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Interstock-Induced Anatomical and Metabolomic Shifts Enhance Growth and Stress Adaptation in Citrus Cultivars

(Perubahan Anatomi dan Metabolomik yang Dipicu oleh Interstok Meningkatkan Tumbesaran dan Penyesuaian terhadap Stres dalam Kultivar Sitrus)

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ABSTRACT

Citrus growers are challenged to be able to produce high yields while being resilient to environmental stress, while maintaining high yield and cultivar potential. However, the use of pummelo as an interstock-despite its growing relevance for cultivar replacement-remains poorly studied in terms of its anatomical and metabolic effects on scion varieties. This study evaluates the impact of Pummelo interstock on the growth, leaf anatomy, and metabolomic profile of three citrus cultivars: Sweet Orange (Manis Pacitan), Tangerine (Siam Pontianak), and Mandarin (Keprok Batu 55). Morphological assessment showed that interstock application increased leaf thickness by 3.9% and palisade layer thickness by 15.2%, with additional adaptations in epidermal and glandular structures suggesting enhanced stress tolerance. Stomatal density rose by 22% in certain combinations. GC-MS metabolomic profiling identified 91 metabolites, with interstock treatments increasing the accumulation of stress-related compounds such as flavonoids and terpenes. Stress-metabolite upregulation reached 59.6% in Keprok Batu 55, 36.2% in Manis Pacitan, and 47.8% in Siam Pontianak. Key pathway modifications involved the stimulation of fatty acid metabolism and the development of glycosylphosphatidylinositol (GPI) anchors in interstocked plants, which led to better morphological and physiological features. These findings highlight the role of interstock technology in promoting vegetative vigor, metabolic reprogramming, and stress adaptation in citrus, with practical implications for improving productivity and sustainability in citrus cultivation. Further research should explore long-term performance and underlying genetic mechanisms.

Keywords: Citrus; interstock; morpho-anatomical traits; pummelo; secondary metabolite

ABSTRAK

Penanam sitrus dicabar untuk menghasilkan hasil yang tinggi dan berdaya tahan terhadap tekanan alam sekitar, sambil mengekalkan potensi hasil dan kultivar yang tinggi. Walau bagaimanapun, penggunaan pummelo sebagai interstok-walaupun semakin relevan untuk penggantian kultivar-masih kurang dikaji dari segi kesan anatomi dan metaboliknya pada varieti scion. Kajian ini menilai kesan interstok pumello terhadap pertumbuhan, anatomi daun dan profil metabolomik tiga kultivar sitrus: Jeruk Manis (Manis Pacitan), Jeruk Keprok (Siam Pontianak) dan Jeruk Mandarin (Keprok Batu 55). Penilaian morfologi menunjukkan bahawa penggunaan interstok meningkatkan ketebalan daun sebanyak 3.9% dan lapisan palisade sebanyak 15.2%, disertai adaptasi pada epidermis dan kelenjar yang mencadangkan peningkatan toleransi tekanan. Ketumpatan stomata meningkat sebanyak 22% dalam beberapa gabungan. Pemprofilan GC-MS mengenal pasti 91 metabolit dengan interstok meningkatkan pengumpulan sebatian berkaitan tekanan seperti flavonoid dan terpena. Peningkatan metabolit tekanan dicatatkan sebanyak 59.6% di Keprok Batu 55, 36.2% di Manis Pacitan dan 47.8% di Siam Pontianak. Perubahan laluan utama termasuk pengaktifan metabolisme asid lemak dan biosintesis penambat glikosilfosfatidilinositol (GPI) dalam tumbuhan saling berstok, sepadan dengan anatomi dan fisiologi yang lebih baik. Hasil ini menyerlahkan potensi teknologi interstok dalam meningkatkan kekuatan vegetatif, pengaturcaraan semula metabolik dan penyesuaian tekanan dalam tanaman sitrus. Penyelidikan lanjut perlu dijalankan untuk menilai prestasi jangka panjang dan mekanisme genetik yang terlibat.

Kata kunci: Citrus; interstok; ciri morfo-anatomi; metabolit sekunder; pummelo

INTRODUCTION

Citrus is among the most economically important fruit crops in tropical and subtropical regions. For the whole of 2021-2022, world citrus production reached 158.5 million metric tons. China alone accounted for 28% of the world production, and Asia represents a little over half of this (World Citrus Organization 2022). In 2013-2023, the Indonesian citrus products industry grew from 1.65 million metric tons to 2.80 million metric tons at a compound annual growth rate of approximately 2.2% (ReportLinker 2025). Despite this growth, Indonesia remains a major importer, spending $234 million on citrus in 2023 - ranking second in Southeast Asia - while exports stayed modest at only $743,000 (Trend Economy 2024).

The decreasing market price of pummelos has been a key factor driving adjustments to the domestic orchard structure, thereby stimulating growers to plant more economically attractive cultivars, such as mandarins and sweet oranges. However, orchard transitions are expensive and require intensive labor. In such a setting, interstock technology offers an economically viable strategy by enabling the grafting of new cultivars onto established rootstocks, thereby preserving root system integrity while avoiding the need for tree removal (Dutt, Mahmoud & Grosser 2023). Interstocks facilitate cultivar replacement without the need to remove mature trees, thereby improving long-term orchard flexibility and profitability.

This method is commonly used in apple, peach, and grapevine production to control canopy size, maximize nutrient uptake, and improve tolerance to salt and other environmental conditions (Dwiastuti et al. 2023; Mahmoud et al. 2021; Rong et al. 2023). In citrus, interstocks are especially useful in non-dwarfing cultivars such as sweet orange, in which they provide management of vegetative vigor and promote higher-density orchard systems (Dutt, Mahmoud & Grosser 2023; Fayek, Ali & Rashedy 2022; Fayek, Rashedy & Ali 2022).

The selection of interstock is crucial for controlling growth and promoting beneficial physiological responses, such as improvements in nutrient uptake and hydraulic conductance, as well as growth (Shen et al. 2019; Vives-Peris et al. 2023). The specific combination of the scion, interstock, and rootstock influences the overall strength and stress resistance of the plant (Bennici et al. 2021; Widyaningsih et al. 2017). Recent studies indicate that interstocks can influence photosynthetic efficiency, leaf morphological plasticity, and metabolic allocation (Wang et al. 2020). Nevertheless, the anatomical and biochemical processes that regulate this response are not well characterized, particularly in tropical citrus systems.

Among different candidates, Pummelo (*Citrus maxima*) is an ideal interstock material, offering advantages such as fast growth, widespread scion compatibility, and a large xylem vessel area to transport water and nutrients effectively. Field studies have demonstrated that pummelo interstocks enhance tree growth rates and canopy volume compared to controls, and may also improve tolerance to Huanglongbing (HLB), particularly in susceptible scions (Dutt, Mahmoud & Grosser 2023). Additionally, it can also activate mechanisms that lengthen fatty acids, which may help grafted plants grow and improve their metabolism (Alves, Setzer & da Silva 2019). However, it may not work the same way with grafting from one cultivar to another; therefore, it needs to be thoroughly examined. Moreover, the impact of pummelo as an interstock on the morphology, architecture, and secondary metabolites of citrus leaves remains inadequately studied, particularly within the Southeast Asian citrus system.

Interstocks can influence not only structural development but also biochemical signaling across graft interfaces. Hormonal transport, nutrient flow, and secondary metabolite translocation - including pathways such as GPI-anchor biosynthesis (a lipid-mediated pathway involved in protein localization and stress signaling) - are all modulated at the scion-interstock-rootstock interface (He et al. 2024). Grafting success depends upon the precise vascular reconnection, callus formation, and cambial and pith alignment (Rasool et al. 2020). While the processes of grafting are anatomical and biochemical complex, most of the current investigations have focused more on the morphological aspects, ignoring the in-depth anatomical and metabolomic influences - especially in tropical production settings.

It has been reported that rootstocks and interstocks can modify leaf thickness, mesophyll architecture, photosynthesis, and antioxidant activity in response to stress (Balfagón et al. 2022; Killiny et al. 2018; Kumar et al. 2019; Li et al. 2020; Toscano-Morales et al. 2016; Yulianti & Agisimanto 2023). The extent to which these anatomical features are involved in the synthesis of stress-associated metabolites, such as flavonoids, terpenoids, and lipidic signaling molecules, is unknown.

To bridge the deficiencies of anatomical and metabolomic aspects, the present study aims to elucidate the influence of pummelo interstock on three economically essential citrus cultivars: Sweet Orange 'Manis Pacitan' (MP), Tangerine 'Siam Pontianak' (SP), and Mandarin 'Keprok Batu 55' (KB55). MP had a high juice yield but moderate drought susceptibility; SP is a commercial favorite with consistent productivity across multiple seasons; and KB55 is a vigorous, highland-adapted Mandarin with early flowering and considerable anatomical flexibility (Devy et al. 2023; Widyaningsih et al. 2017). Nonetheless, despite this complexity, only a few numerical morphological studies have been published, which also encompass anatomy and metabolomics to provide an additional profile for tropical growth environments.

We hypothesize that pummelo interstock acts as a signaling hub that modulates growth, anatomical adaptation, and metabolite composition in a cultivar-specific manner. We tested this through integrated anatomical and GC-MS-based metabolomic analyses comparing interstock versus control grafting. The objective of this integrated approach is to identify the systemic roles of interstocks and to provide directions for managing the sustainable intensification of citrus.

MATERIALS AND METHODS

PLANT MATERIALS AND EXPERIMENTAL DESIGN

The experiment began in January 2020 and continued through December 2024. Pummelo (*Citrus maxima* (Burm. F.) Merr.) cv. Nambangan was used as an interstock on Japanese citron (JC) (*C.* × *limonia* Osbeck) rootstock to bud three scion citrus cultivars: Mandarin cv. Keprok Batu 55 (*C. reticulata* Blanco) (KB 55), Sweet Orange cv. Manis Pacitan (*C. xsinensis* (L.) Osbeck) (MP), and Tangerine cv. Siam Pontianak (*C. nobilis* Lour.) (SP). The corresponding non-interstocked grafts were considered as controls.

JC rootstock seeds were sown in sterile media in January 2020. Seedlings of JC rootstock, four months old, were used for initial budding. Pummelo (*Citrus maxima*) cv. Nambangan was budded onto JC at 20 cm above the soil base using a T-budding technique. After 3-4 weeks, successful unions were confirmed by the bud's green coloration, and the new-shoot rootstocks were pruned 2-3 cm above the graft union to stimulate interstock growth. One year later, budwood from KB 55, MP, and SP were chip budded onto the pummelo interstock 10 cm above the union. Control groups were created by directly budding the scions onto JC rootstocks.

Budding was performed using the T-budding technique and wrapped similarly, with stem diameters of 0.5-0.6 cm, to ensure optimal vascular alignment and promote successful healing. They were about one year old or had a height of approximately 90-100 cm. Each treatment consisted of 12 plants (four replicates × three plants per replicate).

GROWTH CONDITIONS AND PLANT MAINTENANCE

The three-year-old plants were grown in 30 L polybags under full sunlight (8 h/day) at the Punten II Experimental Garden in Batu, East Java (950 m above sea level, with an average annual temperature of 22.4 °C, a humidity of 86.8%, and a rainfall of 1891.8 mm). Plant maintenance was carried out in accordance with standard management practices. The polybags were placed in an open area, with manual watering applied weekly and inorganic fertilizer administered monthly. The soil media was composed of a 2:1:1 ratio of topsoil, sand, and compost. Fertilization was performed using either liquid urea (0.5-1 g/L) or NPK (2-3 g/L), supplemented with biweekly foliar fertilization. Irrigation (1-2 L/polybag) was alternated with fertilizer delivery. Standard orchard pest and disease management was followed.

MORPHOLOGICAL MEASUREMENTS

In this research, plant morphology refers to growth parameters, including plant height, stem diameter, reproductive output, leaf size, and structural characteristics. a) Plant Height (cm) was measured from the base to the highest growing point, b) Rootstock Diameter (cm) was measured 5 cm above the base of the rootstock, c) Interstock and Scion Diameter (cm) were measured 5 cm below and above their joint area, d) The number of flowers and fruits was counted three years after budding, with fruits classified into three categories of their diameter: small (<3 cm), medium (3-5 cm), and large (>5 cm). These categories were developed as a standardized classification system specific to this study to facilitate uniform comparison across treatments, e) Leaf area was estimated as: Length × Width × Correction Factor, using cultivar-specific constants (Susilo 2015), and f) Leaf Texture Measurement (N) was using the penetrometer (Johannes et al. 2022).

ANATOMICAL OBSERVATIONS

Leaf anatomy observation: Fully expanded leaves were fixed in 70% ethanol, dehydrated with ethanol: xylene (1:1), and embedded in paraffin. Microtome cross-sections were mounted on slides and stained for microscopic observation.

GC-MS METABOLOMIC PROFILING

Fully expanded leaves from the 3rd to 5th nodes, located at the apex of citrus plants, were collected, washed, oven-dried at 50 °C for 48 h, and pulverized into a fine powder. The powder (1 g) was suspended in absolute Ethanol (1 mL) in a microtube. Samples were vortexed until they became uniform, and incubated overnight at room temperature. After 24 h, they were centrifuged (9500 rounds per minute) for 3 min to transfer the supernatants into GC-MS vials. GC-MS (Trace™ 1310 ISQ™) system, fitted with an HP-5MS UI column (30 m × 0.25 mm I.D., 0.25 µm film thickness) was employed for analysis. The carrier gas used was Helium (UHP) at a flow rate of 1.00 mL/min. The injector was used in split mode (split ratio, 50:1) at 230 °C. The transfer line temperature was 250 °C, and the ion source temperature was 200 °C for the mass spectrometer; the scanning mass range was 40–500 amu. Data were processed using MS-DIAL and annotated against NIST and GNPS libraries (Abadie, Lalande & Tcherkez 2022).

EXPERIMENTAL DESIGN

A split-plot design was used with four replicates. The main plot factor was rootstock type (with and without pummelo interstock), and the subplot factor was citrus cultivar (KB55, SP, and MP). The model used was:

$$Y_{ijk} = \mu + Rk + Ai + \delta + Bj + ABij + \varepsilon ijk.$$

where μ is the overall mean; *Rk* is the effect of replicate; *Ai* is the main plot effect (interstock); *Bj* is the subplot effect (cultivar); *ABij* is the interaction effect; and *εijk* is the error term.

STATISTICAL ANALYSIS

Data on plant growth and anatomical leaf traits were analyzed using one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$) for post-hoc comparisons. Prior to analysis, normality and homogeneity of variance were tested using the Shapiro–Wilk and Levene's tests, respectively. Fruit count data were square root $\sqrt{x}$-transformed to satisfy ANOVA assumptions. However, the data on flower count were not normally distributed, even after transformation. They were therefore subjected to the non-parametric Kruskal-Wallis test, followed by post hoc comparison with Dunn's. Minitab 16 was used for classical ANOVA, while MetaboAnalyst v6.0 was used for multivariate and pathway analyses.

RESULTS AND DISCUSSION

This study showed that Pummelo interstock influenced vegetative growth, leaf anatomy, stomatal traits, and metabolite expression in three citrus cultivars, namely Keprok Batu 55 (KB55), Manis Pacitan (MP), and Siam Pontianak (SP). These changes indicate cultivar-specific physiological and biochemical reprogramming, as described earlier, that graft compatibility affects morphological and metabolic outcomes (Rong et al. 2023; Wang et al. 2020).

PLANT GROWTH PARAMETERS

Vegetative Growth Parameters

This study showed notable variations in plant growth, including leaf area, scion diameter, and leaf hardness, among different interstock/cultivar combinations. Significant differences were observed in leaf area, stem diameter, and leaf hardness among the scion–interstock combinations (Table 1). Both scion cultivar and the interaction between scion cultivar and interstock treatment significantly influenced leaf area. The largest leaf area was recorded in the Manis Pacitan cultivar (38.47 cm^2) and the Interstock-MP treatment (44.5 cm^2), which were significantly larger than all other treatments ($p < 0.001$). Meanwhile, Control–KB 55 had the smallest (19.3 cm^2). The Scion diameter did not exhibit a similar trend, with Control–SP producing the thickest stems (16.0 mm) and Interstock-SP producing the thinnest (8.47 mm). The leaf hardness was significantly influenced by the interaction between scion cultivar and interstock treatment, with the Interstock-MP treatment producing the highest leaf hardness (4.6 N), a proxy for mechanical resistance (i.e., leaf toughness) (Table 1, Figure 1).

The raised leaf area observed in the interstock treatment in this study supports previous findings, indicating that interstocks affect shoot morphology not by directly promoting leaf elongation, but through modified assimilation distribution and hormonal transfer (David et al. 2023; Wang et al. 2020). These increases in leaf area may indicate an increase in cell expansion and division, which may be affected through auxin gradient signals regulated by the pummelo interstock. Larger leaf size is correlated with the rise of photosynthetic area and developmental rate (Torabi, Majd & Enteshari 2015; Zheng & Van Labeke 2017), which also explains the vigorous growth in interstock-treated plants.

Similarly, increased leaf hardness in Interstock–MP may result from both anatomical thickening and mesophyll fortification, providing mechanical resistance and perhaps enhancing drought tolerance. This result is consistent with Rong et al. (2023), who correlated enhanced leaf mechanical strength with the transcriptional induction of genes related to lignin biosynthesis and cuticle formation. Furthermore, leaf toughness has been linked to nutrient acquisition and to structural responses to fertilization and stress (Khalil 2023). Interestingly, the leaf toughness of Control–MP and Interstock–SP was lower, indicating that anatomical strengthening caused by the pummelo interstock did not apply to all plants. These differences may result from hormone crosstalk and varying scion–interstock compatibility, as hypothesized in prior studies (Cookson et al. 2014; Goldschmidt 2014).

Consistently, plant height was increased by 24.2% in interstock treatments, with Interstock–KB55 plants being the highest at 179.7 cm. This increase suggests improved vascular connectivity, which would facilitate the transport of growth hormones, such as gibberellins and cytokinin, essential regulators of stem elongation and apical dominance, through the graft union (de Lima Costa et al. 2021; Noreen et al. 2021). Improved vascular function is also crucial for nitrate and potassium transport, and for driving protein synthesis and optimal stomatal behavior. However, as the soil pH and temperature were not manipulated in the present study, additional experiments under controlled conditions are required to confirm these trends.

Flowering and Reproductive Output

Since the flower count data were not normally distributed (Shapiro-Wilk $p < 0.05$), we used a non-parametric Kruskal-Wallis test. There were no significant differences in the total number of flowers among the six treatment combinations ($H = 2.49$, $p = 0.778$), as well as between cultivar and interstock treatments. However, Control-KB 55 produced the highest number of flowers (12.3 per plant). At the same time, Interstock-KB 55 yielded significantly fewer (5.3 per plant), suggesting that the pummelo interstock may suppress flowering, possibly due to disrupted hormone or nutrient distribution. Specifically, it may be the essential growth hormones, including auxins and gibberellins, which govern vascular development and floral induction. Furthermore, restricted translocation of

TABLE 1. Effect of interstock and cultivar combination on plant height, leaf area, rootstock diameter, scion diameter, total fruits/plant, and leaf hardness

Treatments	Plant Height (cm)	Leaf Area (cm^2)	Rootstock Diameter (mm)	Scion Diameter (mm)	∑ fruits/ plants	Leaf Hardness (N)
Interstock						
Control	121.6b	25.83a	17.61b	14.94a	15.56a	2.6a
Interstock	151.0a*	29.71a	21.40a	12.89a	8.67a	2.7a
Scion Cultivar						
Keprok Batu55	149.0a	20.01b	20.47ab	12.88a	16.17a	2.4a
Manis Pacitan	131.5a	38.47a**	21.38a	16.62a	3.50b	2.9a
Siam Pontianak	128.3a	24.82ab	16.67b	12.25a	16.67a	2.7a
Interaction						
Control-KB55	118.3a	19.32b	18.03a	10.03b	23.67a	2.5bc
Control-MP	124.3a	32.44ab	18.77a	18.77b	2.33a	1.9c
Control-SP	122.0a	25.72ab	16.03a	16.03ab	20.67a	3.4ab
Interstock-KB55	179.7a	20.70ab	22.90a	15.73ab	8.67a	2.2bc
Interstock-MP	138.7a	44.50a	24.00a	14.47ab	4.67a	3.9a
Interstock-SP	134.7a	23.92ab	17.30a	8.47b	12.67a	2.1c
R^2 (%)	56.8%	64.5%	66.2%	76.0%	54.7%	84.5%

Different letters in the row indicate significant differences according to Tukey's Range Test ($p \leq 0.05$). *, **Significant at the 0.05 and 0.01 probability level, respectively; ^{ns}: nonsignificant.

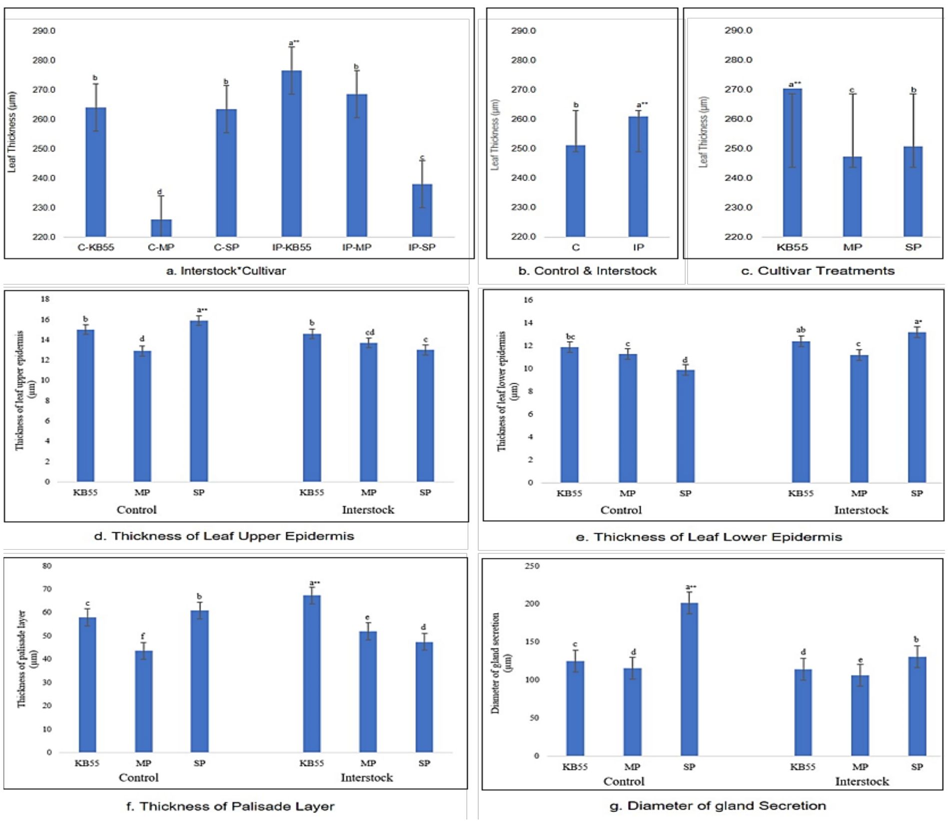


FIGURE 1. The average of leaf thickness in interstock combination (a), control and interstock (b), cultivars treatments (c); the thickness of the upper epidermis (d), the lower epidermis (e), palisade layer (f); and the diameter of gland secretion (g). Error bars represent the standard error, and different letters indicate statistically significant differences ($p < 0.05$). Note: C= Control; IP= Interstock

macronutrients such as nitrogen and phosphorus - crucial for meristematic activity and floral organogenesis - across the graft union may also lead to diminished flowering, as demonstrated by structural plasticity and nutrient-driven leaf development in the referenced studies (de Lima Costa et al. 2021; de Lima Vieira et al. 2014). Although signaling by gibberellin and cytokinin could increase the vegetative characteristics such as the plant height in Interstock–KB55, these same hormonal dynamics - or their imbalance - may suppress floral induction, highlighting a potential trade-off between vegetative vigor and reproductive capacity.

The MP and SP cultivars had lower flowers under the control conditions (0.0 and 1.7, respectively). However, flowering was induced in both cultivars by the interstock (2.0 and 2.7 flowers, respectively). These findings suggest that KB 55 is intrinsically very floriferous. This is likely mediated by hormonal modulation involving gibberellins and abscisic acid, which regulate floral signaling genes such as *CiFT* (Bennici et al. 2021). These findings demonstrate a cultivar-specific response to interstock application: flowering was inhibited in the naturally floriferous KB 55, but it was stimulated in MP and SP, which did not flower under control circumstances. This contrasting pattern highlights the complexity of the interstock–scion relationship. It suggests that reproductive control may not be universally beneficial or detrimental, but rather that it is to some extent context-dependent on the scion genotype and signaling compatibility.

Fruit Set and Productivity Trends

Fruit production among treatments was not significant after transforming the data by taking the square root ($\sqrt{x}$) to meet ANOVA assumptions ($F_{(5, 12)} = 2.83$, $p = 0.065$). Although not statistically significant, a biological trend was evident: Control–KB 55 and Control–SP showed higher fruit numbers than interstock-treated combinations. However, although interstocks enhanced vegetative growth, total fruiting was low because the plants were still juvenile. Keprok Batu 55 was the cultivar with the highest average number of fruits (16.7 per plant), followed by Siam Pontianak (16.2 fruits per plant), which emphasizes the relevance of its selection for early productivity (Marín, Rincón Barón & Montoya-Lerma 2020). This study indicates that the use of interstocks may retard fruit set at early developmental stages, possibly by internal resource redistribution towards vegetative tissues. This trade-off is a well-known phenomenon in grafted citrus, with shoot vigor and early yield being negatively related by competition for assimilates (Goldschmidt 2014; Marín, Rincón Barón & Montoya-Lerma 2020). Collectively, these results illustrate the complex, cultivar-specific effects of pummelo interstock on citrus vegetative and reproductive growth.

ANATOMICAL CHARACTERIZATION OF CITRUS LEAVES IN RESPONSE TO INTERSTOCK TREATMENTS

Total Thickness Trends

There was a significant influence of the interaction between citrus cultivars and interstock treatments on total leaf thickness ($p < 0.01$). The thickest leaves belonged to Interstock–KB55 (IP-KB55, 276.7 µm), and the thinnest were those of Control–MP (C-MP, 226.1 µm) (Figure 1(a)). Irrespective of the scion, plants grafted with pummelo interstock generally had higher total leaf thickness relative to their controls (Figure 1(b)), and KB55 (270.4 µm) always had the best anatomical development (Figure 1(c)). In contrast to the other cultivars, interstock application in *Siam Pontianak* resulted in a significant reduction in leaf thickness—from 263.5 µm in the control to 238.1 µm - indicating possible incompatibility or attenuated signal transduction between scion and interstock. These findings are consistent with the results of other studies that have indicated that interstocks may regulate vegetative growth through hormone signaling, particularly auxin and gibberellin, as well as improved physiological integration (Liao et al. 2022; Mahmoud et al. 2021). Similar outcomes were reported by Devy et al. (2023) in *Citrus reticulata* cv. Borneo Prima.

Epidermis and Palisade Responses

The analysis of tissue layers showed cultivar-related adaptations. The upper epidermis was thickest in Control–SP and thinnest in Control–MP. Interstock–SP also had significantly thinner lower epidermis, and Interstock–KB55 had a thicker palisade layer compared with the other treatments. These phenotypes are indicative of a hormonal gradient - especially auxin and gibberellin transport - across the graft junction, which mediates the growth of epidermal and palisade tissues (Mahmoud et al. 2021). Auxins regulate polarity and elongation of cells, whereas gibberellins promote the creation of chloroplasts, and intercalary growth facilitates the development of palisade cells, increasing photosynthetic capacity. Meanwhile, the increased palisade-to-spongy mesophyll ratio in Interstock–KB55 confirms enhanced CO_2 flow and chloroplast density (Thiesen et al. 2022; Yao et al. 2023), and a thicker upper epidermis may control water retention and gas exchange in plants (Cahyanti et al. 2024).

Secretory Glands and Cultivar-Specific Differences

The diameter of the secretory glands was largest in Control–SP, while in MP and KB55, differences among treatments were minor (Figure 1(d)-1(g)). This indicates that secretory gland characteristics are rather genotype-dependent than interstock-regulated. This supports the idea that both secretion and essential oil biosynthesis are under strong genetic regulation (Alves, Setzer & da Silva 2019; Raveau, Fontaine & Lounès-Hadj Sahraoui 2020). Although the pummelo interstock promoted the mesophyll and epidermis traits, its addition could not lead to remarkable changes in the glandular traits, supporting the effect of the anatomical baseline observed in another cultivar. The prominent secretory structures in SP may contribute to its better defense and stress tolerance.

Stomatal Density

Stomatal density differed significantly among treatments, with the highest density in Interstock–MP and Control–SP and the lowest in Interstock–SP and Interstock–KB55 (Figure 2(B(a-d)). Higher stomatal density in MP is apparently the result of a notably larger leaf area and higher gas-exchange capacity. On the contrary, the low density in Interstock–KB55 and Interstock–SP can be attributed to the feedback inhibition by gibberellins, as they inhibit stomatal initiation and differentiation (Oliveira et al. 2019). Since stomatal density influences gas exchange and photosynthesis efficiency (Zhang et al. 2023b), the high density in Manis Pacitan suggests enhanced photosynthetic capacity. These changes illustrate that interstock treatments can alter stomatal structure according to the hormonal responsiveness and developmental plan of the scion.

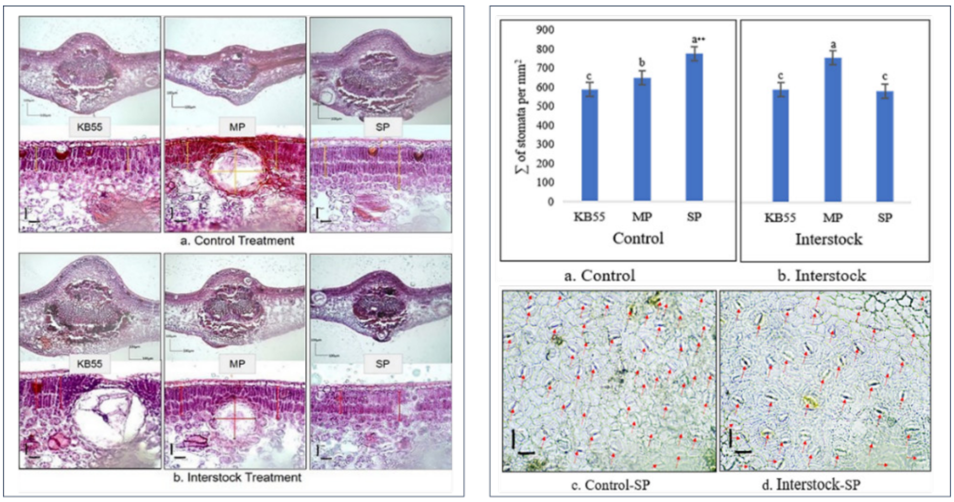
Metabolite–Anatomy Integration and Pathway Comparison

The anatomical improvements in Interstock–MP and Interstock–KB55 were supported by the metabolomic evidence that is depicted in Figure 4(b), comparing with Control in Figure 4(a). It can be seen from the release of information for pathways relative abundances, trajectory analysis and bubble size (Figure 4), specifically, that Pathway #2 (Monoterpenoid biosynthesis) and #3 (Cutin, suberin, and wax biosynthesis) were more robustly activated in Figure 4(b) (i.e., its bubble size and −log(p) value were higher) relative to the counterpart Pathway #4 and #5 in Figure 4(a), indicating that monoterpene production and cuticle-related lipid metabolism primarily were enhanced under interstock treatments, MP and KB55 in particular.

These pathways are involved in epidermal thickening, cuticle formation, and stress tolerance. They are consistent with the observed anatomical characteristics, such as a thicker upper epidermis, palisade tissue, and denser mesophyll, in Interstock–KB55 and Interstock–MP plants. The increase in these biosynthetic pathways further supports the speculation that structural shape development is metabolically synthesized through the action of hormones.

Monoterpenes and wax components are naturally synthesized according to the signals of Jasmonic Acid (JA) and Abscisic Acid (ABA), which are involved in stress and development responses (Khalil 2023; Rong et al. 2023). Their enrichment in metabolically active tissues also implies that hormonal crosstalk is involved in the anatomical as well as biochemical compensation to interstock signaling.

Mechanistically, the pummelo interstocks could work as a supply or carrier of graft-transmissible cues - such as auxin, gibberellins, small RNAs, and secondary metabolites - that modulate the establishment of gene expression networks related to cell wall biosynthesis, vascular development, and cuticle strengthening (Cookson et al. 2014; Goldschmidt 2014; Mahmoud et al. 2021). This



A B

FIGURE 2. A. The cross-section and anatomical variation of the upper epidermis, palisade layer, and gland secretory of the citrus leaf of Control-KB 55, Control-MP, and Control-S of Control Treatment (a), and Interstock-KB 55, Interstock-MP, and Interstock-SP of Interstock Treatment (b). B. Total stomata per mm^2 across the control (a), interstock (b), and the stomata distribution per field of view in Control-SP (c), and Interstock-SP (d).

coordinated response indicates that there is interdependence between the anatomical and metabolic characteristics of citrus grafting systems.

Developmental Plasticity and G × T Interactions

The anatomical differences in citrus scions in response to the pummelo interstock were generated by cultivar-specific regulatory networks, which may result from hormonal flux and metabolic switching. Although interstocks consistently improved the KB55 and MP epidermis and mesophylls, they had the opposite effect in SP, possibly indicating incompatibility or reduced signal transduction. The present results highlight genotype-by-treatment (G × T) interactions between genotype responses in interstock anatomy and the compatibility of each cultivar with cues produced in the interstock. Integration of these anatomic findings with the metabolomic pathway activation, for example, of monoterpenoid and cuticle biosynthesis, solidifies interstocks as major modulators of vegetative resilience in a genotype-specific manner.

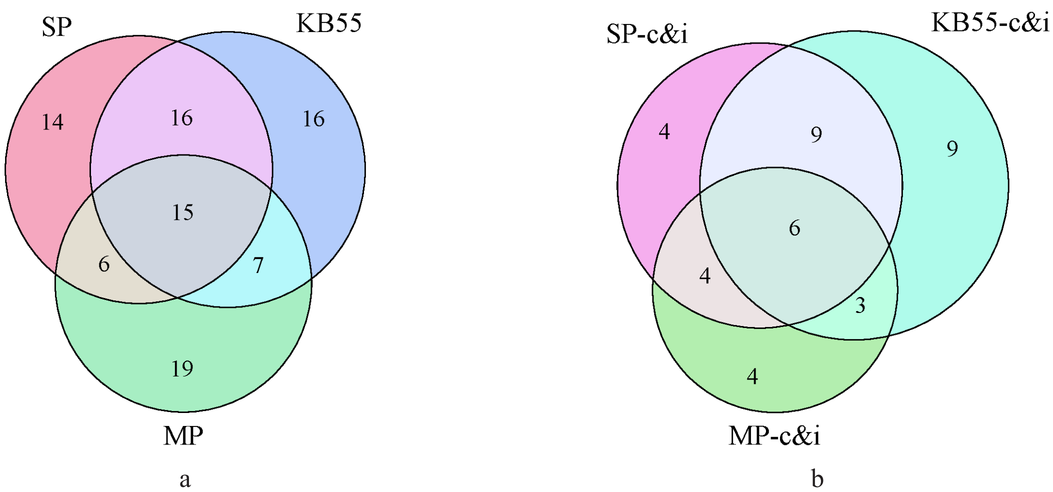
METABOLOMIC SHIFTS IN CITRUS LEAF DUE TO INTERSTOCK BUDDING

Untargeted metabolomic profiling indicated that the pummelo interstock treatments dramatically reprogrammed the leaf metabolome across citrus cultivars. In total, 93 different metabolites were identified (Figure 3(a)), with interstock-treatments containing the most significant number of metabolites: 54 for KB55, 47 for MP, and 51 for SP. Each cultivar had a set of shared compounds between control and interstock - 27 in KB55, 17 in MP, and 23 in SP - but only six compounds were shared in all treatments (Figure 3(b) & Table 2). These products included linalool, ethyl iso-allocholate, caryophyllene, methyl glycocholate, and (22S)-6α,11β,21-trihydroxy-pregnadiene derivatives that serve basal roles in growth regulation, detoxification, and membrane stabilization (Dah-Nouvlessounon et al. 2023; González-Mas et al. 2019; Gutiérrez et al. 2022; Zhang et al. 2023a).

Interstock treatment increased growth-and stress-related compounds. The caryophyllene, quassin, and Linalool were up-regulated in KB55 (contributing to disease resistance, antioxidant, and floral signal) (Rong et al. 2023; Shen et al. 2019). Interstock–MP had higher contents of β-ylangene and D-limonene serving as herbivory defense (Anandakumar, Kamaraj & Vanitha 2021; Ho et al. 2021), whereas the contents of elemene, phytol, and 10,13-octadecadiynoic acid - an important reactive oxygen species (ROS) modulator (Luo et al. 2024; Taj et al. 2021) - were higher in Interstock-SP.

By contrast, defense- and reproduction-associated plant secondary metabolites, including isocaryophyllene, lupeol, 3,9-epoxypregnane, demecolcine, and (R)-lavandulyl acetate (De Carvalho & Caramujo 2018; Ho et al. 2021), were found to be accumulated in the control plants. These signatures indicate a possible reliance of plants on early reproductive signaling and antioxidant buffering signs in the absence of interstocks, which potentially compensates for a lower anatomical robustness (Carrera et al. 2021; Deng et al. 2019; Rong et al. 2023).

Pathway enrichment analysis of *Arabidopsis* mapping found that ten metabolic pathways were up-regulated under the interstock condition. In Interstock–KB55 and Interstock–MP (Figure 4(b)), three major lipid-associated pathways were enriched significantly: fatty acid elongation

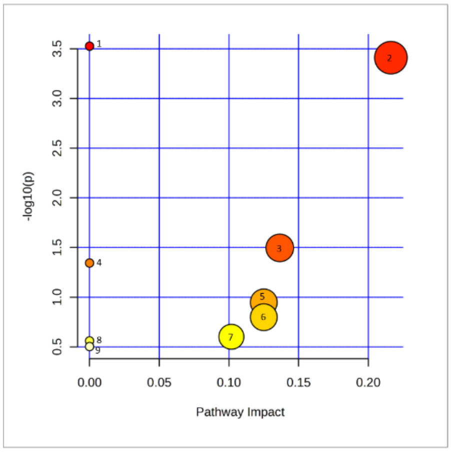


MP: all kinds of compounds found in Manis Pacitan, SP: all kinds of compounds found in Siam Pontianak, KB55: all kinds of compounds found in Keprok Batu 55. MP-c&i: only the same compounds found at the Control and Interstock treatment of Manis Pacitan; SP-c&i: only the same compounds found at the Control and Interstock treatment of Siam Pontianak; KB55-c&i: only the same compounds found at the Control and Interstock treatment of Keprok Batu 55

FIGURE 3. Venn diagram of all kinds of compounds in every cultivar treatment (a) and the total of the same compounds in every cultivar (b)

TABLE 2. The total of identified metabolite compounds and their prominent functional roles

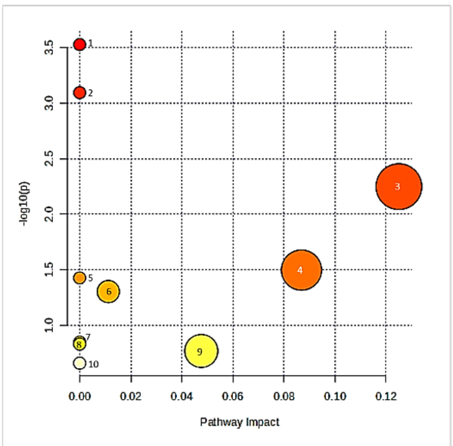
Treatments		∑ Identified compounds	∑ Share compounds	Key Metabolites	Main Functional Roles
KB 55	Control	36	27	Nobiletin, Ponkanetin, β-Cyclogermacrane, Linalool	Antioxidant, floral aroma
	Interstock	45		Nobiletin, Ponkanetin, β-Elemene, α-Sabinene	Plant defense, antifungal
MP	Control	34	17	Squalene, Caryophyllene, α-Tocopheryl Acetate	Antioxidant, stress response
	Interstock	31		β-Elemene, Linalool, D-Limonene	Plant volatile, herbivore deterrent
SP	Control	30	23	β-Pinene, Ponkanetin, Nobiletin	Essential oil, pollinator attraction
	Interstock	44		Caryophyllene, β-Germacrene, α-Sabinene	Stress adaptation, growth regulation



No	Pathway Name	Match Status
1	Biosynthesis of unsaturated fatty acids	3/22
2	Sesquiterpenoid and triterpenoid biosynthesis	3/24
3	Steroid biosynthesis	2/44
4	Monoterpenoid biosynthesis	1/7
5	Cutin, suberine, and wax biosynthesis	1/18
6	Alpha-Linolenic acid metabolism	1/26
7	Carotenoid biosynthesis	1/43
8	Ubiquinone and other terpenoid-quinone biosynthesis	1/48
9	Fatty acid biosynthesis	1/56

The Pathways of Control Treatment

a



No	Pathway Name	Match Status
1	Biosynthesis of unsaturated fatty acids	3/22
2	Monoterpenoid biosynthesis	3/24
3	Cutin, suberine, and wax biosynthesis	1/5
4	Steroid biosynthesis	2/44
5	Ubiquinone and other terpenoid-quinone biosynthesis	1/7
6	Fatty acid biosynthesis	1/18
7	Fatty acid elongation	1/26
8	Sesquiterpenoid and triterpenoid biosynthesis	1/43
9	Glycosylphosphatidylinositol (GPI0-anchor	1/48
10	Fatty acid degradation	1/56

The Pathways of Interstock Treatment

b

FIGURE 4. The names of the pathways of Control (a) and Interstock (b) treatments based on their leaf metabolomes

(map00062), fatty acid degradation (map00071), and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. These pathways are essential for membrane remodeling, jasmonate biosynthesis, and signal transduction, ultimately increasing cellular plasticity, energy homeostasis, and stress resistance (Li et al. 2024; Negi et al. 2018). Their absence in controls also corresponds to anatomically observed decreases in mesophyll development and epidermis thickness.

Furthermore, moderate levels of induction of the biosynthesis of monoterpenoids as well as cutin, suberine, and wax biosynthetic pathways (Figure 4(b)), associated with cuticle integrity, control of transpiration, and defense against the environment, were found in Interstock–KB55 and Interstock–MP. These improvements are not reflected in the controls, where the metabolism of α-linolenic acid, carotenoid biosynthesis, and reproductive-related pathways (e.g., map00906, map00905) were higher (Figure 4(a)), particularly in Control–SP and Control–KB55. These shifts suggest a reallocation of metabolic investment in controls toward reproduction and cuticle repair, as opposed to vegetative growth.

In general, interstock-promoted metabolic changes favor variations in some growth-promoting flavonoids, terpenoids, and lipid-derived hormones, such as jasmonates and gibberellins. These changes were coupled with anatomical improvements, in particular those seen in KB55 and MP, and further support the conclusion that pummelo interstock serves as an active regulator of plant plasticity. The cultivar-dependent enrichment patterns under the diverse treatments provide strong observational evidence indicating the existence of genotype × treatment (G × T) interactions determining metabolic strategies in the grafting of citrus systems.

CONCLUSIONS

This study indicates that *Citrus maxima* (pummelo) interstock is an active modulator of vegetative development of various citrus cultivars. Significant improvements in anatomical traits were observed, primarily in the Interstock–KB55 and Interstock–MP combinations, which exhibited higher plant (23.5%) and leaf area (15.6%), as well as thicker palisade tissue (18.2%). These architectural characteristics were related to enhanced photosynthetic structure (higher palisade-to-spongy mesophyll ratios and thicker epidermal layers).

Metabolomic profiling showed enriched levels of terpenoids, flavonoids, and lipid derivatives - substances associated with stress resistance, cell integrity, and hormone response. Pathway analyses showed that the fatty acid degradation, elongation, and GPI-anchor biosynthesis were the major pathways occurring in the Interstock–KB55 and Interstock–MP pathways, which may be related to the membrane alteration, energy optimization, and jasmonate or gibberellin signaling. These results lend support for the concept of interstocks as biochemical signaling centers linking anatomical development and metabolic reprogramming. In contrast, control plants displayed a higher induction of the α-linolenic acid and carotenoid biosynthesis pathways, which are frequently associated with oxidative stress and the very early events in reproductive signaling, respectively; however, lower vegetative vigor and thinner mesophyll layers, especially under the Control–SP treatment.

Interestingly, despite promoting vegetative growth, the interstock treatment - particularly in KB55 - was associated with reduced flowering, suggesting a potential trade-off between structural vigor and reproductive output. This decline in floral emergence may be driven by hormonal imbalances or nutrient allocation shifts, especially involving auxins, gibberellins, and macronutrient transport across the graft union. Such findings emphasize the need to consider both growth and reproductive traits when selecting interstocks for specific production goals.

These results have direct consequences for the citrus growers and breeders who wish to maximize scion performance under drought-prone and/or suboptimal conditions. Pummelo as an interstock provides a scalable, cultivar-specific means to enhance vegetative vigor, strength, and metabolic efficiency - in highland citrus production systems, especially. To apply these findings in practice, future research needs to verify the detected anatomical and metabolomic improvements in the field condition, and investigate the long-term reproductive status and molecular mechanism of interstock–scion interactions from the perspective of transcriptomic, proteomic, and hormonal analyses. They will enable precision interstock selection based on scion traits, agroecological zones, and production goals.

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