LEAF ANATOMY, PHOTOSYNTHETIC CHARACTERISTICS, FRUIT QUALITY, AND GENETIC CHANGES IN 'BORNEO PRIMA' MANDARIN (*Citrus reticulata* Blanco) GRAFTED ONTO DIFFERENT INTERSTOCKS IN DRY HIGHLAND CONDITIONS

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Abstract. The purpose of this study was to see how interstocks affected the anatomy, physiology, and genetic characteristics of grafted 'Borneo Prima' (BP) trees. 'Tangelo' (*C. reticulata* x *C. paradisi*) (TGL), 'Waturejo' (*C. sinensis* L.) (SW-W), 'Cina Licin' (*C. reticulata* Blanco) (M-CL), and 'Konde Purworejo' (*C. reticulata* Blanco) (M-KP) with Japanese Citroen/JC (*C. limonin* Osbeck) as their rootstocks have been planted at Tlekung Experimental Garden, Batu, Indonesia (950 asl) since 2002. In 2007, the top grafting technique was used to replace these with 'BP' mandarin. BP/TGL/JC, BP/SW-W/JC, BP/M-CL/JC, and BP/M-KP/JC were the new tree combinations, plus control (BP/JC). The TGL, SW-W, M-CL, and M-KP served as interstocks. The observation variables were monitored from January 2021 to May 2022. Interstocks significantly influenced the thickness of leaf, and palisade; oil gland diameter; stomatal density; peel thickness, and titratable acidity (TA) of grafted BP. 'M-CL' as an interstock had a significant impact on several grafted BP variables. Based on DNA analysis using six ISSR markers, the plant was grouped in two main clusters on the 0.63 coefficient, i.e., control and interstock treatments, respectively. In the second one, it was divided into three subclusters i.e., tangelo, mandarin group, and sweet orange interstocks. The genetic alteration was shown between the control (BP/JC), and BP grafted onto interstocks (BP/interstocks).
Keywords: *chlorophyll, dendrogram, ISSR marker, Japansche citroen (JC), rainfall, species, stomata, top working, varieties*

Introduction

In Indonesia, market demand for qualified citrus fruit has increased significantly; therefore, the existence of commercial citrus cultivars cultivated by farmers should be adjusted to meet consumer needs. Nonetheless, farmers do not want to remove all existing trees in orchards with new superior varieties because it would waste time and money for planting the new varieties from seedlings. Top working technology has been introduced, demonstrated, and trained in farmers' citrus orchards in order to replace existing varieties with consumer-demanded varieties. With this technology producing proven results, top working has been adopted and implemented in many farmers' citrus orchards. Top working is accomplished by grafting bud shoots or scions of newly commercial citrus varieties onto existing interstock varieties. Meanwhile, almost all commercial citrus varieties in Indonesia are budded onto Japansche citroen/JC (*Citrus limonia* Osbeck) and/or Rough lemon (*Citrus jambhiri*) (RL) rootstock, which is widely adapted to tropical conditions and resistant to *Phytophthora sp.* disease (Dwiastuti, 2017). Nonetheless, rootstock, interstock, and grafted plant compatibility becomes important factor for the

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success of citrus variety substitution through top working (Melnyk and Meyerowitz, 2015).

Interstock is commonly used in fruit crops such as apple, pear, plum, mango, and citrus to minimize scion-rootstock incompatibility, regulate growth and fruiting, improve fruit quality, and control some pests and diseases (Das and Dhakar, 2016; Oliveira et al., 2019; Gaona-Ponce, 2021). However, the success of those objectives is determined by the crops, rootstock, and/or interstock used (Sosna and Kortylewska, 2013; Hayat et al., 2021; Biniam et al., 2021). According to Karoly et al. (1997), various interstocks in cherry plants can have both positive and negative effects on crop production. Meanwhile, Susanto et al. (2004) stated that when compared to Flying Dragon, Troyer, and Rangpur Lime, the use of Citrumelo as interstock was the most promoted scion growth of Pummelo. Furthermore, Sugiyatno et al. (2013) stated that the C/N ratio of Mandarin cv. Batu 55 grafted onto pummelo, sweet orange, tangerine, and lime as interstock produced more fruit than the control. Volkameriana as a rootstock and Sour Orange (SO) as an interstock could improve the physical and chemical fruit quality of sweet oranges (El-Sayed, 2017a).

Many studies have also shown that different varieties used as interstock can affect the physiological aspects of lemon trees such as salt tolerance (Gimeno et al., 2012), uptake and leaf mineral content of its scion (Yılmaz et al., 2014, 2015; El-Sayed, 2017; Yahia et al., 2019), and photosynthetic characteristics and gas exchange such as chlorophyll content, net photosynthetic rate, transpiration rate, stomatal density, and stomata conductance (Kamiloglu and Yesiloglu, 2014; Wang et al., 2020). Several phytohormones may play a role in the grafted union (Sharma and Zheng, 2019) and physiological processes (Asahina and Satoh, 2015; Zhai et al., 2021).

Yin et al. (2022) and Hertle et al. (2021) discovered that during genetic changes, proteins and RNA molecules can transport between scion and stock. Many studies have also revealed that different interstock varieties can influence the physiological aspects of lemon trees, such as salt tolerance. This means that the transfer of plastid, mitochondrial, or nuclear genomes during the grafting process would result in new nuclear and organellar genome combinations in grafted plants. Horizontal Gene Transfer (HGT) of chloroplast DNA was detected in grafted tobacco plants via plasmodesmata that connected cells in the junction area (Stegemann et al., 2012; Thyssen et al., 2012).

The 'Borneo Prima' mandarin, which is mostly grown in dry highland areas, is one of the commercially superior varieties, and it has recently gained popularity among consumers. Nonetheless, there is no information on the performance of this variety when grafted onto various interstoks. The hypothesis is that using different interstocks could have a positive or negative impact on grafted plants. The current study will assess the effect of several interstock varieties on the leaf anatomy, photosynthetic traits, and genetic characteristics of 'Borneo Prima' mandarin.

Materials and Methods

Location and plant materials

This research was conducted at Tlekung Experimental Garden (950 m above sea level), Batu, East Java, Indonesia under Indonesian Citrus and Subtropical Fruit Research Institute (ICISFRI) from January 2021 to May 2022. Tangelo (*C. reticulata* x *C. paradisi*), Waturejo (*C. sinensis* L.), Cina Licin (*C. reticulata* Blanco), and Konde Purworejo (*C. reticulata* Blanco) with Japanese Citroen/JC (*Citrus limonia* Osbeck) as

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their rootstock have been planted in the field with a planting distance of 3 x 3 m since 2002. Five years later, in 2007, 'Borneo Prima' mandarin (*Citrus reticulata* Blanco) was top grafted onto these interstock varieties. The citrus species and interstcok combinations are shown in *Table 1*. As a control, we used a five-year-old mother plant of 'Borneo Prima' mandarin grafted onto JC rootstock (no interstock) grown in the nursery house. The control plants were virus-free mother plants that provided budwoods for all interstock treatments.

Table 1. *Citrus species and interstock combination*

No	Kind of interstocks	Scientific name	Accession's interstock-code	Scion+ interstock/rootstock
1	Control *) ('Borneo Prima'Mandarin)	*C. reticulata* Blanco	C	BP/JC
2	Tangelo	*C. reticulata x C. paradisi*	TGL	BP/TGL/JC
3	'Waturejo' Sweet orange	*C. sinensis* L.	SW-W	BP/SW-W/JC
4	'Cina Licin' Mandarin	*C. reticulata* Blanco	M-CL	BP/M-CL/JC
5	'Konde Purworejo'Mandarin	*C. reticulata* Blanco	M-KP	BP/M-KP/JC

*) The control plant was cultivated in the nursery without interstock

The study used three replications, each with two trees per combination treatment per replication. The trees were hand-irrigated and fertilized in accordance with Good Agricultural Practices (GAP). Except for genetic traits, all observation parameters were evaluated without the control tree because the age of the treatment and control trees differed.

Variable measurements

Rainfall

Rainfall data for Batu City, East Java, Indonesia were obtained from BPS Statistic Batu Municipality (2020) and Punten Experimental Garden, under ICISFRI (2022).

Leaf anatomy

Leaves that had been completely opened were used as samples. These leaves were taken from one tree per replication. These samples were fixed in FAA solution (90 mL of 95% ethyl alcohol + 5 mL of formalin + 5 mL of glacial acetic acid) for 48 h. After that, samples were washed into 50% ethyl alcohol, dehydrated and cleared in a tertiary butyl alcohol series before being embedded in paraffin wax at 54-56 °C. Leaf samples were sliced transversely with a rotary microtome to a thickness of 20 µ, then adhered with Haupt's adhesive and stained with the crystal violet-erythrosin combination. The final step was to clear in carbolxylene and mount in balsam. An upright light microscope was used to observe and document the results of the slices. A micrometer eyepiece was used to take measurements, and an average of three readings was calculated. The thickness of the upper and lower epidermis, the thickness of the lamina, the diameter of the oil gland, the thickness of the palisade, and the density of stomata were measured. Except for the control, information on all treatments was gathered. The samples for each replication were obtained from a single plant.

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Photosynthetic traits

Analysis of leaf chlorophyll a, b, and total carotene were done based on Sumanta et al. (2014). The observed leaves came from three trees per replication; they were a composite of four main branches and five fully expanded leaves derived from non-fruiting terminal branches. They were retrieved in December 2021 that due to represent the annual average condition, with average humidity and temperature (2019 and 2020) nearing the annual average value (Batu City Statistics Agency, 2022). Except for the control, information on all treatments was gathered. The samples for each replication were obtained from a single plant.

A fresh plant leaf sample weighing 0.5 g was homogenized in a tissue homogenizer with 10 mL of 95% ethanol. The homogenized sample mixture was centrifuged at 40 ºC for 15 minutes at 10,000 rpm. The supernatant has been separated, and 0.5 mL of it has been combined with 4.5 mL of this solvent. In a spectrophotometer, the solution mixture was tested for the presence of chlorophyll-a, chlorophyll-b, and carotenoids (Parkin). Chlorophyll-a, Chlorophyll-b, and carotenoids were quantified using the following equation:

$$\text{Ch-a}=13.36\text{A664} - 5.19\ \text{A649} \quad \text{(Eq.1)}$$

$$\text{Ch-b}=27.43\text{A649} - 8.12\ \text{A664} \quad \text{(Eq.2)}$$

$$\text{C x+c}=(1000\text{A470} - 2.13\text{Ca-} - 97.63\text{Cb})/209 \quad \text{(Eq.3)}$$

Ch-a = Chlorophyll a, A664 = Absorbance at wavelength 664 nm, A649 = Absorbance at wavelength 649 nm in *Equation 1*. The formula for calculating chlorophyl b was shown in *Equation 2*. Ch-b = Chlorophyll b, A649 = Absorbance at 649 nm, and A664 = Absorbance at 664 nm. *Equation 3* was used to calculate the amount of carotenoid in the sample. C x+c = Carotenoids; A470 = Absorbance at 470 nm (Sumanta et al., 2014).

Stomata density was determined by making an impression or cast of the lower side of the leaf surface with clear nail polish. With sticky tape, the cast was removed and placed on a microscope slide. In a 609 μm^2 area, the stomata were examined under a microscope at 100x magnification.

Trunk cross-section area (TCSA)

Trunk girth was measured with a flexible ruler 15 cm above the upper graft union for both rootstock (JC)-interstock and interstock-scion relationships (BP). It is measured at the end of January 2022. The data were then converted into trunk diameter (Girth/π) and TCSA (calculated as $\text{Girth}^2/4\pi$) (Kumar et al., 2019). Except for the control, data was collected on all treatments.

Total number of generative components

All generative component data are in the plant's principal growth stage; they range from inflorescence emergence to fruit maturity, or stages 5 to 8 (Meier, 2001). Flowers, fruit set, small and large fruits are among the stages. These components were recorded between October 2021 and the end of January 2022. All rootstock-interstock-scion combinations were observed weekly. Data were collected from four main branches as

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samples per plant. In January 2022, the total number of fruits per sample branch was counted.

Fruit quality

In May 2022, ten mature fruits from each tree were collected at random. Variables of physical and chemical nature were measured. A tension gauge was used to measure the tension in the fruit. The pH of the juice was determined with a pH meter with an accuracy of 0.01. Using a portable refractometer and a few drops of juice, the total soluble solids (TSS) content was determined. The juice's titratable acidity (TA) was determined by titrating 5 mL of juice with 0.1 N sodium hydroxide (NaOH) using phenolphthalein. The Titrimetric method was used to determine the vitamin C content of juice (mg/100 mL juice).

Genetic alterations

Twenty young expanding healthy leaf samples (6 - 8 weeks) were collected from all interstock treatments and control plants for DNA extraction. The CTAB method was used to extract DNA from 'Borneo Prima' mandarines (Doyle and Doyle, 1990). *Table 2* shows the results of DNA amplification using 5 ISSR markers. 2 L (50 ng) DNA, 10 L Taq Green PCR 2x Master Mix, 4 L primer, and 4 L water nuclease-free were mixed together. The mixed PCR was spun down at 60 rpm for 15-30 seconds before being placed on a PCR machine for 40 cycles. A PCR (Thermocycler-Biometra) machine was used for amplification.

Table 2. *ISSR primer agents*

No	Code for Primer	5'-3' sequence
1	ISJ 5	$VHV(GT)_7G$
2	ISJ 7	$HVH(TCC)_5$
3	ISJ 10	$(AG)_8YT$
4	ISJ 18	$(AG)_8YC$
5	ISJ 20	$(AG)_8T$
6	ISJ 21	$(GA)_8T$

The ISSR marker was amplified after one cycle of denaturation at 94 ºC for 5 minutes, followed by 28 cycles of denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1.5 minutes, and extension at 72 ºC for 2 minutes. The PCR cycle for this ISSR marker ended with one final extension cycle at 72 ºC for 5 minutes, followed by cooling at 4 ºC. The amplified DNA bands were separated by electrophoresis on 2.5% agarose gel (Vivantis, # PC0701) containing ethidium bromide (10 mg/L) in 0.5 x TBE solution for 60 minutes at 100 volts. Using a biodocumentation system, DNA bands were detected (BioDoc Analyzer-Biometra).

The availability of DNA bands on plants was used to score and dendrogram the plants. SAHN was used to analyze grouping within a dendrogram on NTSys-PC version 2.10.

Experimental design

The rootstock-interstock-scion combinations were assigned at random. Three replications of a randomized completely block design experiment were used, each with two trees per combination treatment.

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Statistical analysis

LSD was used to test the observed differences in values of all components. Duncans' multiple range test determined that differences were statistically significant at the $p < 0.05$ level. Minitab 16 statistical software was used for all statistical analyses. Before analyzing the percentage of the generative phase, the data was transformed into arcsine.

Results

Rainfall

During the year 2021, the main daily temperature ranged between 15 ºC and 31 ºC, with the average being 31 ºC. *Fig. 1* depicts the rainfall pattern in Batu and the surrounding areas in 2020 and 2021. The value for 2021 was higher than the value for 2020. The dry season (rainfall of <100 mm month^{-1}) began in April and lasted until September.

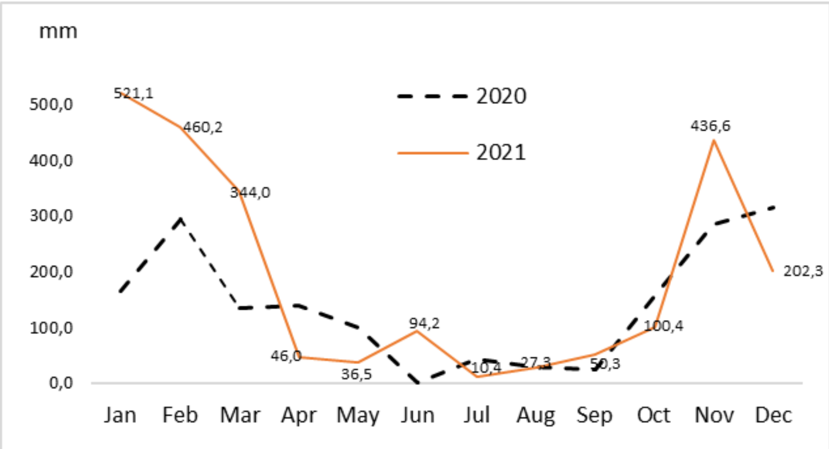

Figure 1. *Diagram average monthly rainfall in Batu, East Java, Indonesia in 2020 and 2021*

Effect of interstock treatments on leaf anatomy

Interstock had a significant effect on the thickness of the leaf and palisade, the diameter of the oil glands, and the stomatal density of grafted 'Borneo Prima' Mandarin. Oil gland diameter (15.15 µm) was found to be greatest in BP grafted onto 'Cina Licin' interstcok (BP/M-CL/JC). The same result was also obtained in terms of palisade thickness (8.18 µm). In fact, BP grafted onto 'Konde Purworejo' (BP/M-KP/JC) as an interstock had the lowest leaf thickness (22.5 µm) than other interstock combinations, whereas; BP grafted onto 'Tangelo' (BP/TGL/JC), 'Waturejo' (BP/SW-W/JC, and 'Cina Licin' (BP/M-CL/JC) gave the same responses.

BP citrus leaves grafted onto 'Tangelo' (BP/TGL/JC) had the lowest stomata density per unit area of 630 µm^2 (21.3), while SW-W, M-CL, and M-KP as interstocks did not differ significantly. Furthermore, the diameter of the oil glands of BP grafted onto 'Waturejo' (BP/SW-W/JC) could not be measured. The results of leaf anatomy variables are shown in *Table 3*, and *Fig. 2* shows the stomata density of grafted 'Borneo Prima' onto Konde Purworejo (BP/M-KP/JC) and Tangelo (BP/TGL/JC).

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Table 3. *Effect of interstocks on some leaf anatomy of 'Borneo Prima' Mandarin*

Kind of interstocks	Upper epidermis (µm)	Lower epidermis (µm)	Diameter of oil gland (µm)	Thickness of leaf (µm)	Thickness of palisade (µm)	Stomata density (/630 µm²)
BP/TGL/JC	1.07±0.03 a	1.03±0.02 a	13.54±0.11 b	27.06±1.26 a	5.20±0.40 c	21.3±0.47 b
BP/SW-W/JC	1.27±0.01 a	1.02±0.04 a	-*)	27.34±0.34 a	6.47±0.10 b	33.3±2.05 a
BP/M-CL/JC	1.30±0.14 a	1.01±0.04 a	15.15±0.09 a	25.12±0.61 a	8.18±0.40 a	31.3±1.25 a
BP/M-KP/JC	1.14±0.03 a	1.04±0.00 a	9.09±0.03 c	22.57±0.34 b	5.54±0.16 c	35.0±2.45 a
R^2 (%)	74.5	33.3	99.9	91.4	96.3	90.9

*) could not be found. According to DMRT α<0.05, numbers with the same letters in one column are not significantly different at 0.05. Data are means ± standard deviations from 9 samples

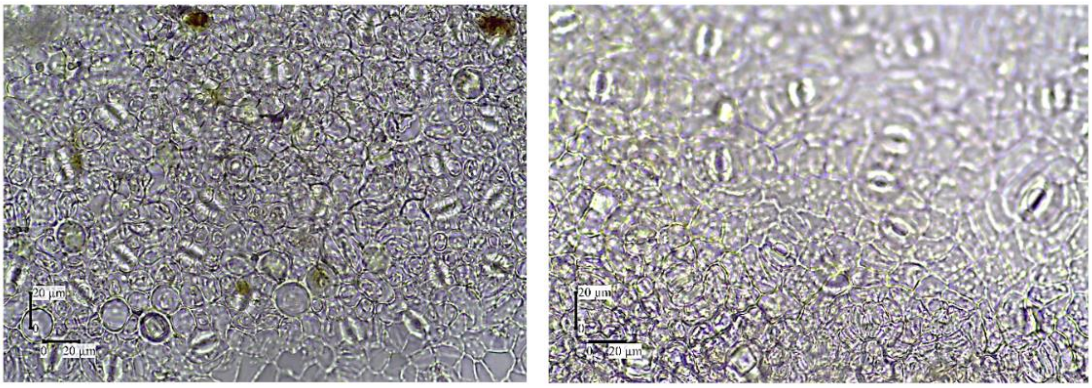

Figure 2. *Performance stomata density of Konde Purworejo (BP/M-KP/JC) and Tangelo (BP/TGL/JC) treatments (scale: 20 µm; magnification: 40x10)*

Effect of interstock treatments on leaf chlorophyll and carotene contents

Leaf chlorophyll and carotene content did not differ significantly between treatments. The average concentrations of chlorophyll a, chlorophyll b, and carotene were 0.18, 0.43, and 0.43 µg ml^{-1}, respectively. The effect of interstocks on chlorophyl a, b, total chlorophyl, and carotene content in grafted 'Borneo Prima' leaves is shown in *Table 4*.

Table 4. *Effect of interstocks on chlorophyll a, b, total chlorophyll and carotene content in leaves of 'Borneo Prima' Mandarin*

Kind of interstocks	Chl a (µg ml^{-1})	Chl b (µg ml^{-1})	T- Chl (µg ml^{-1})	Carotene (µg ml^{-1})
BP/TGL/JC	0.225±0.017 ns	0.528±0.046 ns	0.754±0.063 ns	0.520±0.045 ns
BP/SW-W/JC	0.159±0.027	0.371±0.064	0.530±0.091	0.365±0.062
BP/M-CL/JC	0.152±0.012	0.345±0.027	0.497±0.039	0.309±0.025
BP/M-KP/JC	0.200±0.060	0.475±0.140	0.675±0.201	0.471±0.145
p	0.095	0.077	0.081	0.052

Note that ns beside means indicate that there are not significantly difference between treatments at p<0.05. Data are means ± standard deviations) from three replications

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Effect of interstock treatments on plant trunk cross-sectional area (TCSA) of interstock and scion

The interstock and grafted 'Borneo Prima' stem diameters did not differ significantly (data not shown), nor did the interstock and scion TCSA values. The TCSA of the interstock and scion ranges from 197.1 to 375.7 cm^2 and 113.6 - 202.2 cm^2, respectively, with an average of 280.7 cm^2 and 164.6 cm^2. The minimum and maximum TCSA values in the interstock and scion parts are at 'Borneo Prima' grafted onto 'Cina Licin' (BP/M-CL/JC) and 'Waturejo' (BP/SW-W/JC) interstocks, respectively. *Table 5* shows the result of the effect of interstock on the TCA of 'Boreno Prima'.

Table 5. *Effect of interstocks on TCA value of interstock and scion of Keprok cv. Borneo Prima*

Kind of interstocks	Interstock (cm^2)	Scion (cm^2)
BP/TGL/JC	336.2±55.2 ns	196.4±28.9 ns
BP/SW-W/JC	375.7±87.6	202.2±26.4
BP/M-CL/JC	197.1±36.9	113.6±18.6
BP/M-KP/JC	213.9±29.1	146.0±34.5
p	0.080	0.052

Note that ns beside means indicate that there are not significantly difference between treatments at $p<0.05$. Data are means ± standard deviations from three replications. (±SD) from 3 replications

Effect of interstock treatments on total number of generative components

Fig. 3 depicts the total number of flowers per month from October 2021 to January 2022, as well as the average flower number of grafted 'Borneo Prima' under different rootstocks. From October 2021 to January 2022, the total number of flowers per month varied. The average values did not differ significantly ($p<0.05$) between treatments. In plants with interstock 'Cina Licin' (BP/M-CL/JC) and 'Konde Purworejo' (BP/M-KP/JC), the average number of flowers $month^{-1}$ ranged from 9 to 17, with 'Waturejo' (BP/SW-W/JC) being the lowest.

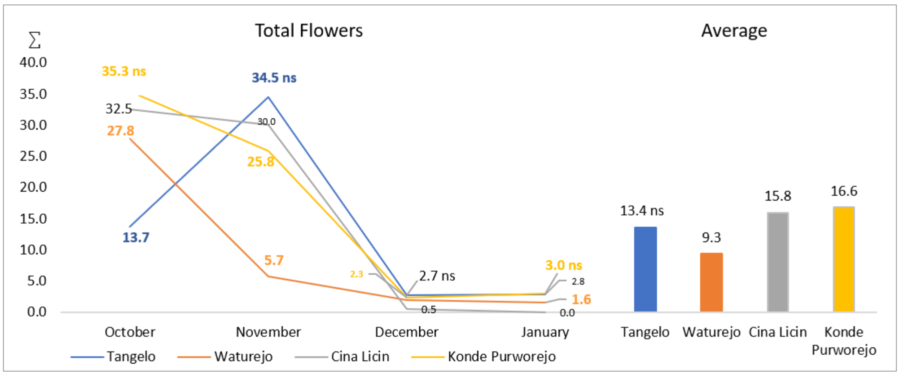

Figure 3. *The average of total flowers $plant^{-1}$ top grafted onto four interstocks from October 2021 to January 2022. The ns beside means denotes that there are not significanty differences between treatments at $p<0.05$*

From October to January 2022, other generative components such as fruit set, small fruits, and large fruits emerged in addition to flowers. These variables did not differ significantly in October and November but did in December and January. Grafting

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'Borneo Prima' onto CL as an interstock (BP/M-CL/JC) produced the highest value, outperforming 'Tangelo' (BP/TGL/JC), 'Waturejo' (BP/SW-W/JC), and 'Konde Purworejo' (BP/M-KP/JC) by 122.4, 39.2, and 14.8%, respectively. *Fig. 4* depicts the total and average of generative components of 'Borneo Prima' grafted onto four interstocks.

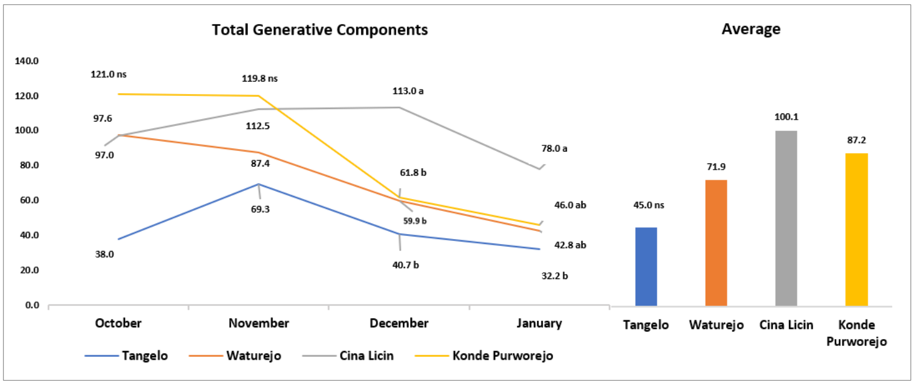

Figure 4. *The average of total generative components of topgrafted plants onto four interstocks (October 2021 to January 2022. The ns beside means denotes that there are not significantly difference between treatments at p<0.05*

From October 2021 to January 2022, the average of dominant phase at generative components varied greatly between treatments. Except for October, the development phase of 'Bornoe Prima' flower and fruit was not significantly different (p<0.05%) across all interstock treatments. However, the generative components that reached the fruit stage earlier were discovered at the BP/SW-W/JC treatment in October 2021. The effect of interstocks on the generative phase percentage was shown in *Table 6*.

Table 6. *The effect of interstocks on the percentage of generative phase (October 2021 to January 2022)*

Date	Treatment/ Phase	BP/M-KP/JC (%)	BP/M-CL/JC (%)	BP/TGL/JC (%)	BP/SW-W/JC (%)
October-2021	Flower	28.8 ± 1.8 a	66.8 ± 27.1 a	39.3 ± 8.9 a	38.4 ± 7.1 a
	Fruit set	71.2 ± 1.8 a	33.2 ± 27.1 ab	38.7 ± 16.3 ab	0.10 ± 0.1 b
	Small Fruit	0.0 ± 0.0 b	0.0 ± 0.0 b	22.0 ± 15.8 b	61.5 ± 7.2 a
November-2021	Flower	30.2 ± 12.2 a	51.2 ± 39.8 a	53.3 ± 4.7 a	17.8 ± 12.9 a
	Fruit set	36.6 ± 7.9 a	2.0 ± 1.1 a	23.3 ± 2.8 a	33.8 ± 6.2 a
	Small Fruit	33.2 ± 9.4 a	27.8 ± 22.7 a	23.4 ± 3.8 a	48.4 ± 6.8 a
December-2021	Flower	3.3 ± 2.4 a	0.5 ± 0.4 a	7.8 ± 2.5 a	13.7 ± 12.7 a
	Fruit set	42.2 ± 19.8 a	49.5 ± 40.4 a	63.6 ± 17.7 a	7.5 ± 5.1 a
	Small Fruit	54.5 ± 19.3 a	50.0 ± 40.8 a	28.6 ± 18.5 a	78.8 ± 14.7 a
January-2022	Flower	4.2 ± 2.9 a	0.0 ± 0.0 a	6.1 ± 8.6 a	2.6 ± 2.0 a
	Fruit set	25.6 ± 16.2 a	42.5 ± 34.7 a	54.5 ± 19.9 a	8.4 ± 6.2 a
	Small Fruit	65.6 ± 14.1 a	55.9 ± 33.4 a	38.6 ± 11.6 a	88.4 ± 4.7 a
	Big Fruit	4.7 ± 3.6 a	1.6 ± 1.3 a	0.8 ± 0.1 a	0.6 ± 0.5 a

Note: the same letters in one row at every month are not significantly difference between treatments at p<0. Data are means (±SD) from 3 replications

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Effect of interstock treatments on fruit quality

Interstock had no effect on the fruit-peel thickness of 'Borneo Prima,' with Konde Purworejo (BP/M-KP/JC) producing the highest value (2.8 mm) and Cina Licin (BP/M-CL/JC) producing the lowest (1.9 mm). The results of this study revealed that grafted BP onto Cina Licin (BP/M-CL/JC) produced the highest TA (13.9%). *Tables 7* and *8* present data on fruit texture and fruit quality.

Table 7. *Effect of interstock on fruit morphology of ‘Borneo Prima’*

Interstock Treatments	Weight (g)	Diameter (mm)	∑ segments	Peel thickness (mm)	Hardness (N)	
					Side	Top
BP/TGL/JC	213.9±37.7 ns	75.9±4.2 ns	10.9±0.9 ns	2.1±0.2 ab	19.1±4.6 ns	22.3±6.5 ns
BP/SW-W/JC	193.6±47.4	74.6±6.2	11.0±0.7	2.7±0.6 ab	11.8±3.2	42.0±16.3
BP/M-CL/JC	138.2±25.5	66.9±3.9	10.3±0.8	1.9±0.3 b	10.8±1.3	133.0±89.5
BP/M-KP/JC	98.1±60.7	66.7±3.8	11.4±1.1	2.8±0.4 a	35.3±23.6	43.9±32.6
p	0.140	0.101	0.162	0.034	0.212	0.146

Note: the same letters or ns in one row are not significantly difference between treatments at p<0.05. Data are means ± standard deviations from 9 samples

Table 8. *Effect of interstock on fruit juice quality of ‘Boreno Prima’*

Interstock Treatments	TSS (ºBrix)	pH	vit C mg 100 g^{-1}	TA (%)
BP/TGL/JC	8.8±0.3 ns	3.9±0.1 ns	70.4±7.2 ns	8.4±0.4 b
BP/SW-W/JC	8.4±0.6	4.2±0.1	73.1±15.4	8.0±0.5 b
BP/M-CL/JC	8.7±0.1	3.5±0.5	73.3±15.9	13.9±4.1 a
BP/M-KP/JC	8.9±0.6	3.8±0.5	58.3±8.0	7.1±1.9 b
p	0.779	0.110	0.398	0.002

Note: the same letters or ns in one row are not significantly difference between treatments at p<0.05. Data are means ± standard deviations from 9 samples

The effect of interstock treatments on the scion's genetic trait profile

The ISSR polymorphism analysis revealed genetic diversity in 'Borneo Prima' grafted onto different interstock and without interstock (control) treatments. It produces 100% polymorphic bands from the total of 6 ISSR markers used. Polymorphism analysis yielded 236 alleles, with a mean of 38.5 alleles per marker and a range of 1-6 alleles per locus. *Table 9* shows a statistical summary of the total 6 ISSR markers tested in 6 types of treatment on 'Borneo Prima' mandarin. *Figure 5* depicts an example of an ISSR marker's DNA band pattern.

Table 9. *Profile of the amplified bands from 5 interstock types and 1 control of Borneo Prima tangerine using six ISSR primers*

ISSR Primers	Band Size (bp)	∑ bands	∑ polymorphic bands	Percentage of polymorphic bands (%)
ISJ 5	200-1000	9	3	33.3
ISJ 7	300-1300	9	9	100.0
ISJ 10	200-1500	12	9	75.0
ISJ 18	250-1600	13	8	61.5
ISJ 20	300-1200	12	7	58.3
ISJ 21	250-1000	10	7	70.0

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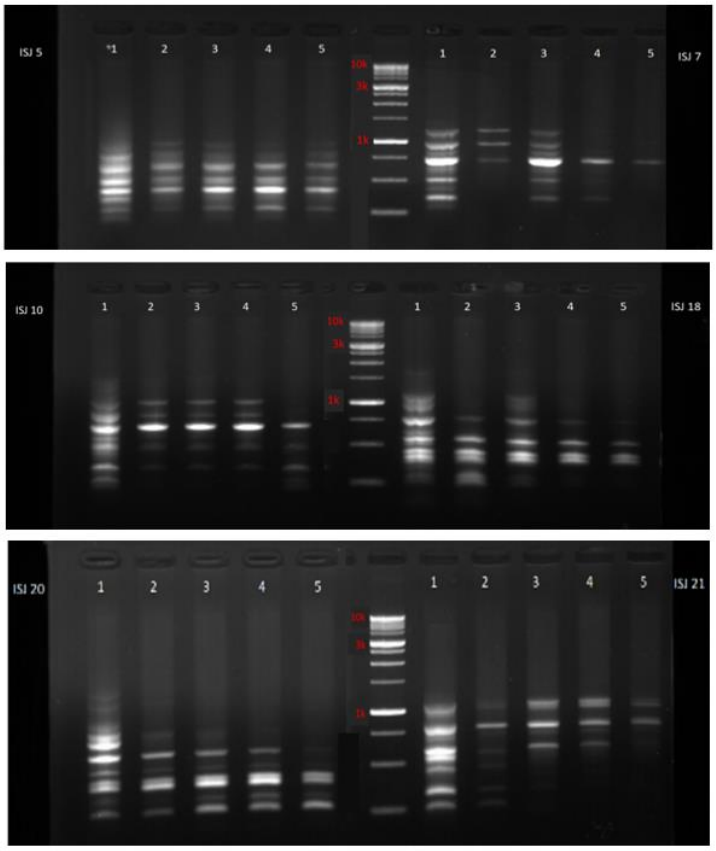

Figure 5. *ISSR polymorphism resulted from ISJ 5, 7, 10, 18, 20 and 21 primers. The column numbers are related to the interstock treatments of Table 1*

Phylogenetic analysis showed that the four interstocks and control treatments on Borneo Prima mandarin separated into two main clusters at a coefficient of 0.63 based on the presence or absence of its interstock. The dendrogram is presented in *Figure 6*.

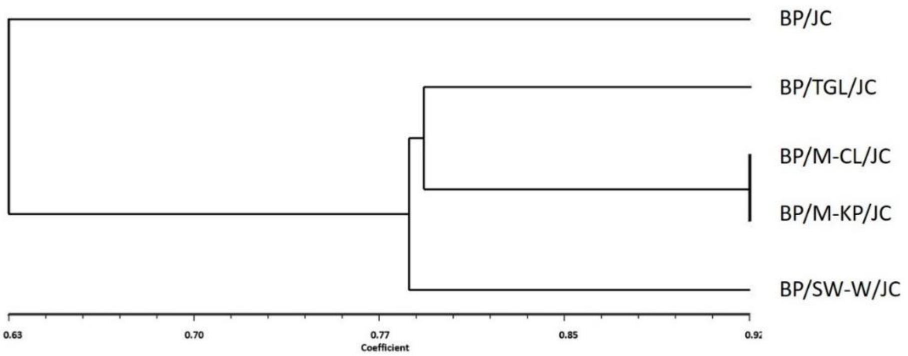

Figure 6. *Dendrogram showed relationships among interstock treatments at mandarin Borneo Prima based on six ISSR markers*

The first cluster consisted only the control treatment, namely mandarin cv. Borneo Prima (BP) without interstock or control (BP/JC), while the second one was the rest of them. The control plants have the highest percentage similarity to plants using 'Waturejo'

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(BP/SW-W/JC) (76.8%) and the lowest percentage similarity to plants using 'Konde Purworejo' (BP/M-KP/JC) (50.9%), respectively.

The plants were divided into three groups for the second cluster, each with a different type of interstock: 'Tangelo' (*C. reticulata* x *C. paradisi*): BP/TGL/JC, mandarin (*C. reticulata* Blanco): BP/M-Cl/JC & BP/M-KP/JC, and sweet orange (*C. sinensis* L.): BP/SW-W/JC. *Table 10* shows the genetic similarity of 'Borneo Prima' in four interstocks.

Table 10. *Genetic similarity matrix of Borneo Prima tangerines in four interstocks and controls*

	Control (BP/JC)	Tangelo (BP/TGL/JC)	Waturejo (BP/SW-W/JC)	Cina Licin (BP/M-CL/JC)	Konde Purworejo (BP/M-KP/JC)
Control (BP/JC)	1.000				
Tangelo (BP/TGL/JC)	0.643	1.000			
Waturejo (BP/SW-W/JC)	0.768	0.778	1.000		
Cina Licin (BP/M-CL/JC)	0.589	0.788	0.868	1.000	
Konde Purworejo (BP/M-KP/JC)	0.509	0.796	0.714	0.921	1.000

Discussion

Batu City, East Java, Indonesia is located in a tropical area, with an average daily temperature of 31 degrees Celsius throughout the year. *Fig. 1* depicts a rainfall pattern in 2020 and 2021, with the 2021 value being slightly higher than the 2020 value. The dry season in those years lasted from April to September. If the dry season rainfall is <100 mm month^{-1}, the plants will flower immediately after receiving rainwater (Srivastava et al., 2000). The plants that flowered in October-November would be harvested around June to July of the following year. Plants that flowered between October and November would be harvested between June and July of the following year. Flowering in potted lime plants was caused by continuous water stress for 4 to 5 weeks, according to Southwick and Davenport (1986). The dry-stress condition activated the signaling cascade, increasing the expression of the FLOWERING LOCUS T (*CiFT*) gene (Bennici et al., 2021).

The effect of interstock treatments on 'Borneo Prima' leaf anatomy, pigment constituents, and TCSA

The BP/M-CL/JC combination had the highest values of upper epidermis thickness and pallisade. Different interstock treatments also had a significant impact on the BP leaf stomata density (*Table 3, Fig. 2*). This anatomical difference in mandarin leaves was caused by the interstock used, though the difference is only slightly different in the upper epidermis. Despite the fact that the difference is only slight in the upper epidermis, the difference is presumably in the cuticle layer, which can remove excess sunlight (Panawala, 2017).

In terms of stomata density, the findings of this study were consistent with the findings of Wang et al. (2020) on 'Yuanxiaochun', a new hybrid citrus in China. The stomata

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density of the plants with the five interstocks used in this citrus varied. Differences in stomata density in plants are permitted to be caused by genetic and physiological influences. Differences in the use of their rootstocks, for example, in Pistachio (*Pistacia vera*) and Ambrosia Apple (*Malus domestica* var. Ambrosia) plants, could cause the physiological process that causes these changes (Çağlar, 1999; Xu and Danielle, 2021). Aside from rootstock, the plant environment has a significant impact on this value. Different stomata density values are provided by *Aleurites montana* plants grown in different locations (Hong et al., 2018). Stronger wind speeds encourage leaf growth with a greater number of stomata per area in grapes than plants protected by natural windbreaks (Gokbayrak et al., 2008).

The chlorophyl of 'Borneo Prima' leaf did not significantly different among interstocks used. Previous research found that the chlorophyl content of citrus leaves could be influenced by the rootstocks used (El-sayed, 2017a; Kumar et al., 2018). It appears that these effects were caused by efficiently absorbed nutrients such as Fe, which acts as an important cofactor of several enzymes and is involved in the chlorophyll biosynthetic pathway (Kumar et al., 2018). El-sayed (2017a) demonstrated that using Volkamer Lemon as a rootstock increased chlorophyll a, b, and total content of four types of sweet orange when compared to Sour Orange, and vice versa when Sour Orange was grafted onto Volkamer Lemon as an interstock and rootstock, respectively. According to Wang et al. (2020), the time or period for collecting the leaf samples also has a significant impact on the content of leaf-citrus chlorophyll.

Plant size reflects plant growth performance. The trunk cross-sectional area (TCSA) value is determined by the diameter of the tree. In this study, the TCSA of interstock and scion were not significantly different between treatments (*Table 5*). However, a positive and linear relationship has been discovered between the interstock diameter and its scion diameter, as well as between TCSA-interstock and TCSA-scion (*Fig. 7*). It was concluded that rootstock diameter growth could influence interstock diameter growth.

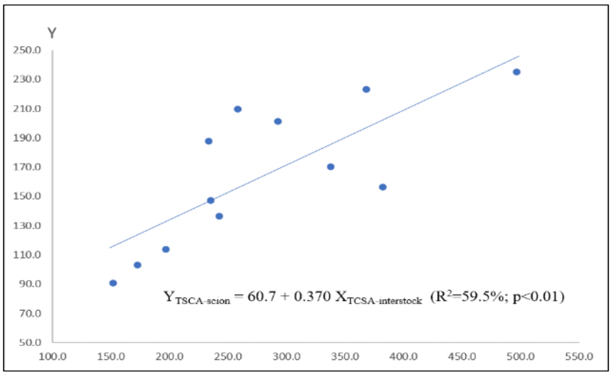

Figure 7. *The relationship between TCSA-interstock and TCSA-scion values*

The diameter and weight of 'Borneo prima' mandarin fruit were also influenced by the size of the interstock diameter (*Fig. 8*). TCSA's beneficial effect on grafted plant growth and fruit production may be related to nutrient transport from the root to the grafted plant. Dalal and Brar (2012) found that TCSA had a significant effect on canopy volume, leaf area, yield, production efficiency, and TSS. The same results were observed in guava, apricot, and banana (Kumar et al., 2008, 2014; Kumar and Pandey, 2010).

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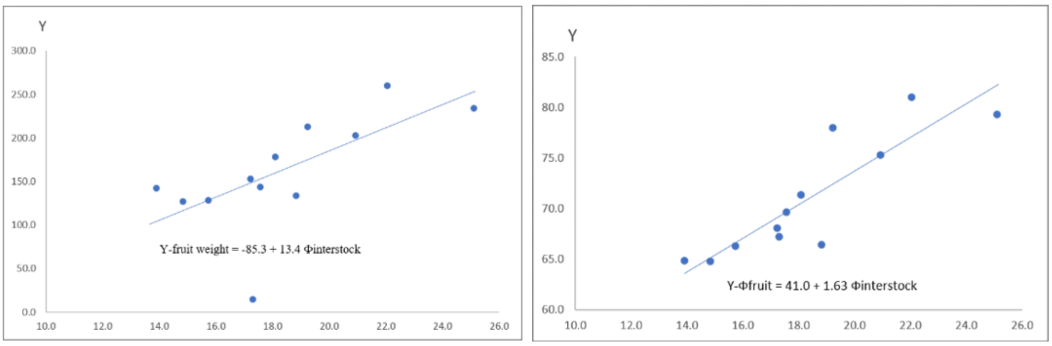

(a) *(b)*

Figure 8. *The relationship between diameter interstock values and fruit weight (a) and diameter interstock values and fruit diameter (b)*

Interstock treatments have an effect on the generative components and fruit quality of 'Borneo Prima'

Citrus trees in tropical climates, such as Indonesia, typically bloom at the start of the rainy season after the dry season. According to Chica et al. (2013), Blooming was also triggered by water stress, low temperature, and combinations of the two. When sweet orange was exposed to water stress, the accumulation of flower-promoting gene *CsFT* transcripts increased; however, this condition reduced the transcripts of others, including *CsSL1*, *CsAP1*, and *CsLFY*. The upregulation of *CsFT* caused plants to flower.

The use of different rootstocks may also influence flower gene expression (Bennici et al., 2021). Through complex mechanisms, the expression levels of the floral promoter *CiFT2* in the leaves of the sweet orange 'Tarocco Scirè' were affected not only by temperature but also by rootstock type. Plants grafted onto the 'C35 citrange' rootstock induced higher levels of expression for this gene than plants grafted onto the 'Swingle citrumelo' rootstock, and this condition was related to the plant's flowers and yield. In this study, grafted 'Borneo Prima' mandarin onto Waturejo (BP/SW-W/JC) produced flowers earlier than other interstocks and the control (BP/JC, without interstock), indicating that this plant could reach the fruit stage earlier than others (*Table 6*).

The interstock treatments did not produce any significant differences in fruit juice quality. Physically, those interstocks only affected fruit-peel thickness (*Tables 7* and *8*). Many studies on the effect of rootstock and interstock on fruit quality have been conducted, with varying results. The combination of *C. macrophylla* and *C. aurantium* rootstocks grafted with three Spanish lemon varieties had the varying physicochemical and compositional characteristics. The rootstocks were affected significantly on the morphological and fruit qualitis characterization in term of TSS, TTA, and maturity index of those plants (Aguilar-Hernández et al., 2021). Besides the rootstcoks, the interstocks treatment also gave different response on the grafted plant. According El-sayed (2017b), all four sweet oranges grafted on Sour Orange/SO rootstock and an interstock on volkamer lemon rootstock (SO/VL) were not significantly different on their physical fruit characters (fruit length, diameter, shape, volume, juice volume, rind thickness and segments number). According to El-sayed (2017b), the physical fruit characteristics of all four sweet oranges grafted on Sour Orange/SO rootstock and an interstock on volkamer lemon rootstock (SO/VL) were not significantly different (fruit length, diameter, shape,

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volume, juice volume, rind thickness and segments number). This interstock improved most fruit quality properties, such as higher TSS and lower acidity, over VL rootstock.

In other plants such as mango and plum, the effect of interstock on TA was also reported by Casierra-Posada and Guzmán (2009) and Oliveira et al. (2019). Interstock influenced fruit firmness, pH, diameter, and TTA in mango, but fresh weight, fruit length, TSS, and TSS/TAA ratio remained unaffected. Interstock treatments, on the other hand, had no effect on plum fruit firmness, TSS content, or vit C. The contribution of interstock to fruit quality and performance appears to be limited, and these variables may be influenced by external factors such as harvesting time, fruit position, or climatic conditions.

The effect of interstock treatments on the genetic trait profile of 'Borneo Prima'

The use of three citrus varieties (sweet orange, tangelo, and mandarin) as interstock for the 'Borneo Prima' mandarin resulted in genetic differences in its plants. According to the dendogram, the genetic character of five treatments on Borneo Prima mandarin separated into two main clusters with a coefficient of 0.63 (*Fig. 7)*. It clearly shows the control and interstock treatments separately. Interstock appears to influence the genetic characteristics of their scions. This phenomenon could be caused by genetic material being transferred from the rootstock/interstock to the scion, or vice versa. According to Thyssen et al. (2012) and Stegemann et al. (2012), grafting between two Nicotiana species causes cell-to-cell migration of DNA-containing organelles such as plastid and chloroplast genomes in these plants. The movement process starts with the appearance of dedifferentiate plastids and the formation of new intercellular connections, which allow the plastid to move into neighboring cells (Hertle et al., 2021).

Grafting between *C. clementina* and *C. trifoliata* rootstock caused the flow of over 4000 genes transporting mRNAs across its graft junctions in citrus plants. These genes are involved in biological processes such as metabolism. Furthermore, there are 3326 differentially expressed genes (DEGs) on the scions and rootstocks after grafting (Liu et al., 2018). In another study, using *Poncirus trifoliata* as a citrus rootstock caused DNA demethylation and a decrease in 24-nt small RNAs (sRNAs) in sweet orange scions (Huang et al., 2021).

Conclusion

The thickness of leaf, and palisade; oil gland diameter; stomatal density; as well as fruit quality (peel thickness, and TA) of grafted 'Borneo Prima' mandarin were all influenced by the interstocks used. The mandarin group as interstocks had a significant impact on several variables of grafted mandarin Borneo Prima.

The genetic responses of grafted 'Borneo Prima' under different interstoks were classified into two clusters based on ISSR markers, with a coefficient of 0.63. The first cluster was occupied solely by the control (BP/JC) without interstock, and the second cluster was occupied by interstocks consisting of three species (Tangelo, Mandarin, and Sweet orange). The highest level of similarity (92.1%) was found when 'Borneo Prima' was grafted onto both 'CL' (BP/M-CL/JC) and 'KP' (BP/M-KP/JC). Meanwhile, the genetic similarities of grafted 'Borneo Prima' between the control (BP/JC) and interstocks used were 76.8 (BP/SW-W/JC) and 50.9% (BP/M-KP/JC), respectively.

Based on primers used, genetic alteration was occurred between BP/rootstock and BP/interstock/rootstock.

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