon (*P. trifoliata*). Smaller but more vessel elements were found in stem cross-sections than root cross-sections of three month's old rootstock seedlings. The possible mechanism of vascular differentiation induced by young leaf primordia, following the original polarity and developing toward the roots is discussed.

Key words: Citrus rootstock, anatomical features, stem, root, leaves.

INTRODUCTION

Citriculture is a dynamic industry and rapid strides in the husbandry of citrus in different regions of the world have resulted in a range of rootstocks being introduced. The potent effects of rootstock on the growth and fruiting of trees are widely recognised. Rootstock development is an open-ended process, because success depends on the interaction of genetic potential and agronomical manipulation. Control of vigour is a characteristic being sought in most citrus rootstock breeding programmes. Size controlling rootstocks are important for higher density orchards. Nonetheless, new rootstocks must also lead to excellent yields of high–quality fruit and possess other key tolerances (Castle, 2010). One of the main problems in evaluating citrus rootstocks for commercial use is the time needed to determine the horticultural characteristics and their commercial performance. Thorough horticultural testing of citrus rootstocks usually takes at least 20 - 25 years. Environmental abnormalities often disrupt these tests and the results may only be of value to the immediate area, a given scion and the particular selection of the rootstock variety.

The physical properties of stem and root are related to their anatomy and there is no way to interpret their role without sufficient knowledge of their structure. Root and stem anatomy have been investigated as a tool for selecting dwarfing apple root-stocks (Beakbane and Thompson, 1939; Beakbane and Thompson, 1947; McKenzie, 1961 and Miller et al., 1961). Majumdar et al. (1972) concluded that stem growth, proportion of bark and vessels in roots can be used to classify mango (*Mangifera indica* L.) rootstocks into various vigour classes in the nursery stage and found a negative correlation between bark percentage in roots and plant vigour. The roots and stem of dwarfing rootstocks had lower hydraulic conductance than the semi-dwarfing rootstocks (Atkinson et al., 2003; Olmstead et al., 2006).

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This may be associated with the factors like lower xylem, phloem ratios and abnormalities in xylem anatomy of dwarfing rootstocks.

It is possible from our existing knowledge, to do much of preliminary screening of potential rootstock in the laboratory and growth chambers, to determine the relationship between stem, leaf and root anatomical characteristics and vigour of a range of citrus rootstocks. Utilization of such techniques should greatly aid the selection of better rootstock for citrus and reduce the time involved in their development. In paper 2, the relationships are explored further to include tree vigour when grafted with a scion variety.

MATERIALS AND METHODS

Seeds of 7 citrus rootstock varieties were imported from the USA to provide plants for experimentation. The varieties were 'V_1' Troyer citrange (*Citrus sinensis* × *Poncirus trifoliata*), 'V_2' Rough lemon (*Citrus jambhiri*), 'V_3' Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*), 'V_4' Sweet lime (*Citrus limettioides*), 'V_5' Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*), 'V_6' Sour orange (*Citrus aurantium*) and 'V_7' Flying dragon (*Poncirus trifoliata*).

Seeds were sown in trays of vermiculite and put into growth cabinets maintained at a constant temperature of 26°C. After two weeks, seedlings started emerging. The seedlings were transplanted into 10 cm perforated base, plastic pots containing perlite. The pots were placed into trays containing water at pH 6.0 and EC 1.0 dSm^{-1} in the glasshouse and a temperature range between 20 and 30°C was maintained throughout the experiment.

The pots were subsequently transferred to a Nutrient Film Technique (NFT) system as soon as the roots emerged from their base. The NFT system was used to facilitate root sampling. The EC of water was gradually increased and finally maintained at 2.5 dSm^{-1} and the pH of the solution was adjusted to 6.0 with the addition of a 5% mixture of nitric and phosphoric acids mixed at a ratio of 3:1. A completely randomised design with five single plant replicates was used for further studies on plant growth and anatomical characteristics.

Plant height

The height of plants was recorded fortnightly from the stem base to the apical meristem.

Collection of samples for anatomical studies

Root stem and leaf samples were collected from three month's old seedlings of different citrus rootstock varieties and the following anatomical characteristics were quantified using an image analysis system attached to a microscope (Seescan Plc., Cambridge, United Kingdom) as described by (Atkinson and Taylor, 1996):

I) Xylem percentage.
II) Phloem percentage.
III) Number of vessel elements in the xylem.
IV) Area of individual vessel elements in the xylem.
V) Total area of vessel elements in the xylem.

An analysis field of 0.4 mm^2 was calibrated and the low magnification (X2) microscopic images were captured from root and stem sections consisting of the whole transverse area. The area occupied by phloem and xylem was measured using image inversion and false colour to the corresponding area. For each root and stem section, transverse low magnification (x10) microscope images were taken from the cambium to the edge of the pith. The software used in this system (Sonata II) was all menu driven and the images of samples were taken using a video camera attached to a microscope. The grey level in the monochrome images was adjusted and the images manipulated to ensure that only vessel elements were included in the analysis and adjoining vessel elements were recorded as individuals. Using image inversion and false colour, only vessels were identified. This was achieved by using an analysis field and that was accurately measured after calibration, from 0.3613 mm^2 (for larger radial cross-sectional area) to 0.0903 mm^2 (for smaller radial cross-sectional area). The data was stored in a format that was compatible with MS EXCEL. The vessel elements and their cross-sectional area within the field were numbered, measured and used to calculate the total number of vessel elements and their total area.

Collection and preparation of root samples

Root segments, 1.0 cm long, were collected from each seedling 5.0 cm below the stem base. These samples were fixed in F.A.A (formaldehyde-acetic acid-alcohol) solution (ethanol 95%, 50 ml, acetic acid (glacial) 5 ml, formalin 40 v/v, 10 ml, water, 35 ml) for 3 days. The subsequent processes, including dehydration in ascending concentrations of ethanol followed by Histoclear, infiltration with wax, embedding in wax and mounting were done in the standard way as described by Purvis et al. (1966). Transverse sections, 12 µm thick were cut with a sledge microtome and mounted on glass slides. All slides were labelled with variety and replication number. These slides were dipped in Histoclear for 3 min to dissolve the wax followed by rehydration in descending strengths of ethanol (70 - 100%) for 1 min each. The samples were then stained with Safranin O (biological stain used in histology) (3% solution made up in 50% ethanol) for one hour followed by 5 min gentle washing in running tap water. They were then transferred to crystal violet (0.25% solution made up in 90% ethanol) for 2 min followed by a dip in distilled water for 1 min and several dips in 90% ethanol until no more violet stain was removed. Several dips in fast green (1% solution made up in distilled water) followed until no more violet stain was removed when they were transferred into two separate isopropanol solutions for 3 min each, respectively. Before covering with coverslips, the slides were placed in histoclear for 5 min.

Collection and preparation of stem samples

The stem segments, 1.0 cm long, were collected from each seedling at the height of 5 cm above the pot. The same procedure as described for the preparation of root samples was applied for dehydration, embedding, sectioning, staining and mounting with cover slips of stem samples for anatomical studies.

Collection and preparation of leaf samples

In this study, the fourth or fifth leaf down from the terminal leaf was used for anatomical observations. From each leaf, single portions of about 10 × 5 mm were taken from the area mid-way between the leaf apex and base and mid-way between the mid-rib and margin of the leaf lamina. These portions were kept in separate and labelled vials containing excess F.A.A (formaldehyde-acetic acid-alcohol). All manipulations were carried out quickly to avoid wilting of leaves.

The further procedures like dehydration and embedding were the same as described for root samples. Transverse sections 8 µm thick were prepared with the sledge microtome and mounted on glass-slides. These sections were stained and permanent mounts

were prepared in a similar way to that described previously.

The leaf sections were examined under a light microscope and representative fields were selected and photographed with the camera attached to microscope at a magnification of X40. The micro-measure was used to digitise different anatomical features of each leaf. Each negative was placed on a glass platform and the image was captured on screen and the following measurements were taken:

I. Leaf thickness (adaxial to abaxial).
II. Mesophyll thickness (without either epidermis).
III. Depth of palisade parenchyma.
IV. Percentage of palisade parenchyma.

The data regarding morphological and anatomical characteristics of different rootstock varieties were analysed using a SAS programme. Statistical significance of differences in means were evaluated using the t test and differences in variance using the F test. If the F test indicated a significant difference in variance between populations, the approximate t test for different varieties with unequal variances was used to test significance of means. The analysis for coefficient of correlation (*r*) was done, correlating all parameters. The *r* value for each correlation was compared with critical value for *r* at n-2 and P=0.05, P=0.01 and P=0.001.

RESULTS AND DISCUSSION

Different anatomical features of citrus rootstock varieties were studied and attempts made to find correlations between plant vigour and anatomical characteristics at the seedling stage. The coefficient of correlation (Table 1) indicates how well anatomical characters correlate with plant height. Significant correlation suggests that such features could be used as indices for predicting vegetative growth of the rootstock. Plant height was found to be significantly correlated (P=0.001) with most anatomical characteristics with the exception of the area of vessel elements in stem transverse sections. However, plant height had a negative correlation with the number of vessel elements in the xylem of stem (Figure 2) and root (Figure 1) as well as phloem percentage in stem and root transverse sections. More vigorous rootstocks varieties tended to have fewer vessels and comparatively narrower phloem in stems and roots than less vigorous ones (Table 2). The diameter of the vessels in long conducting lateral roots, increased with increasing distance from the stem. Leaves are known to induce and control the development of vascular tissues along the plant axis by means of a steady polar flow of auxins. Auxins control the rate of cell enlargement by affecting the extensibility of the cell wall. Relatively high auxin levels near the young leaves induce numerous vessels to form and these remain small because of their rapid rate of differentiation. Lower auxin concentrations towards the plant base, result in slower differentiation and therefore fewer and larger vessels (Aloni and Zimmermann, 1983; Aloni, 1987a; Aloni, 1987b; Aloni, 1988). The concentration of auxin is positively correlated with vessel density, which decreases from shoot to roots.

The frequency distribution of vessel elements in stem xylem is presented in Table 3. This data reveals that vessel elements occurred in a size range between >100 and <1100 μm^2 (Figure 2). Rough lemon (V2) had the largest vessel elements in stems closely followed by Sweet lime (V4). The smallest vessels were recorded in flying dragon (V7) followed by troyer citrange (V1). Along the plant axis, there is generally a gradual increase in vessel size and a decrease in vessel density from leaves to root (Aloni, 1987a). The frequency distribution of vessel elements in xylem of roots revealed in Table 4, showed that wider vessels were differentiated in xylem of roots as compared to stems. Rough lemon (V2) had a range of vessel elements >200 - <1300 μm^2, whereas flying dragon (V7) had a size range of >100 - <400 μm^2. The slower growth and reduced height of dwarfing rootstocks like flying dragon (*P. trifoliata*) were also attributed to a high bark: wood ratio both in root and stem. Similar observations were recorded in dwarfing apple (Mosse, 1952; Lockard, 1976) and peach rootstocks (Yadava and Doud, 1978).

In rootstocks, less vigorous growth was characterised by smaller vessel elements in the stem and root xylem. It has been suggested that lower levels of auxin/higher cytokinin favour differentiation of phloem (Digby and Wareing, 1966) and higher auxin and lower level of cytokinin favour xylogenesis (Roberts, 1969). The greater transport of auxin in invigorating rootstocks is consistent with the observation that invigorating rootstock stems possess higher ratios of xylem to phloem while less vigorous rootstocks have higher phloem to xylem ratios. Transverse sections of different citrus rootstocks leaves were examined and maximum leaf thickness was recorded in sour orange (V6) closely followed by sweet lime (V4) and rough lemon (V2), whereas, it was lowest in flying dragon (V7) leaves. Varieties with thicker leaves also had more mesophyll. Stomata were not present in the upper epidermis of leaves whilst immediately below the epidermis two or three layers of palisade parenchyma were found. The cells of third layer were smaller than those in the first and second layers. The palisade cells were tightly packed with little air space between adjacent cells.

The depth of palisade tissue varied between different rootstock varieties. The densest parenchyma occurred in leaves of in flying dragon (V7). Leaf characteristics like leaf thickness, mesophyll thickness, depth of palisade parenchyma and density of palisade tissues were significant sources of variation between different rootstock varieties (Table 5). It is generally considered that leaves are the most likely source of the stimulus that promotes development of the vascular tissue and that vascular differentiation is influenced by a hormonal (auxin) signal from the developing leaf (Shininger, 1979; Lyndon, 1990; Sachs, 1991; Nelson and Dengler, 1997). It has been suggested that the amount of transport tissue (vessels) is determined by the quantity of leaf surface that must be supplied (Atkinson and Taylor, 1996). As soon as the

Table 1. The correlation matrix showing the relationship between height of plant and anatomical characteristics of transverse section from roots and stems of different citrus rootstock varieties.

The parameters											
Height of plant	1.000 ***										
No. of VEs in stem	-0.811 ***	1.000 ***									
Size of VEs in stem	0.875 ***	-0.686 ***	1.000 ***								
Total VEs area in stem	0.141 NS	0.381 ***	0.320 **	1.000 ***							
Phloem % in stem	-0.900 ***	0.815 ***	-0.780 ***	-0.080 NS	1.000 ***						
Xylem % in stem	0.900 ***	-0.815 ***	0.780 ***	-0.080 NS	-1.000 ***	1.000 ***					
No. of VEs in root	-0.600 ***	0.651 ***	-0.419 ***	0.023 NS	0.779 ***	-0.779 ***	1.000 ***				
Size of VEs in root	0.800 ***	-0.738 ***	0.733 ***	0.061 NS	-0.762 ***	0.762 ***	-0.571 ***	1.000 ***			
Total area of VEs in root	0.446 ***	-0.336 ***	0.478 ***	0.020 NS	-0.207 **	0.207 **	0.252 ***	0.607 ***	1.000 ***		
Phloem %in root	-0.897 ***	0.810 ***	-0.717 ***	-0.063 NS	0.923 ***	-0.923 **	0.738 ***	-0.757 ***	-0.283 **	1.000 ***	
Xylem % in root	0.897 ***	-0.810 ***	0.717 ***	0.063 NS	-0.923 ***	0.923 **	-0.738 ***	0.757 ***	0.283 **	-1.000 ***	1.000 ***
	1	2	3	4	5	6	7	8	9	10	11

new vascular elements become active, the capa-city of the system in-creases and more substrate is available for plant growth.

The variable height of different rootstocks sug-gests that invigorating rootstocks conduct water at a faster rate than dwarfing ones. The reduced transport of nutrients to dwarfing rootstocks may relate to differences in absorption, transport or a higher utilisation by their roots or a combination of the three. Dwarfing rootstocks possess a high bark: wood ratio as well as highly parenchyma-tous xylem and phloem, and thus they contain more living tissues per unit volume of stem and root than invigorating rootstocks (Beakbane, 1952). These parenchymatous tissues have a sto-rage function and therefore might be expected to have a high respiration rate and heavy demand of nutrients. The metabolic requirements of roots may therefore, vary according to the rootstock. Thus, competition is likely to occur between sites at which carbohydrates are utilised, and the roots may utilise a relatively greater proportion of the total assimilates in dwarfed than in vigorous trees (Beakbane, 1956). Bark to wood ratio is commonly used as a marker of vigour in apple rootstocks. As a quick, conve-nient and reliable assessment of potential vigour, especially when dealing with large root-stock populations, the per-centage bark in roots and stems is most suitable. Once the population has been reduced to a manageable size, assess-ment of other anatomical characteristics of stem and root can be ascertained to provide additional information and

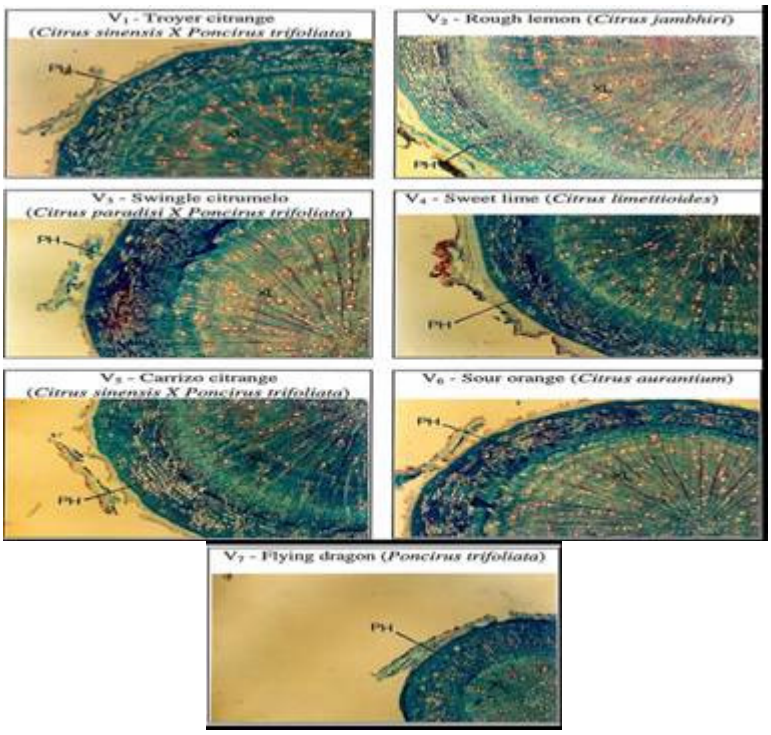

Figure 1. Photomicrographs of cross-sections of root showing the proportion of secondary phloem and xylem of different citrus rootstock varieties (scale = × 20). XL = Xylem, PH = Phloem.

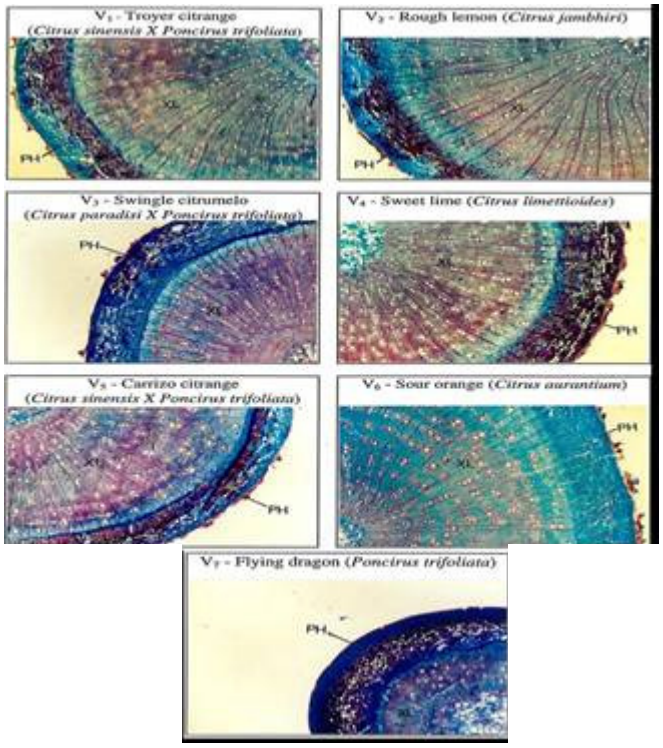

Figure 2. Photomicrographs of cross-sections of stem portions showing the portion of secondary phloem and xylem of different citrus rootstock varieties (scale = × 20). XL = Xylem, PH = Phloem.

Table 2. Height of plant vs. anatomical characteristics (transverse section of stem and root) of different rootstock varieties.

		Stem transverse section					Root transverse section				
Variety	**Height of plant (cm)**	**No. of VEs/mm^2**	**Size of VEs (µm^2)**	**Total area of VEs/mm^2**	**Phloem %**	**Xylem %**	**No. of VEs/mm^2**	**Size of VEs (µm^2)**	**Total area of VEs/ mm^2**	**Phloem %**	**Xylem %**
V1	26.8	86.4	280.5	24271.2	39.4	60.6	65.9	380.5	24559.4	48.7	51.3
V2	43.3	48.7	609.5	29633.2	33.5	66.5	73.6	627.8	46140.4	42.4	57.6
V3	26.6	74.2	294.6	21834.4	42.7	57.3	74.2	474.2	35132.6	48.9	51.1
V4	35.5	94.1	457.4	42843.2	36.0	64.0	57.6	490.2	27925.4	42.7	57.3
V5	28.8	72.5	384.2	27862.2	36.9	63.1	41.5	469.8	19221.8	48.6	51.4
V6	34.2	54.3	457.0	24852.0	36.0	64.0	45.9	484.7	22170.4	44.1	55.9
V7	9.8	140.6	222.4	31250.8	53.5	46.5	112.9	219.1	24410.8	69.3	30.7
$LSD_{5\%}$	3.33	10.78	51.36	56.91.7	2.05	2.05	11.8	94.68	4527.6	2.64	2.64
R- square	0.949	0.928	0.932	0.737	0.957	0.957	0.887	0.754	0.879	0.962	0.962
F value	83.75	68.73	57.25	12.68	92.22	92.22	34.48	14.82	35.60	105.82	105.82
P (6, 24 d.f.)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

VEs = vessel elements, NS = non-significant, ** and *** = significant at 1% and 0.1% respectively.

Table 3. The mean vessel elements size and frequency distribution determined from five basal stem segments of different citrus rootstock varieties.

VEs size range	**V1**		**V2**		**V3**		**V4**		**V5**		**V6**		**V7**	
	No.of VEs.	**SE**	**No. of VEs.**	**SE**	**No. of VEs.**	**SE**	**No. of VEs.**	**SE**	**No. of VEs.**	**SE**	**No. of VEs.**	**SE**	**No. of VEs.**	**SE**
100	4.2	0.6	0.4	0.4	4.6	0.2	0.6	0.4	0.4	0.4	0.0	0.0	21.6	3.4
200	14.8	0.9	1.0	0.8	12.6	1.1	5.4	1.4	6.6	1.0	3.2	0.4	21.6	1.6
300	9.8	1.4	1.4	0.2	5.4	1.2	8.8	1.7	8.6	0.9	3.0	1.1	6.8	2.2
400	2.2	0.7	2.8	0.5	3.0	0.0	6.0	0.7	7.0	1.1	5.8	1.1	0.8	0.6
500	0.2	0.2	5.8	0.9	0.8	0.6	6.0	1.1	2.8	0.5	5.4	1.1		
600			0.8	0.4	0.4	0.2	4.4	0.9	0.6	0.4	1.6	0.4		
700			1.0	0.5			2.0	1.1	0.2	0.2	0.6	0.2		
800			1.6	0.7			0.6	0.2						
900			2.2	0.4			0.2	0.2						
1000			0.6	0.2										

VEs = vessel elements, SE = standard error.

Table 4. The mean vessel elements size and frequency distribution determined from five root segments of different citrus rootstock varieties.

VEs size range	V1		V2		V3		V4		V5		V6		V7	
	No. of VEs.	SE	No. of VEs.	SE	No. of VEs.	SE	No. of VEs.	SE	No. of VEs.	SE	No. of VEs.	SE	No. of VEs.	SE
100	2.8	0.9	0.0	0.0	0.2	0.2	0.2	0.2	1.4	0.5	0.2	0.2	24.0	4.2
200	5.6	1.7	1.8	1.3	4.8	0.7	3.6	2.2	2.2	1.0	1.4	0.6	8.8	0.8
300	6.2	1.0	4.0	0.7	6.6	0.5	3.6	1.5	2.4	0.4	4.8	1.5	8.0	2.2
400	4.4	1.0	3.8	0.2	4.2	0.5	4.4	1.4	2.4	0.8	2.8	0.9		
500	2.8	1.4	4.2	0.9	5.0	0.3	3.4	1.3	2.0	0.9	3.4	0.7		
600	1.4	0.9	4.0	0.7	2.8	0.6	2.4	0.8	3.0	0.5	2.0	1.0		
700	0.6	0.2	1.6	0.2	1.8	0.5	1.8	0.4	1.2	0.4	1.8	0.9		
800			2.4	0.9	1.2	0.4	0.8	0.4	0.4	0.4	0.2	0.2		
900			2.6	0.8	0.2	0.2	0.6	0.4						
1000			1.4	0.5										
1100			0.6	0.2										
1200			0.2	0.2										

VEs = vessel elements, SE = standard error.

Table 5. Anatomical characteristics of leaf (transverse section) of different citrus rootstock varieties.

Variety	Leaf thickness (µ)	Mesophyll thickness (µ)	Depth of palisade parenchyma (µ)	Per unit area of palisade parenchyma (%)
V1	295.8	273.0	96.2	35.2
V2	315.4	278.6	85.6	30.7
V3	269.4	242.2	93.8	38.8
V4	330.8	296.0	67.8	22.8
V5	292.0	257.4	90.0	35.1
V6	335.0	311.4	94.0	30.2
V7	249.4	220.4	90.2	40.9
$LSD_{5\%}$	1.71	1.48	2.10	0.59
R- square	0.998	0.999	0.975	0.995
P (6, 24 d.f.)	<0.0001	<0.0001	<0.0001	<0.0001

further refine rootstock selection. The research suggests that a similar approach can be taken when screening citrus seedlings for rootstocks. Once rootstocks have been grafted with a scion, their characteristics and the relationships between them may change.

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