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Research Article

High-quality off-season mulberry fruit (*Morus laevigata* Wall.) induced by summer pruning

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Abstract

The growing recognition of mulberries as a potent source of bioactive and nutritional compounds, coupled with their increasing global consumption, underscores the need for efficient off-season cultivation. This study explores the influence of both in-season and off-season cultivation on the yield, bioactive components, and antioxidant activity of 'Taiwan Changguosang' (*Morus laevigata* Wall.) in a greenhouse setting. Despite the lower fruit yield during the off-season, the off-season fruit exhibits higher levels of bioactive compounds, including total anthocyanins, polyphenols, and flavonoids compared to its in-season counterpart. Additionally, the off-season fruit demonstrates enhanced DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and FRAP (ferric-reducing antioxidant power), attributed to climatic conditions during fruit development, particularly air temperature and solar radiation. Moreover, the off-season fruit proves to be more palatable, showcasing a favorable balance between sugar and acid. Principal Component Analysis (PCA) and correlation analysis revealed a close association between antioxidant activity and the chemical contents of total polyphenols and flavonoids. This study underscores the feasibility and benefits of off-season cultivation for enhancing the nutritional profile and antioxidant potential of mulberries, providing valuable insights for optimizing cultivation practices.

Keywords: antioxidant capacity; bioactive components; mulberry fruit; off-season

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Introduction

Mulberry fruits, distinguished by their diverse colors and morphologies, are a rich source of health-promoting bioactive compounds and can be consumed either fresh or in various processed forms. Prior investigations have demonstrated the protective association between bioactive compounds found in mulberry fruit and a range of chronic diseases, including the reduction of specific cancers and cardiovascular ailments (Gungor and Sengul, 2008; Zhang *et al*., 2018). This evidence has sparked an interest among planters, consumers, and breeders across diverse countries, underscoring the growing recognition of mulberries as a valuable contributor to overall health and wellness.

Owing to minor variations in phenology among different cultivars, mulberry fruits are available for only a brief period, typically from April to May in Wuhan, China (Mo *et al*., 2022a). This limited availability poses a significant obstacle to the widespread commercial cultivation of mulberries. The current harvest duration falls short of meeting the escalating demand for mulberry fruits. Additionally, the fruits' perishable nature and the challenges associated with storage and transportation further hinder the extension of the market supply period.

To address these constraints, greenhouse cultivation technology has been employed to create an optimally controlled growth environment for mulberry plants. Notably, Kim *et al*. (2012) observed a substantial advancement in mulberry fruit maturation within greenhouse conditions, occurring approximately 18−19 days earlier than outdoor cultivation. Th is technique proves effective in overcoming bud dormancy and is widely adopted for early maturing fruit culture, particularly in early spring. However, despite these advancements, there remains a persistent shortage of fresh mulberry fruit in the off-season, highlighting the continued challenge of ensuring a year-round supply for consumer tables.

The discovery of plant growth regulators capable of inducing flowering and fruiting in perennially subtropical fruit trees has opened the door to off-season fruit production (Manochai *et al*., 2005; Liu *et al*., 2022b). This breakthrough has allowed for the year-round cultivation of fruits, diversifying market offerings, and contributing to economically sustainable development. The application of off-season production techniques has seen success in various subtropical fruit trees, such as Satsuma oranges in Japan and fresh longan in Thailand, facilitating continuous production (Subhadrabandhu and Yapwattanaphun, 2001; Poerwanto *et al*., 2008). However, despite these advancements, off-season production for mulberry fruit remains in its early stages and confronts challenges associated with diminished fruit quality and low induction efficiency. Liu *et al*. (2022b) observed that the yield and quality of off-season mulberry fruit induced by paclobutrazol and monocyandiamide in autumn were inferior to in-season counterparts. This disparity was linked to the impact of short-day length and photosynthesis. It is important to note that the application of paclobutrazol raises concerns about abnormal plant growth due to its prolonged residual presence in the soil (Poerwanto *et al*., 2008), and monocyandiamide, while effective, poses toxicity risks to humans and is listed as a potential human carcinogen by the US Environmental Protection Organization (EPO) (information sourced from the Pesticide Properties Database, available online: *http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/184.htm*). Therefore, there is a pressing need for additional research and refinement of treatments aimed at enhancing the yield, quality, and safety of off-season mulberry fruit. Numerous methods, excluding the use of growth regulators, have been explored to encourage off-season flowering, each yielding varying degrees of success. These methods include grafting (Kulkarni, 1986), girdling (Lavee *et al*., 1983), pruning (Oliveira *et al*., 1996; Bagchi *et al*., 2008), and inducing drought stress (Poerwanto *et al*., 2008).

While the technique of summer pruning has been previously applied and reported to successfully induce flowering in the long-fruit cultivar 'Taiwan Changguosang' (*Morus laevigata* Wall.) (Liu *et al*., 2022a), comprehensive large-scale investigations assessing its impact on both in-season and off-season fruit yield and quality are notably absent. Thus, the primary objective of this study is to compare the fruit yield, bioactive components, and antioxidant activity of 'Taiwan Changguosang' between in-season and off-season cultivation.

Moreover, our research delves into the seasonal variations in microclimate within the greenhouse, aiming to discern the influence of environmental factors and horticultural techniques on the yield and quality of in-season and off-season mulberry fruit. This study is designed to fill the existing gap in knowledge and provide insights that could contribute to a better understanding of inducing off-season mulberry production, ultimately leading to advancements in fruit yield and quality.

Materials and Methods

Plant materials and experimental design

Seventy-two 4-year-old 'Taiwan changguosang' (TW-CGS) (*Morus laevigata* Wall.) trees were selected for cultivation in a greenhouse, spaced at 2 m × 4 m intervals. The TW-CGS is a seedless and parthenocarpic cultivar, so there are no pollinating trees in the greenhouse. The mulberry trees were trained to a horizontal trellis at a height of 2 m. In 2022, the plant height and crown width was 2.0 m and 3.5 m, respectively. Mulberry trees were grafted on 'Guisangyou 12' (*Morus atropurpurea* Roxb.) and located at the mulberry germplasm resource nursery of the Industrial Crops Institute of Hubei Academy of Agricultural Sciences in Wuhan, China, at latitude 30°48'7"N and longitude 114°33'4"E.

The experiment comprised two treatment groups: in-season production (without pruning) and off-season production (using summer pruning). Summer pruning in off-season cultivation was carried out on June 22, 2022, retaining 3-5 axillary buds at the base of the new shoot for short pruning to promote axillary bud germination, flowering, and fruiting. Each treatment has nine biological replicates, with one tree representing one replicate. In-season and off-season mature mulberry fruits in the greenhouse were harvested on April 7, 2022, and July 29, 2022, respectively. Meteorological data were recorded using three temperature and relative humidity recorders (GSP-6, Elitech Technology Co., LTD, Hangzhou, China) from November 2021 to October 2022. Climate records for this horticulture facility show an average annual temperature of 20.13 °C, and an average annual relative humidity of 80.14%, respectively, an average maximum temperature of 38.04 °C in July, and an average minimum temperature of 3.53 °C in February (Figure 1A, B).

Three main phenological stages were recorded throughout 2021-2022 and presented in Supplementary Table S1.

Fruit yield and firmness measurements

Fruits, harvested at the commercially ripe stage, were randomly selected and transported to the laboratory. Fresh fruits were randomly weighed using an electronic balance (ME203E, Mettler Toledo Technology Co., LTD, Shanghai, China). Fruit length (FL, mm) and fruit diameter (FD, mm) were determined by using digital vernier calipers (3V Battery Digital Caliper, Guilin Guanglu Measuring Instrument Co., LTD, Guilin, China). To measure the fruit yield, the average number of fresh fruit number per plant (AFFN, N°·plant^{-1}) and the total number of buds per plant (TNBs, N°·plant^{-1}) were logged at the inflorescence emergence stage and fruit setting stage. The total production per plant (TP, kg·plant^{-1}) was calculated according to the formula: TP = TNBs × AFFN × FW/1000. The fruit firmness was measured using a CT3 texture analyzer (Brookfield Engineering Laboratories, Inc., USA), and expressed as Newtons (N).

Nutritional composition determination

Total soluble solid (TSS) was determined using an ATAGO PAL-1 digital refractometer (Tokyo, Japan), and expressed in items of °Brix. Total titratable acidity (TTA) was performed by NaOH-titration method (Mo *et al.*, 2022b). Soluble sugar content (SSC) was assessed using the anthrone reagent method (Morris, 1948), and the SSC/TTA ratio was estimated. The reducing sugar content (RSC) was determined by the DNS method (Miller, 1959).

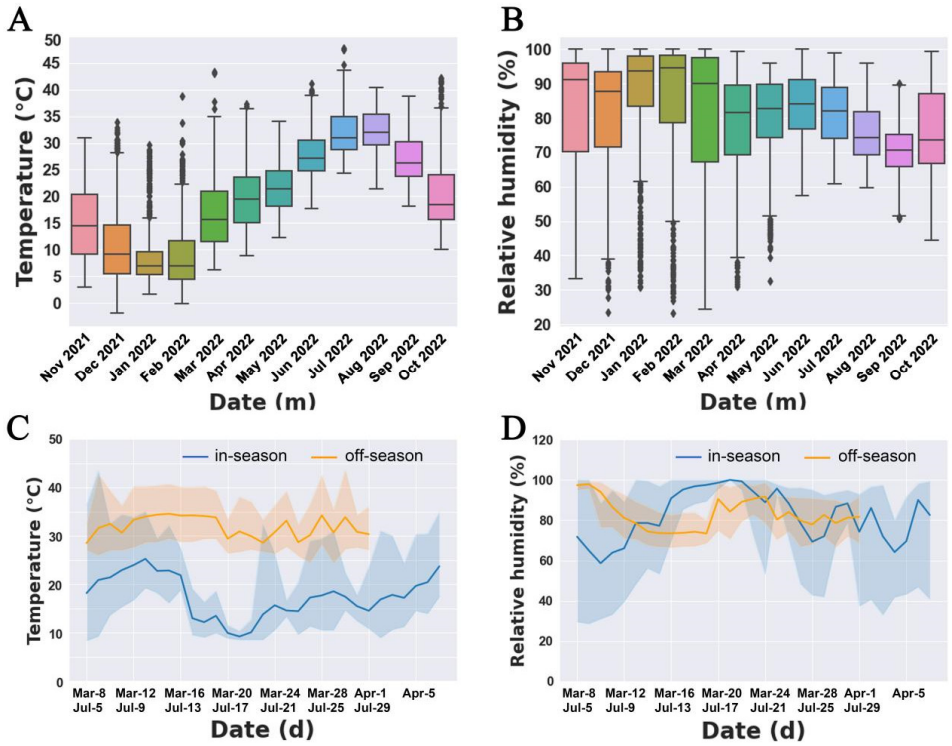

Figure 1. Climate factors in the greenhouse

(A, B) Temperature and relative humidity variation. Each box plot represents 720 replicates, with the inner line showing the median, the ends of the box representing the first and third quartiles, and the dots representing outliers. (C, B) Daily mean, maximum, and minimum temperature and relative humidity recorded from flowering to fruit ripening

Athocyanin and Vc determination

Total anthocyanin (TA) content was measured using a pH differential method (Giusti and Wrolstad, 2001). Briefly, 1.0 g of freeze-dried fruit was added to 10 mL of extracting solution (95% C_2H_5OH: 1.5 M HCl = 85: 15) for 40 minutes at 4 °C. The 2.0 mL supernatant from centrifugation was mixed and brought to the volume of 25 mL with KCl-HCl solution (0.2 M, pH 1.0) and NaAc-HAc solution (0.2 M, pH 4.5), respectively. The absorbance at 510 nm and 700 nm was measured using A = (A510 nm−A700 nm) pH 1.0−(A510 nm−A700 nm) pH 4.5. The results were based on a cyanidin-3-glucoside extinction coefficient of 26, 900 expressed as mg of CGE (cyanidin-3-glucoside equivalent) per gram of fresh weight. Vitamin C (VC) content was determined using xylene extraction colorimetric method (Zhang, 2000).

Total polyphenols and flavonoids

One gram of freeze-dried fruit was used for total polyphenols (TPO) extraction. The samples were soaked in 10 mL of precooled 80% methanol and extracted with ultrasound for 20 minutes, then centrifuged at 10,000 × g for 20 minutes (4 °C).

The prepared supernatant was used to determine the TPO using the Folin-Ciocalteau colorimetric method (Singleton *et al.*, 1999). Specifically, the 1.0 mL extract solution was diluted into 3 mL with 80% methanol. One milliliter diluted sample was combined with 0.5 mL of Folin-phenol reagent (1 M), and 1.5 mL of 20% (w/v) $NaCO_3$. The mixture was brought to a volume of 10 mL using distilled water. The absorbance of the mixture was examined at 760 nm after 2 hours of reaction at room temperature. The content of total polyphenols was expressed as mg of GAE (gallic acid equivalents) per gram of fresh weight.

Total flavonoids (TF) content was determined using $AlCl_3$ colorimetric method with minor modifications (Lin and Lay, 2013). The reaction mixture contained 1.0 mL extract solution of 80% methanol, 1.0 mL of alcohol solution (95%), 0.1 mL of $AlCl_3$ (10%, w/v), 0.1 mL of potassium acetate (1 M), followed by the addition of 2.8 mL of distilled water. The absorbance at 415 nm was measured after the reaction for 40 minutes at room temperature. The final findings were calculated and expressed as mg QE (quercetin equivalent) per 100 grams of fresh weight.

Antioxidant capacity analysis

Hydroxyl radical scavenging activity (HRSA) was determined using a previously published method (Chen *et al*., 2016). The results were determined and expressed as mg of AAE (L-ascorbic acid equivalents) per 100 g of fresh weight.

The scavenging ability of DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined using the method of Chen *et al*. (2016). The final reaction mixture consisted of 0.1 mL extract solution of 80% methanol and 3.9 mL of DPPH• methanol solution (0.1 mM). After a 30-minute reaction in a dark environment, absorbance was measured at 517 nm. An 80% methanol solution instead of mulberry extract was used as the blank. DPPH radical scavenging was calculated as follows: scavenging ability (%) = (Ablank−Asample)/Ablank × 100. The final findings were computed and expressed as mg of TE (Trolox equivalents) per 100 g of fresh weight.

The ferric-reducing antioxidant power (FRAP) of mulberry fruit was determined according to the method of Chen *et al*. (2016) with minor modifications. The FRAP reagent consisted of 0.3 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mM ferric chloride (10: 1: 1). 0.1 mL of diluted 80% methanol extracts (3-fold) was mixed with 3.9 mL of FRAP reagent. After incubation for 10 minutes at 37 °C water, the absorbance was read at 593 nm. $FeSO_4$ solution was used as a standard, and the ferric-reducing ability was expressed as mmol of $FeSO_4$ equivalents per 100 g of fresh weight.

Statistical analysis

Evaluation of statistical significance was performed using paired Student's t-test with SPSS (version 26.0, IBM, USA) for Windows software to compare the differences between in-season and off-season production. The relationships among 13 attributes investigated in our study were evaluated using the 'corrplots' package implemented in RStudio version 4.3.1 (*http://cran.r-project.org/*). Principal component analysis (PCA) was performed using the 'FactoMineR' and 'factoextra' packages in RStudio (version 4.3.1). The radar figure was generated using the package 'pyecharts' in Python version 3.9.2 (*https://doi.org/10.21105/joss*).

Results

Comparison of fruit yield and firmness of in-season and off-season mulberry

Figure 2 illustrates hanging fruit trees of two mulberry varieties. A visual assessment revealed a notably lower number of mulberry fruits in off-season production compared to in-season production. Statistical analyses confirmed a substantial 80% reduction in total production (TP) for off-season cropping compared to in-season cropping (Table 1). Specifically, in-season and off-season mulberry exhibited mean TP values of 7.39 $kg{\cdot}plant^{-1}$ and 1.47 $kg{\cdot}plant^{-1}$, respectively. The diminished fruit yield in off-season mulberries was attributed to reductions in both the total number of buds per plant (TNBs) and the average fresh fruit number per plant (AFFN). As a consequence of summer pruning, TNBs and AFFN in off-season mulberries were markedly lower than those in in-season mulberries. Additionally, the fruit firmness of off-season mulberries was significantly lower than that of in-season mulberries. Despite the decrease in fruit production and firmness, off-season

mulberries demonstrated higher individual fruit fresh weight and longer fruit length. However, there was no significant difference in fruit diameter (FD) between in-season and off-season fruits.

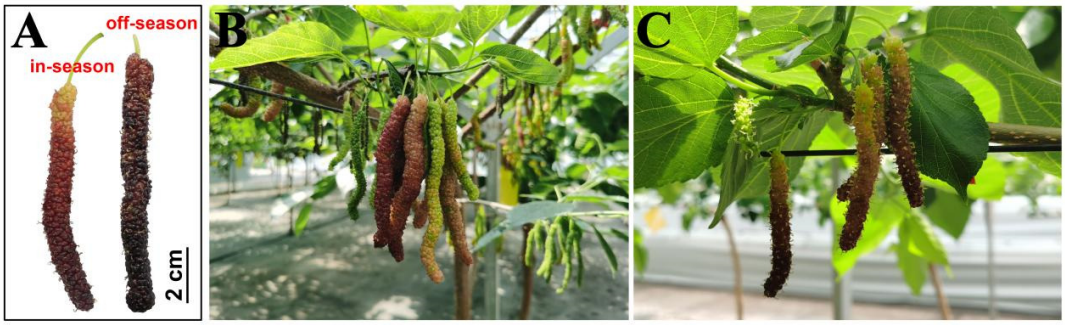

Figure 2. Photographs of mulberry fruit at in-season (B) and off-season (C) maturation stages

Table 1. Comparison of fruit yield, firmness, and size of in-season and off-season production

Item	TNBs (N°·plant^{-1})	AFFN (N°·bud^{-1})	TP (kg·plant^{-1})	Firmness (N)	FW(g)	FL (mm)	FD (mm)
In-season	328.00 ± 94.41	3.71 ± 0.26	7.39 ± 2.20	0.58 ± 0.02	6.07 ± 0.77	88.73 ± 6.69	9.90 ± 0.71
Off-season	68.11 ± 13.55***	3.14 ± 0.23**	1.47 ± 0.21***	0.55 ± 0.02**	6.96 ± 0.99*	103.33 ± 6.20***	9.81 ± 0.27

Data were presented as mean ± standard deviation (n = 9). The symbol "***", "**" or "*" represents a significant difference between in-season cultivation and off-season cultivation at 0.001 level ($p < 0.001$), 0.01 level ($p < 0.01$), and 0.05 level ($p < 0.05$), respectively. FW (fruit fresh weight), FL (fruit length), FD (fruit diameter), TNBs (total number of buds), AFFN (average fresh fruit number), TP (total production).

Sugar and acid comparison of in-season and off-season fruit

Significant differences were identified in the sugar and acid content of mulberry fruit (Table 2). In comparison to in-season fruit, off-season fruit exhibited higher SSC (16.03 g·100 g^{-1} FW), TTS (23.54 °Brix), and lower RSC at 7.75 g·100 g^{-1} FW. Notably, there was an inverse relationship between SSC and RSC in in-season and off-season mulberry fruit. Regarding acidity, the average total titratable acidity (TTA) in off-season mulberry fruit was 0.35 g·100 g^{-1} FW, significantly lower than the TTA in in-season fruit. Moreover, as a key indicator of mulberry fruit ripening, the ratio of SSC/TTA in off-season fruit was significantly higher compared to that in in-season fruit. Consequently, the off-season mulberry fruit exhibited a notably sweeter profile than the in-season fruit.

Table 2. Comparison of sugar and acid content between in-season and off-season production

Item	TSS (°Brix)	SSC (g·100 g^{-1} FW)	RSC (g·100 g^{-1} FW)	TTA (g·100 g^{-1} FW)	SSC/TTA
In-season	18.74 ± 0.72	12.84 ± 0.29	11.08 ± 0.24	0.43 ± 0.02	30.14 ± 1.74
Off-season	23.54 ± 0.56**	16.03 ± 0.24***	7.75 ± 0.48***	0.35 ± 0.01***	45.79 ± 1.60***

Data were presented as mean ± standard deviation (n = 9). The symbol "**" and "***" represent a significant difference between in-season cultivation and off-season cultivation at 0.01 level ($p < 0.01$) and 0.001 level ($p < 0.001$), respectively. TSS (total soluble solid), SSC (soluble sugar content), RSC (reducing sugar content), TTA (total titratable acidity), SSC/TTA (soluble sugar content/total titratable acidity).

Bioactive components and antioxidant activity comparison between in-season and off-season fruit

Bioactive components play a crucial role as contributors to the antioxidant activity of mulberry, with TA being recognized as an important antioxidant prevalent in human food. Additionally, TA is a key factor

influencing the distinct coloration of mulberry fruit. As depicted in Figure 3, the mean value of TA in off-season fruit was 0.34 mg CGE·g^{-1} FW, marking a 1.7-fold increase compared to in-season fruit. Figure 2A further illustrates that off-season mulberry fruit exhibited a darker color than in-season mulberry fruit. The intensified red coloration in off-season fruit may be associated with the elevated temperature and solar radiation experienced during the summer. Similarly, TPO and TF also showed increases in off-season fruit compared to in-season fruit, measuring 1.31 mg GAE·g^{-1} FW and 25.75 mg QE·100 g^{-1} FW, respectively (Figure 3). However, a contrasting trend was observed for VC.

Mulberry has received increasing attention due to its high antioxidant properties, which contribute to the reduction of certain disease risks. In order to comprehensively evaluate the in vitro antioxidant capacity of mulberry fruit, we used three determination methods including DPPH•, •OH scavenging, and FRAP assays. The results are presented in Figure 3. Notably, there was no significant difference in HRSA between in-season and off-season fruits. However, off-season fruit exhibited higher FRAP (2.01 mmol $FeSO_4$·100 g^{-1} FW) and DPPH radical scavenging activities (183.72 mg TE·100 g^{-1} FW) compared to in-season fruit.

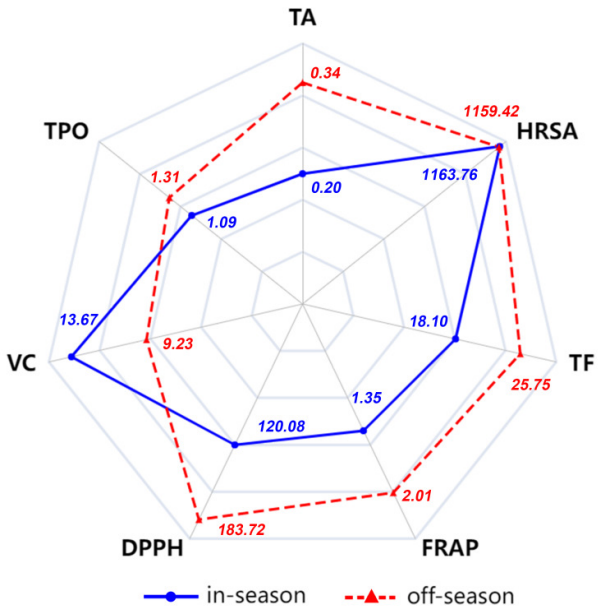

Figure 3. Radar plots depicting the differences in bioactive components and antioxidant activity between in-season and off-season production

TA (total anthocyanin, mg CGE·g^{-1} FW), HRSA (hydroxyl radical scavenging activity, mg AAE·100 g^{-1} FW), TF (total flavonoids, mg QE·100 g^{-1} FW), FRAP (ferric reducing antioxidant power, mmol FeSO4·100 g^{-1} FW), DPPH (DPPH radical scavenging activity, mg TE·100 g^{-1} FW), VC (vitamin C, mg·100 g^{-1} FW), TPO (total polyphenols, mg GAE·g^{-1} FW)

Correlation analysis of fruit yield and quality traits

A Pearson correlation analysis was performed to explore the relationships between different evaluated indexes of mulberry fruit (Figure 4). The results revealed significant correlations among various fruit traits. Notably, strong positive correlations (correlation coefficients above 0.9) were observed among bioactive components, including TA, TPO, and TF. The positive association between TA and TPO suggests that darker-colored mulberry fruit generally contains higher levels of polyphenols. Furthermore, a significantly positive correlation (r = 0.98, p < 0.01) was found between DPPH radical scavenging and FRAP, both of which exhibited highly significant positive correlations with the aforementioned bioactive components. Additionally, SSC demonstrated a highly positive correlation (r = 0.96, p < 0.01) with TSS, but significant negative correlations with RSC and TTA. Strongly significant positive correlations were observed among AFFN, TNBs,

and TP, with the highest correlation coefficient (r = 0.99, p < 0.01) observed between TNBs and TP. The results of the average linkage hierarchical clustering indicated two distinct clusters of linked traits. Cluster 1 primarily comprised TA, TPO, TF, FRAP, and DPPH radical scavenging, reflecting their strong ties to bioactive components and antioxidant activity. On the other hand, Cluster 2 encompassed yield indexes and nutritional compositions.

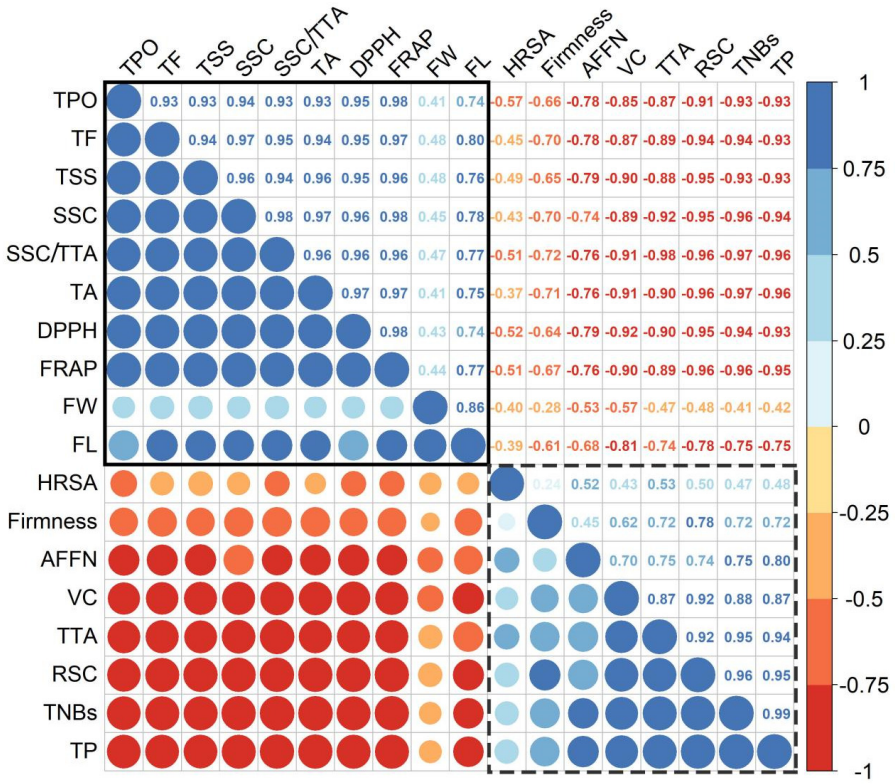

Figure 4. Correlation test among eighteen performance variables with significant differences
'Average' linkage was employed for the hierarchical clustering. Cluster 1 and 2 are denoted in solid and dashed lines, respectively. TNBs (total number of buds), AFFN (average fresh fruit number), TP (total production), FW (fruit fresh weight), FL (fruit length), TSS (total soluble solid), SSC (soluble sugar content), RSC (reducing sugar content), SSC/TTA (soluble sugar content/total titratable acidity), TTA (total titratable acidity), TA (total anthocyanin), HRSA (hydroxyl radical scavenging activity), TF (total flavonoids), FRAP (ferric reducing antioxidant power), DPPH (DPPH radical scavenging activity), VC (vitamin C), TPO (total polyphenols)

Principal component analysis (PCA) of fruit yield and quality traits

PCA was performed on all indexes of mulberry fruits, and the eigenvalues, cumulative contribution rates, and component loading matrix are presented in Supplementary Table S2. The first two principal components explained 76.85% and 8.21%, respectively, accounting for 85.06% of total variability, signifying a statistically significant discrimination. Given the high cumulative contribution rate of the first two PCs, the subsequent PCs were deemed negligible, and the results and discussion will focus on PC1 and PC2.

In the first principal component (Figure 5A and Supplementary Table S2), TSS, SSC, SSC/TTA, TA, TPO, TF, DPPH, and FRAP exhibited negative values. Conversely, positive values were observed for TNBs, AFFN, TP, firmness, RSC, TTA, and VC. FW and FD contributed to PC2. The score plot of PCA visually discriminates all samples in a 2D space. It is evident that the two types of samples cluster in different areas without overlap, indicating a clear distinction in fruit yield and quality between in-season and off-season mulberries.

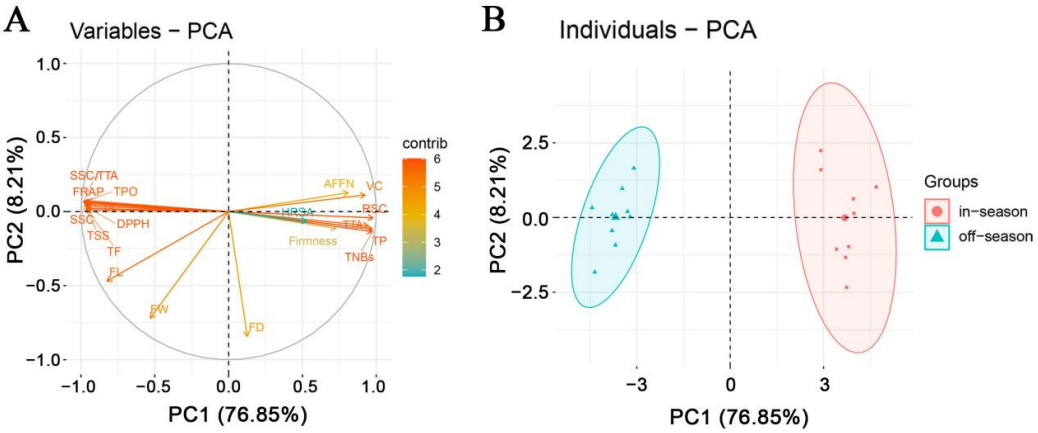

Figure 5. Principal component analysis (PCA) of the different variables factor (A) and active individuals (B) of fruit yield and quality traits in-season and off-season production

TNBs (total number of buds), AFFN (average fresh fruit number), TP (total production), FW (fruit fresh weight), FL (fruit length), FD (fruit diameter), TSS (total soluble solid), SSC (soluble sugar content), RSC (reducing sugar content), SSC/TTA (soluble sugar content/total titratable acidity), TTA (total titratable acidity), TA (total anthocyanin), HRSA (hydroxyl radical scavenging activity), TF (total flavonoids), FRAP (ferric reducing antioxidant power), DPPH (DPPH radical scavenging activity), VC (vitamin C), TPO (total polyphenols)

Discussion

The increasing recognition and utilization of functional components in mulberry fruit have driven a growing demand for both fresh mulberries and mulberry-based food products. Consumers are not only seeking access to mulberries during the traditional in-season period but also yearn for availability in the off-season. However, the limitation of harvesting mulberry fruit at a specific time poses a clear challenge for large-scale commercial production. Off-season production has emerged as an effective means to address this limitation and achieve year-round availability. Currently, there is a dearth of information on the variations in bioactive compounds and antioxidant activity of off-season mulberry fruit. This study successfully induced flowering in 'Taiwan Changguosang' (*Morus laevigata* Wall.) within a greenhouse environment using summer pruning. The investigation aimed to uncover the distinctions in bioactive compounds and antioxidant activity in mulberries cultivated during different seasons.

In various studies, whether employing girdling, pruning, or growth regulator treatments, the primary objective has consistently been to suppress vegetative growth (Poerwanto *et al*., 2008). In the current study, all branches of mulberry trees in the greenhouse were pruned, retaining 3-5 buds, and cut off from the base on June 22, 2022. It is noteworthy that the interval between pruning and bud-break was surprisingly short, lasting only 9 days, aligning with previous findings (Liu *et al*., 2022a, 2022b). A similarly brief interval of 20-25 days between $KClO_3$ treatment and flowering of longan was also observed in a previous study (Manochai *et al*., 2005). These results contribute valuable insights into determining the optimal timing of induced treatments for off-season fruit production based on market demand.

Furthermore, the duration of fruit development and maturation in off-season cultivation was notably shorter than that in in-season cultivation (Supplementary Table S1), consistent with earlier research findings (Liu *et al*., 2022b). In our study, the daily average temperature during the period from flowering to fruit ripening in off-season cultivation (summer) was higher compared to that in in-season cultivation (spring) (Figure 1), along with increased solar radiation. The elevated temperature and solar radiation in summer expedited fruit ripening. This phenomenon is analogous to grapes maturing faster when exposed to solar radiation compared to those in shaded areas within the canopy (Kliewer and Lider, 1968). Furthermore, it was

observed that the ripeness of mulberry fruit in off-season cultivation correlated with lower fiber content and higher SSC, contributing to a softer texture.

The economic benefits of mulberry cultivation are closely tied to fruit yield. However, off-season cultivation demonstrated a marked decrease in yield compared to in-season cultivation. This decline in off-season fruit production can be attributed to lower values of TNBs and AFFN (Table 1), influenced by the efficacy of induction methods. Previous studies by Liu *et al.* (2022a) emphasized the significant impact of different summer pruning methods on off-season fruit yield. Additionally, Liu *et al.* (2022b) noted that the highest off-season yield was achieved through growth regulator treatment on August 9. Furthermore, the lower fruit yield in off-season cultivation resulted in a smaller fruit load, contributing to higher individual fruit weight.

The maturation of fruit involves the accumulation of sugars and the catabolism of acids in the majority of species (Lee and Hwang, 2017; Batista-Silva *et al*., 2018). Sugar and acid content are influenced by genotypes and environmental conditions. Increasing temperatures have been reported to favor sugar accumulation (Fukuoka *et al*., 2008; Jiang *et al*., 2022), while contributing to a decrease in acidity (Kliewer, 1973; Gautier *et al*., 2008). Therefore, the higher SSC and TSS, as well as the lower TTA in the off-season mulberry fruit, align with expectations given the seasonal temperature increase. Additionally, it was evident that RSC in off-season fruit was significantly decreased compared to in-season fruit, consistent with previous studies (Liu *et al*., 2022b, 2017a). Fructose and glucose, as reducing sugars, are major components in long mulberry (Mahmood *et al*., 2012). In this study, we observed a negative correlation between SSC and RSC, which contrasts with the findings of our previous study. Hence, we hypothesized that off-season cultivation induces a significant accumulation of non-reducing sugars (such as sucrose), leading to a reduction in the proportion of reduced sugar. This hypothesis warrants confirmation through further analysis of individual sugar components using HPLC. These intriguing findings open avenues for proposing and addressing more scientific questions.

The pivotal role of bioactive components in the onset and progression of diseases is widely acknowledged. Although common key bioactive components, including anthocyanins, polyphenols, flavonoids, and vitamin C, were detected in mulberry fruit across different seasons, notable differences in concentration were observed. Anthocyanin, recognized as a key contributor to mulberry fruit color, exhibited higher levels in off-season fruit. It appears that the accumulation of TA is influenced by various climatic conditions in the greenhouse, particularly temperature and solar radiation. Previous studies have demonstrated that light positively influences anthocyanin biosynthesis at both mature and postharvest stages (Ubi *et al*., 2006; Zhou *et al*., 2020). Additionally, sugar has been identified as a signaling molecule for anthocyanin biosynthesis, regulated by hot air temperature and UV in various plants, such as Arabidopsis (Das *et al*., 2012), apples (Liu *et al*., 2017b), and peaches (Zhou *et al*., 2020). Consequently, the higher SSC in off-season mulberry fruit likely contributes to the enhancement of TA. The strong correlation observed between TA and SSC (r = 0.97, $p < 0$) reinforces this connection.

Significantly, a positive correlation ($r = 0.93$, $p < 0$) between TA and TPO was identified in the current study (Figure 4), aligning with the findings of Guerrero *et al.* (2010) and Aramwit *et al.* (2010). These results suggest that deeply colored fruits, especially red and black mulberry fruits, often contain higher levels of polyphenol content. This provides a straightforward reference for consumers to make healthier choices when selecting fruits and vegetables. In addition to TA, TFO and total TF were also notably increased in off-season fruit compared to in-season fruit, consistent with findings reported by Prasad *et al.* (2015). The increased levels of TA, TPO, and TF in off-season mulberry fruit may be indicative of the plant's adaptability to the environment.

These bioactive phytochemicals feature multiple hydroxyl groups in their molecular structures, providing them with antioxidant potential and radical-scavenging properties. Notably, antioxidant activity is closely linked not only to the type and concentration of components but also to their structural properties and synergistic effects (Sun *et al*., 2023). Evaluating the antioxidant capacity of each bioactive ingredient

individually is complex, slow, and costly. Therefore, widely used methods such as DPPH and hydroxyl scavenging activity, as well as FRAP, are popular choices for assessing antioxidant activity.

In this study, the DPPH scavenging activity and FRAP of off-season fruit were 1.5-fold higher than those in in-season fruit (Figure 3). Additionally, a significantly positive correlation was observed between DPPH scavenging activity and FRAP (Figure 4), indicating that these two assays were better suited than the HRSA assay for detecting antioxidant activity. Furthermore, consistent with previous studies in different plants (Alizadeh and Fattahi, 2021; Sawicki *et al*., 2022; Sun *et al*., 2023), TPO and TF exhibited positive correlations with antioxidant activity in the current study. These findings suggest that the antioxidant activity of mulberry fruit may primarily be attributed to the bioactive components of TPO and TF. Thus, it is not surprising that the antioxidant activity, as measured by FRAP and DPPH radical scavenging, in off-season mulberries was significantly higher than that in in-season fruit.

Conclusions

In summary, the mulberry fruit cultivated in a greenhouse during in-season and off-season exhibited distinct differences in yield, physicochemical characteristics, and antioxidant activity. The higher daily average temperature and solar radiation in the summer contributed to superior overall fruit quality in the off-season compared to mulberry fruit produced in the in-season of spring. These findings highlight the potential for high-quality off-season mulberry production strategies. However, further research is urgently needed to explore and optimize cultivation technologies for off-season production under greenhouse or alternative cultivation conditions, including considerations such as treatment timing, methods, and water and fertilizer management.

Authors' Contributions

Conceptualization, RLM, CYQ and NZ; methodology, RLM, CYQ and NZ; software, RLM, CYQ and NZ; validation, SH, WW and CHZ; formal analysis, DL and YL; investigation, RLM and NZ; resources, SH, WW and CHZ; data curation, DL and YL; writing - original draft preparation, RLM and NZ; writing - review and editing, CYQ, YL and QL; visualization, CYQ, QL and NZ; supervision, CYQ and QL; project administration, QL and NZ; funding acquisition, CYQ and QL.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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