

Using physiological and morphological methods for comparison: is mixed *Phyllostachys edulis* and *Carya illinoensis* a reasonable mixed forest model?

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Research Article

Keywords: anatomy, correlation analysis, JIP test, light response curve, photosynthesis

Posted Date: June 8th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3020614/v1>

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Abstract

The purpose of this study was to investigate whether the *Phyllostachys edulis*-*Carya illinoensis* co-plantation is a feasible forest model. Two treatments and one control were evaluated. The control was a low-density *C. illinoensis* forest (CK), and the treatments were a high-density *C. illinoensis* forest (DF), and a *C. illinoensis*-*P. edulis* co-plantation forest (MF). Gas exchange and chlorophyll fluorescence parameters, leaf physiology, macromorphology, and anatomical structure of *C. illinoensis* were measured and principal component analysis (PCA) was used to evaluate treatment effects. The highest net photosynthetic rate (Pn), which was $13.72 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, was recorded for *C. illinoensis* under the CK treatment, while the corresponding values for MF and DF treatments were 8.98 and $5.25 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, respectively. The JIP test revealed that plastoquinone libraries were inhibited under both MF and DF, particularly in the latter. Compared with CK, antioxidant substances in MF and DF leaves increased to a certain extent, again, particularly in the latter. Leaf macromorphology and anatomical structures under the different treatments also changed to acclimated to different environments. The leaf area of MF became lower, and the vascular tissue of DF petiole became larger. Finally, based on the main data, the order of the PCA scores was CK > MF > DF. The results indicated that both co-plantation and high-density planting caused both interspecific and intraspecific competition. Photosynthesis was inhibited in *C. illinoensis* to varying degrees under both cultivation models. Nonetheless, the stress levels in *C. illinoensis* were significantly lower under the co-plantation forest than under high-density planting. These findings indicate that *C. illinoensis* growth was not severely inhibited by co-plantation with *P. edulis*, as it still developed well. Hence, co-plantation of *P. edulis* and *C. illinoensis* is a promising mixed-forest model.

Introduction

Carya illinoensis (Wangenheim) K. Koch (pecan; Order Fagales; Family Juglandaceae, C₃ plants) is a high value fruit-bearing and ornamental tree species. *Phyllostachys edulis* (Carriere) J. Houzeau (Moso bamboo; Order Poales; Family Poaceae, C₃ plants) is an economically important, fast-growing tree species that is widely planted in southern China, where there are numerous *P. edulis* forests [1, 2]. *P. edulis* is highly valued because of its large carbon fixation capacity, which has contributed substantially to carbon storage in the forest ecosystems of China [3, 4]. Nonetheless, as their ecological diversity is low [5, 6], the economic and ecological value of *P. edulis* forests are severely threatened [7]. However, mixed *P. edulis* forests can effectively compensate for this weakness. Additionally, mixed forest construction improves forestland productivity while lowering the incidence and severity of insect pest and microbial pathogen attacks [8–10]. Mixing of *C. illinoensis* with many other species has also showed a good growth trend [11, 12]. Furthermore, mixed forest construction improves *C. illinoensis* fruit yield. Hence, a mixture of *P. edulis* and *C. illinoensis* might help transform forestlands in southern China to both economic and ecological advantages.

Mixed forests have high ecological, economic, societal, and ornamental value [13]. The method of constructing mixed forests has been applied to various tree species in different regions [14, 15]. However, as monoculture forests are structurally simpler than mixed forests, building this type of forest is more complex and challenging than the former type [16, 17].

Intraspecies and interspecies competition occurs in complex ecosystems such as mixed forests [18], and competitive environments reportedly stimulate a part of plant growth [19, 20]. Photosynthetic capacity serves as a convenient parameter to measure tree growth in successful, mixed-forest ecosystems [21, 22], as it provides the photoassimilate that is the basis of yield formation [23, 24]. Additionally, photosynthetic energy can be directly expressed as gas exchange and chlorophyll fluorescence parameters [25, 26]. Among the former, the photosynthetic rate is reflected in photosynthetic activity intensity, while stomatal conductance, transpiration rate, and other parameters are reflected in photosynthetic capacity [27]. Chlorophyll fluorescence parameters are obtained from the polyphasic fluorescence transient (OJIP) curve, which reflects photosystem performance and has a feedback effect on chloroplast status [28].

Leaf macromorphology and anatomical structure significantly affect photosynthesis. Thus, plants may modify their leaf area and weight, palisade layer thickness, and geometric morphology in order to acclimated to changing environments [29–31]. Additionally, photosynthetic capacity might change accordingly [32–34]. Particularly in complex environments, leaf physiological indices such as chlorophyll content, photosynthetic rate-limiting and antioxidant enzyme levels, as well as leaf stoichiometry, must be measured to evaluate photosynthesis and plant growth status [35–38].

China started the introduction of *C. illinoensis* in the 1920s, and currently, the species occupies a large planting area in China and is an important economic tree species [39]. However, due to the small area of cultivated land in China, most of the plain areas must be used for crop cultivation, and the use of cultivated land is strictly controlled [40]. With most of its territory comprising hills, it was difficult for Zhejiang Province to support the massive plantations of *C. illinoensis* on an already scarce plain terrain. Therefore, some farmers tried to plant *C. illinoensis* on the hillside. There are many forests in the hills of Zhejiang Province, of which a large proportion is composed of bamboo forests [41]. Due to the difficulty in management and the decline in economic benefits, a large number of *P. edulis* forests are in a state of waste [42–44]. However, as *P. edulis* is a clonally propagated plant [45], *P. edulis* forests are almost impossible to completely eradicate, whereby, building a mixed forest of *P. edulis* and *C. illinoensis* has become almost the only alternative.

In this study, the experimental groups included, co-plantation of *P. edulis* and *C. illinoensis* and high-density *C. illinoensis* forests. Although mixed *P. edulis* and *C. illinoensis* forest was our main research focus, the high-density *C. illinoensis* forest has a high canopy density after maturity, which can greatly reduce the interference of the *P. edulis* forest, presenting a feasible method for planting *C. illinoensis* on hillsides and hence was included in the study. Low-density *C. illinoensis* forest served as the control. Gas exchange and chlorophyll fluorescence parameters, leaf physiology, macromorphology and anatomical structure, and other indices were measured. The parameters were dimensionally reduced and

scored by principal component analysis (PCA). Photosynthetic capacity and growth status of *C. illinoensis* under the various cultivation patterns were evaluated to establish whether the *P. edulis*-*C. illinoensis* mixed-forest is a feasible and viable afforestation management strategy. Our study aimed to provide a sound theoretical basis for the sustainable management of *C. illinoensis* and the transformation of *P. edulis* forests in Zhejiang; furthermore, it will provide a basis for future research.

Materials and Methods

Plant materials

C. illinoensis trees were uniformly planted in 2018 in Mazhai Town, Dongyang City, Jinhua City, Zhejiang Province, China (120° 37' E, 29° 09' N). Average daily minimum and maximum temperatures at the study site were 13 and 22 °C, respectively. Average annual precipitation was 943 mm, and average annual number of precipitation days was 74. Trees were assigned to (1) high density planting = 4,500 ha⁻¹ (densely planted forest; DF), (2) low density planting = 1,500 ha⁻¹ (control group; CK), or (3) mixed planting with *P. edulis* at low density (mixed forest, MF) treatments. The experiment was performed in 2022. The experimental area size for each treatment is 0.1 ha. Manual weeding and pruning were conducted once annually. Neither herbicides nor fertilizers were used, and there was minimal manual intervention. At the time of the experiment, the experimental *C. illinoensis* young trees were approximately 4–6 m tall, while *P. edulis* stalks were over 15 m tall. According to the principle of diagonal sampling, before the experiment, one disease-free *C. illinoensis* with good growth was selected at each of the four corners and the center of each plot. These five *C. illinoensis* specimens were used for data collection in the subsequent experiments.

Gas exchange and chlorophyll fluorescence measurements

Photosynthetic parameters were measured in July 2022. Specifically, gas exchange parameters were measured with the LI-6400XT console (LI-COR Biosciences Inc., Lincoln, NE, USA) on sunny days between 08:30 and 11:30, under the following conditions: CO₂ concentration = 400 ± 5 μmol CO₂ mol⁻¹, block temperature = 35 ± 1 °C, and relative humidity = 35%±1%. Mature leaves were used as the test objects and the light response curves were measured. Leaves used for measurements were selected such that they received 850 ± 150 μmol·m⁻²·s⁻¹ of natural light intensity to ensure that the photosynthetic characteristics between sampled leaves were comparable. The light intensities used to construct light response curves were 2,000, 1,500, 1,200, 1,000, 750, 500, 250, 100, 60, 20, and 0 μmol·m⁻²·s⁻¹. Each light intensity was stable for 2 min. During that time, five consecutive measurements were made and recorded. The light response curve was fitted according to a model proposed by Ye [46].

$$P(I) = \alpha \frac{1 - \beta I}{1 + \gamma I} (1 - I_C)$$

where α is the apparent quantum yield and I_C is the light compensation point. Dark respiration intensity, light compensation and saturation points, and other indices were also calculated.

After gas exchange parameters were measured, chlorophyll fluorescence parameters were measured for three mature leaves per target plant during the afternoon using an OS-30p+ (Opti-Sciences Inc., Hudson, NH, USA). Each leaf was dark-treated for 20 min, chlorophyll fluorescence parameters measured immediately after, and the values for the three leaves were averaged. To eliminate interference from other factors, the original OJIP curve was standardized and the start and end points of all curves were harmonized (Formula (2)). A curve difference operation was simultaneously conducted to demonstrate the influences of the various cultivation models on leaf fluorescence (Formula (3)) [47]:

$$V_t = \frac{F_t - F_0}{F_P - F_0}$$

2

$$\Delta V_t = V_{t-X} - V_{t-CK}$$

3

where V_t is the amplitude of the OJIP curve at time t , F_0 is the amplitude of the OJIP curve at 20 μ s, F_P is the highest value of the OJIP curve, and V_{t-X} and V_{t-CK} are the amplitudes for the standardized experimental group X and the control group, respectively, at time t . The corresponding time of phase O is 20 μ s, the corresponding time of phase J is 2 ms, the corresponding time of phase I is 30 ms, and the time corresponding to the F_P is defined as phase P.

Leaf shape measurements

Six mature leaves on the target plants with uniform healthy growth were selected. Their lengths and widths were measured with a ruler. Photographs were taken and the leaf perimeters and areas were calculated with ImageJ2 (National Institutes of Health, Bethesda, MD, USA). The OpenCV module in Python v. 3.10 (<https://www.python.org/downloads/release/python-3100/>) was used to extract the blade contour and draw the minimum bounding box and the circumscribed and inscribed circles of each leaf blade. The geometric characteristics of each leaf blade were calculated to describe its shape according to the method of Wang et al. [48].

Six leaves were taken from each treatment for weight and physiological measurements, Three of them are used for weight measurement, and the fresh weight (FW) is measured before drying. Afterwards, the leaves were quickly inactivated at 105 °C and dried to constant weight at 80 °C for measuring the dry weight of the leaves (DW). Then, relative leaf water content (WC) was calculated. The three remaining leaves per plot were stored at – 80 °C until use in the subsequent physiological index determinations in the laboratory. In addition, three mature and healthy leaves from the same plant were cut at the center of the leaf blade into 1 cm × 1 cm segments, fixed in formaldehyde-acetic acid-ethanol (FAA; 70% (v/v)), and

anatomically examined. Similarly, the petioles were excised, cut to a particular length, fixed in 70% (v/v) FAA, and anatomically examined.

Anatomical structure measurements

One of the three FAA-fixed leaves and its petiole were embedded in paraffin, sliced into 4- μm sections with a microtome (RM2016, Leica Microsystems GmbH, Wetzlar, Germany), and double-stained with safranin and fast green.

An Olympus BX53 light microscope equipped with a QImaging MicroPublisher 5.0 RTV imaging system (Olympus (China) Co. Ltd., Beijing, China) fitted with a camera was used to observe and photograph the anatomical structures of the leaves, midveins, and petioles. The anatomical structures of six fields of view per section were evaluated and the averages were calculated. As the petioles were large, the local fields of view were first viewed and ImageJ was used to splice the images into a single complete picture. ImageJ was also used to measure the images and calculate the geometric characteristics of the leaf blades, midveins, and petioles.

Physiological index measurements

The measured leaf physiological indices included chlorophyll a (Chl a), chlorophyll b (Chl b), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), proline (Pro), total nitrogen (TN), total phosphorus (TP), and total potassium (TK). Chlorophylls a (F8042-A) and b (F50015-A), Rubisco (F4938-A), POD (F5058-A), SOD (F5056-A), CAT (F4957-A), MDA (F7878-A), and Pro (F7973-A) were measured with an enzyme-linked immunosorbent assay (ELISA) kit (FANKEW, Shanghai, China) and the data were standardized by protein quantification (D1001-A). The numbers in parentheses represent the number of the reagent kit. TN was determined by the semimicro Kjeldahl method. TP was determined by molybdenum blue colorimetry and TK was determined by flame photometry [49]. Photosynthetic nitrogen use efficiency (PNUE, $\mu\text{mol CO}_2 \cdot \text{mol N}^{-1} \cdot \text{s}^{-1}$), photosynthetic phosphorus use efficiency (PPUE, $\mu\text{mol CO}_2 \cdot \text{mol P}^{-1} \cdot \text{s}^{-1}$), and photosynthetic potassium use efficiency (PKUE, $\mu\text{mol CO}_2 \cdot \text{mol K}^{-1} \cdot \text{s}^{-1}$) were calculated according to the methods of Hidaka et al. [50].

Data analysis

Principal component analysis (PCA) was performed using the essential functions and plug-ins of SPSS Statistics v. 26 (IBM Corp., Armonk, NY, USA). Before PCA, the data were standardized to eliminate the influence of various data dimensions on the results as follows [47, 51]:

$$x_n = \frac{x - \mu}{\sigma}$$

All data were analyzed by one-way analysis of variance (ANOVA) and Duncan's new multiple-range test in SPSS Statistics v. 26. Graphs were plotted with Origin 2023 (OriginLab, Northampton, MA, USA). Correlation analysis used Pearson correlation coefficient for analysis. PCA used correlation matrix analysis. The P value less than 0.05 was considered statistically significant. The P value in the range of 0.05 to 0.1 was considered to have a weak difference between different treatments, There is a certain indicative effect, but it did not reach a significant level.

Results

Leaf blade differences

Leaf shape affects photosynthesis. Leaf morphology significantly differed among cultivation models. Compared with CK and DF, MF showed the smallest leaves. Values for FW, DW, length of leaf (LL), area of leaf (LA), and perimeter of leaf (LP) under the MF treatment were 51.76%, 50.75%, 73.20%, 50.47% and 72.76%, respectively, of the corresponding values recorded in CK plots. Similarly, the same indicators in MF plots were 54.17%, 49.53%, 71.50%, 45.23% and 71.01%, respectively, of the values registered in DF plots. However, LW did not differ among the cultivation models tested herein. Thus, there were no differences between MF and CK in terms of leaf morphology. Various parameters were non-significantly higher in MF than in CK plots. Although trees in MF plots had a small leaf area, their leaf water content was the highest of all three cultivation models. In contrast, leaf water content was significantly lower under DF than MF treatment. The water content under the three cultivation models was 53.84% (CK), 54.97% (MF), and 51.00% (DF). Further, there were no differences in leaf appearance or geometry (aspect ratio, circularity, eccentricity, and form factor) among the three cultivation models.

The leaves of *C. illinoensis* were composed of the upper epidermis, the palisade mesophyll layer (chlorenchyma), the spongy mesophyll layer (aerenchyma), and the lower epidermis. Crystals occasionally appeared in the upper epidermis. *C. illinoensis* commonly had two palisade layers; however, a few leaves had only a single palisade layer. Additionally, there were small alternate vascular bundles connected to the upper epidermis via one or two rows of cells. In general, the vascular tissue was largest in the midvein and comprised a crescent-shaped central pith with peripheral xylem, phloem, and phloem fibers. One large and one small vascular bundle in the petiole consisted of pith, xylem, phloem, and phloem fibers. Certain small vascular bundles did not fully develop while others fused or merged (Fig. 1).

The anatomical structures of *C. illinoensis* leaves differed among cultivation models. Differences were mainly in terms of the widths of their palisade layers. The palisade layers of MF were significantly wider than those of leaves from CK and DF ($P < 0.05$), specifically, 120.08% of CK and 106.85% of DF. Upper epidermis, intercellular space, and vascular bundle width also differed among cultivation models ($0.05 < P < 0.1$).

Cultivation models differed in terms of the mid vein size and vascular bundle morphology. Leaf vein height and area differed among the different cultivation models ($P < 0.05$). The leaf vein height in leaves

sampled from CK plots was $403.58 \pm 24.77 \mu\text{m}$ and the leaf vein area was $119,525.98 \pm 12,567.18 \mu\text{m}^2$. In contrast, the leaf vein height in leaves sampled from MF plots was $552.70 \pm 37.34 \mu\text{m}$ and leaf vein area was $82,361.08 \pm 8,824.47 \mu\text{m}^2$. Lastly, the vein height of leaves sampled from DF plots was $463.28 \pm 27.11 \mu\text{m}$ and leaf vein area was $155,726.54 \pm 18,163.40 \mu\text{m}^2$. The vascular bundle area, phloem fiber area, phloem area, xylem area and pith area of different cultivation methods also showed significant differences ($P < 0.05$). The above indicators of DF reached 115.4–218.9% of CK. The vascular bundle area of leaves sampled in DF plots was significantly larger than that of leaves from MF plots ($P < 0.05$), reaching 225.45% of the vascular bundle area in leaves from MF plots. The leaf veins showed a relatively regular geometric morphology across cultivation models. Nevertheless, the area convexity was significantly higher for MF than for CK or DF plots ($P < 0.05$), reaching 6.83, whereas values of 5.57 and 5.17 were characteristic for CK and DF plots. The geometric morphology (aspect ratio, area convexity, and eccentricity) of the leaf vein vascular bundle differed among cultivation models (Fig. 1D–1F).

Only the medulla area of the extensive vascular bundle in the petiole differed significantly among cultivation models ($P < 0.05$). The medulla area of the vascular bundles in petioles of leaves collected from DF plots was 251.62% that of those from CK and 371.07% that of those from MF plots. Certain indices (petiole, large vascular bundle, and small vascular bundle widths, large vascular bundle phloem fiber area, and small vascular bundle phloem area) significantly differed among cultivation models ($0.05 < P < 0.1$). The DF treatment had the highest values for these metrics. The differences between petioles and veins were similar in terms of their geometric indices. The aspect ratio did not differ significantly among petioles under the three cultivation models. The perimeter convexity of the petiole was significantly lower for leaves from MF than from CK or DF treatment plots ($P < 0.05$). The perimeter convexity in leaves from CK and DF was 1.02, while that of MF was 1.01. In contrast, the form factor of the petiole was considerably higher for MF than for CK or DF. Nonetheless, the absolute values of the foregoing parameters did not differ significantly among cultivation models. The geometric indices of the large vascular bundles in the petioles did not differ significantly among cultivation models ($P > 0.1$). The area convexity of MF was significantly greater than that of DF ($0.05 < P < 0.1$) but did not substantially differ from that of CK.

As each cultivation model generated its own unique environment, *C. illinoensis* leaf physiology markedly differed among models. Chlorophyll and Rubisco contents differed among cultivation models (Table 1). Chlorophyll a and Rubisco contents were relatively low in tress leaves collected in MF plots. However, chlorophyll b content was the same for both MF and CK. In contrast, chlorophyll a and Rubisco contents were significantly higher for DF than for CK ($P < 0.05$), whereas chlorophyll b content was considerably lower for tree leaves from DF than for those in the CK plots. Additionally, there were significant differences in chlorophyll a/b ratio among the three cultivation models ($P < 0.05$). The antioxidant enzyme (POD, SOD, and CAT), Pro, and MDA levels were low in plants growing in the MF treatment plots (Table 2). Hence, leaves under this treatment may not have been under stress. In contrast, all indices increased under DF, implying that high-density planting may have induced stress in *C. illinoensis* trees. Foliar NPK content also differed among the three cultivation models (Table 3). Trees in

DF treatment plots consistently showed relatively higher N contents, while MF seemingly promoted significantly higher K content than CK or DF. The foregoing results suggest considerable differences in the NPK ratios among the cultivation models.

Table 1
Photosynthetic physiological indexes of leaves

	Chl a ($\mu\text{mol}\cdot\text{g}^{-1}$)	Chl b ($\mu\text{mol}\cdot\text{g}^{-1}$)	Chl a/b	RubisCO ($\text{ng}\cdot\text{g}^{-1}$)
CK	29.13 $\pm$ 0.17Bb	22.61 $\pm$ 0.31Aa	1.29 $\pm$ 0.02Bb	43.74 $\pm$ 0.65Bb
MF	24.02 $\pm$ 0.52Cc	22.12 $\pm$ 0.29Aa	1.09 $\pm$ 0.02Cc	39.44 $\pm$ 0.37Cc
DF	33.56 $\pm$ 0.29Aa	20.26 $\pm$ 0.30Bb	1.66 $\pm$ 0.01Aa	48.1 $\pm$ 0.70Aa

Capital letters in the same column represent significant differences at 0.05 level, and small letters in the same column represent substantial differences at 0.1. The value is expressed as mean $\pm$ standard error(SE). The same is below.

Table 2
Antioxidant system index of leaves

	Pro ($\text{ng}\cdot\text{g}^{-1}$)	POD ($\text{ng}\cdot\text{g}^{-1}$)	SOD ($\text{ng}\cdot\text{g}^{-1}$)	CAT ($\text{ng}\cdot\text{g}^{-1}$)	MDA ($\text{nmol}\cdot\text{g}^{-1}$)
CK	562.73 $\pm$ 5.89Bb	13.87 $\pm$ 0.16Bb	9.4 $\pm$ 0.09Bb	17.15 $\pm$ 0.20Aa	1.24 $\pm$ 0.01Bb
MF	579.58 $\pm$ 8.99Bb	12.6 $\pm$ 0.20Cc	8.74 $\pm$ 0.10Cc	15.65 $\pm$ 0.32Bb	1.14 $\pm$ 0.01Cc
DF	614.76 $\pm$ 9.23Aa	15.41 $\pm$ 0.14Aa	10.1 $\pm$ 0.11Aa	17.06 $\pm$ 0.21Aa	1.63 $\pm$ 0.02Aa

Table 3
Stoichiometry of leaves

	N ($\text{mg}\cdot\text{g}^{-1}$)	P ($\text{mg}\cdot\text{g}^{-1}$)	K ($\text{mg}\cdot\text{g}^{-1}$)	P/N	P/K	K/P
CK	18.25 $\pm$ 0.41ABab	1.70 $\pm$ 0.12Aa	8.82 $\pm$ 0.43Bb	10.98 $\pm$ 0.74ABb	2.09 $\pm$ 0.09Bb	5.24 $\pm$ 0.18Bb
MF	16.61 $\pm$ 0.62Bb	1.75 $\pm$ 0.1Aa	10.73 $\pm$ 0.5Aa	9.67 $\pm$ 0.77Bb	1.57 $\pm$ 0.11Bc	6.18 $\pm$ 0.3Aa
DF	19.31 $\pm$ 0.81Aa	1.48 $\pm$ 0.12Aa	6.71 $\pm$ 0.45Cc	13.38 $\pm$ 0.83Aa	2.94 $\pm$ 0.23Aa	4.62 $\pm$ 0.3Bb

Differences in photosynthetic capacity

The net photosynthetic rate of *C. illinoensis* trees did not differ among cultivation models in response to increasing PAR (Fig. 2A). At PAR < 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, Pn in CK trees was slightly higher than those in trees growing in MF and DF treatment plots. Further, although Pn of trees in CK and MF plots were similar in the low light range (PAR = 100–750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), the gap between them widened in the mid- and high light range (PAR > 750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). However, Pn in trees growing in CK and DF plots significantly differed across the entire illumination range ($P < 0.05$). The difference in Pn between trees in MF and DF plots was highest in the low light range ($P < 0.05$) but Pn was substantially lower in the low (PAR < 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and mid-high (PAR > 750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light ranges. The model by Ye [38] (Table 4) was used to calculate gas exchange parameters. Trees in CK plots showed weak Rd and reached LCP first. However, the PAR level corresponding to LSP and Pm were higher for trees in CK than those in MF and DF plots. The Rd values recorded in trees growing in MF plots was higher than that recorded in CK trees. Nevertheless, the corresponding φ_{c0} was at least as high as that observed in CK plots. Pn decreased first and LSP was lowest in the MF model, and φ_{c0} and Pm were lowest for the DF model.

Table 4
Comparison of photosynthetic characteristics under different planting patterns

	φ_{c0} ($\mu\text{mol CO}_2\cdot\mu\text{mol}^{-1}$)	Rd ($\mu\text{mol O}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LCP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LSP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Pm ($\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
CK	0.0464 ± 0.0051Aa	0.0303 ± 0.0498Bb	2.2 ± 0.57Bb	1358 ± 70.82Aa	13.72 ± 0.56Aa
MF	0.0497 ± 0.0017Aa	1.1833 ± 0.3298Aa	24.1 ± 6.75Aa	958.7 ± 121.55Ab	8.98 ± 0.95Bb
DF	0.0338 ± 0.0082Bb	1.0104 ± 0.2484Aa	27.5 ± 5.16Aa	1153.89 ± 143.25Aab	5.25 ± 0.84Cc

φ_{c0} means the initial apparent quantum yield; Rd implies the dark respiration rate; LCP means the Light compensation point; LSP means the Light saturation point; Pm means Maximum photosynthetic rate.

At nearly all PAR intensities, trees in MF plots showed significantly higher Cond, Ci, and Tr than trees in CK and DF plots (Fig. 2B–2D). At very low PAR intensity (< 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), the Cond of trees in the CK group did not significantly differ from that of trees in the MF group ($P > 0.05$) (Fig. 2B). Under mid-high PAR intensity (> 750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), Ci in CK trees did not significantly differ from that in trees growing in MF plots ($P > 0.05$) (Fig. 2C). As MF trees showed high Tr, their WUE was lower than that observed in trees in the CK and DF treatments. In fact, WUE of CK trees was significantly higher than those of trees in MF and DF plots, at all PAR intervals (Fig. 2D). The LS of CK trees was also high. Particularly, LS of MF trees was initially significantly lower than those of CK and DF trees ($P < 0.05$). This gap gradually narrowed

with increasing PAR. The gaps among the three cultivation models virtually disappeared at high PAR intensity ($> 1,500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Fig. 1C). The utilization rates of the three photosynthetic elements were higher for trees in MF and CK plots than those for trees in DF plots. These along with the preceding data suggest that the MF cultivation model did not induce stress in *C. illinoensis*. In contrast, the poor performance of *C. illinoensis* under the DF cultivation model suggests a considerable level of stress in this case.

Chlorophyll fluorescence trends were similar for all three cultivation models during the interval from Phase O to Phase J (Fig. 3A–3B). After Phase J, however, chlorophyll fluorescence of leaves in DF plots began to differ from that of trees in CK and MF plots. The leaf chlorophyll fluorescence in DF plots changed more significantly than that CK plots. While leaf chlorophyll fluorescence values in MF and DF changed in a similar manner, the latter changed after the former. The fluorescence in DF plots was closer to that of CK plots, and the difference with respect to MF plots was greater. However, in general, the fluorescence of leaves in MF and DF plots was less indicating that the transport efficiency of electrons in the photosynthetic electron transport chain might be higher. For a long period after phase J, the fluorescence yield in leaves from MF and DF plots was lower than that in leaves from CK plots, but then, before phase I, the fluorescence yield increased to a certain extent. Fluorescence yield in MF plots showed a narrow peak near phase I, and that in DF plots showed a very low peak.

Among their fluorescent kinetic parameters, the three cultivation modes differed mainly in terms of quantum yield and reaction center density (Fig. 4). The F_v of MF was significantly higher than those of CK and DF. The ϕ_{P_0} and ϕ_{D_0} of MF substantially differed from that of CK. ET_o/CS_m , ABS/CS_m , TR_o/CS_o , and TR_o/CS_m were considerably higher for MF than CK and DF. Hence, the electron transport and light energy harvesting abilities were higher under MF than CK and DF. The ψ_o of DF was significantly higher than that of CK. Overall, though, there were no significant differences between DF and CK in terms of their hydrodynamic parameters.

Correlation analysis

Correlation analysis revealed that practically none of the chlorophyll fluorescence parameters was significantly associated with P_n ($P > 0.05$). However, several of them, were significantly correlated with C_i , Cond, Tr, WUE, and LS ($P < 0.05$). Further, there was an apparent threshold near the light compensation point for the factors correlated with Cond and LS.

On the other hand, leaf physiology correlated with photosynthetic capacity in a light intensity-dependent manner, and there were apparent boundaries. There was seemingly a threshold near the light compensation point. Chl a, Rubisco, POD, SOD, MDA, and N were strongly significantly and negatively correlated with photosynthetic capacity at $PAR < 60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ($P < 0.05$), below the light compensation point (Fig. 5). In contrast, PNUE, PPUE, and PKUE all showed a strong and significantly positive correlation with photosynthetic capacity above the light compensation point ($P < 0.05$). Conversely, the

correlations between absolute P and K contents, and photosynthetic capacity, were weak and non-significant.

In addition, photosynthesis was related to some other indicators. Fresh and dry leaf weight and leaf length, width, area, and perimeter were significantly and positively correlated with LSP ($P < 0.05$), while area convexity was significantly and negatively correlated with it ($P < 0.05$). When light intensity was $> 250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, C_{ir} was significantly and negatively correlated with photosynthetic capacity. Aspect ratio, rectangularity, sphericity, and eccentricity were somewhat correlated with photosynthetic capacity in the mid- to high light intensity levels ($0.05 < P < 0.1$).

Further, among the physiological indices measured, N was significantly correlated with most fluorescent dynamic parameters ($P < 0.05$), and, except for P and K contents, other physiological indices were somewhat correlated with most fluorescent dynamic parameters. V_i , RE_o/RC , δRo , reaction center density, and the performance indices of the chlorophyll fluorescence parameters were correlated with leaf morphology and anatomy ($P < 0.05$). Lastly, some geometric parameters, such as petiole perimeter conductivity, circularity, and form factor were also significantly correlated with numerous gas exchange parameters ($P < 0.05$).

Principal component analysis (PCA)

Apparent photosynthesis, chlorophyll fluorescence, photosynthetic physiological substances, stress resistance-related physiology, and leaf shape were the main indices selected for principal component analysis (PCA). Two principal components were extracted from apparent photosynthesis, which included 97.11% of the original data. Five principal components were extracted from the chlorophyll fluorescence indices and included 97.14% of the original data. Two principal components were extracted from the photosynthetic physiological substances and included 79.98% of the original data. Only one principal component was extracted from stress resistance-related physiology and included 73.13% of the original data. Three principal components were extracted from the leaf morphology indices and included 80.66% of the original data.

The values obtained for P_n and WUE under various PAR levels during daytime ($< 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were the main apparent photosynthetic indices used for PCA. Principal component 1 and principal component 2 represented most of the information of P_n and WUE under medium and high PAR. The differences between the CK and MF treatments were mainly reflected in the first quadrant; similarly, the differences between the CK and DF treatments were mainly reflected in the first quadrant, but the differences between the MF and DF treatments were mainly reflected in the second quadrant. (Fig. 6A). Based on principal component expression, the scores for the apparent photosynthetic parameters under the various cultivation models were in the order $CK > MF > DF$.

Except for chloroplast fluorescence corresponding to the O point, all chloroplast fluorescence within the 0–1 s time interval comprised the main chlorophyll fluorescence index for PCA. Principal component 1 and principal component 2 contained the majority of the information of fluorescence kinetic parameters.

The main difference between the three cultivation methods was reflected in principal component 1. Treatments CK, MF, and DF showed an obvious separation on principal component 1, while the three cultivation methods showed no obvious separation on principal component 2 (Fig. 6B). Based on principal component expression, the scores for the apparent photosynthetic parameters under the various cultivation models were in the order DF > MF > CK.

The main leaf physiology parameters, including Chl a, Chl b, Rubisco, and chemo-metrics, constituted the central photosynthetic physiological substances for PCA. Principal component 1 integrated nearly all parameters except N and P. In turn, principal component 2 integrated certain data for N and P. The three cultivation patterns significantly differed in terms of principal component 1, and CK, MF, and DF differed from CK in terms of principal component 2 (Fig. 6C). Based on principal component expression, the scores for the photosynthetic physiological substances under the various cultivation models were in the order DF > MF > CK. In addition, CK, MF, and DF are separated from each other on the only principal component of the physiological index of stress resistance. Based on principal component expression, the scores for the stress resistance-related physiology under the various cultivation models were in the order MF > CK > DF.

The main leaf morphological parameters, including fresh weight, area, perimeter, and anatomical structure comprised the main leaf shape index for PCA. The first principal component integrated the leaf macro parameters and the leaf vein and petiole parameters. Meanwhile, the second principal component integrated leaf, palisade layer, and spongy layer thickness. The third principal component integrated the leaf epidermis thickness and petiole parameters. The fourth principal component integrated the leaf small vascular bundle parameters. There were significant differences among the three cultivation models in terms of their principal components 1 and 2. CK was significantly different from the other two models in terms of principal component 3 (Fig. 6D). Based on principal component expression, the scores for the morphological parameters under the various cultivation models were in the order DF > CK > MF. In terms of a comprehensive score, the total score of CK was the highest among the three cultivation models, and the total scores of MF and DF were similar, but MF performed better on the more important indexes of apparent photosynthesis and stress resistance-related physiology. The total score of MF was also slightly higher than that for DF; therefore, the MF was considered to perform better than the DF cultivation model.

Discussion

Changes in photosynthetic capacity under various complex environments

A mixed forest is more complex than a pristine forest. In the former case, light [13, 54], nutrients [54], space [18], and other resources change to varying degrees. Mixed forests generally increase tree productivity and/or resilience [55–57]. However, competition occurs in mixed forests because of the complex environmental changes associated with them [18, 58]. Mixed forest productivity may

significantly improve under benign competition. Nevertheless, irreversible losses may occur in the entire forest system under adverse conditions [58].

Plant photosynthetic capacity is directly related to plant productivity [59]. Here, Pn of *C. illinoensis* markedly varied with cultivation model. PCA revealed that CK trees showed the highest apparent photosynthetic capacity, a suitable environment, and no interspecific competition. In contrast, the photosynthetic capacity of trees in MF plots was lower than that of CK trees but stronger than that of trees in DF plots. Further, PCA showed that the difference between CK and MF was nearly equal to the difference between MF and DF. This may mean that the intensity of interspecific competition between *C. illinoensis* and *P. edulis* has not reached a very high level. The addition of the latter partially lowered the photosynthetic capacity of the former, especially at high light intensity. Nevertheless, the competition was not particularly strong. In contrast, the intense intra-specific competition brought by high-density planting will cause greater damage to *C. illinoensis*. Thus, interspecific competition was significantly lower under MF than under DF. This finding is consistent with previous reports [58]. When species are mixed in a relatively stable environment, its complementary effect may not entirely offset the impact of competition. It was nonetheless appropriate to mix *C. illinoensis* with *P. edulis*, although the productivity of *C. illinoensis* somewhat decreased in co-plantation with *P. edulis*, the productivity of the latter must also be considered. Moreover, the photosynthetic rate of *C. illinoensis* declined in MF treatment plots. Mixed *P. edulis* forests have ecological advantages over *P. edulis* monoculture forests, as the latter are poor with respect to biodiversity [60].

The impact of the photosystem on photosynthetic capacity must also be regarded. The OJIP curve has been widely applied to express the photochemical quantum yield and electron transport activity of PS [61–63]. This study showed that the main changes in the OJIP curves of trees in MF and DF plots occurred near Phase I. The fluorescence intensities under MF and DF treatments rapidly increased before and after Phase I, and slowly increased during Phase I. Before Phase J, the trends were nearly identical for all three cultivation models. Phase J is the result of QA- accumulation. Thus, neither interspecific nor intraspecific competition affected electron transfer from Pheo to QA- [64–66]. It is generally believed that PQ heterogeneity causes Phase I. Before Phase I, rapidly decreasing PQ is being restored. After Phase I, slowly decreasing PQ is being restored [66]. The sharp increases in fluorescence intensity before and after Phase I and the relatively moderate change in fluorescence during Phase I indicate that interspecific and intraspecific competition impede PQ reduction. Figure 3B shows that MF slightly was different from CK before Phase I, The fluorescence yield at this stage of MF is lower compared to CK, and sharply increased during Phase I. By contrast, DF sharply increased both before and after Phase I. Therefore, interspecific competition only strongly affects slow PQ reduction. Strong intraspecific competition will fully restore damaged PQ. Here, the Cond and Ci of MF were higher while Pn in MF plots was lower than that under CK, possibly because PQ restoration was hindered. Until the causes of Phase I formation are fully elucidated, no further benchmarking can be performed [62].

Responses of leaf morphology to various cultivation patterns

As plants acclimation to different environments, their leaf morphology changes and becomes heterogeneous. Leaf shape is consistent with the plant ecological niche [29, 31, 67]. Specifically, leaf macromorphology is determined by light, temperature, water, and other factors [68]. Leaf shape and size are closely related to light protection and competition [69]. Further, leaf veins and petioles transport water and photoassimilate between leaves and stems [70–73]. Here, we observed changes in leaf morphology under the various cultivation models tested. The leaf area was relatively lower under MF treatment as interspecific competition occurred in those plots. A reduction of leaf area can alleviate the pressure of interspecific competition, as small leaves reduce self-shading and increase light access [69]. Nevertheless, DF did not show this effect despite the strong intraspecific competition under this cultivation model. It is proposed that individual leaves may become prominent, such that they can acquire more light, to become more photocompetitive [74]. The diametrically opposite performances of these types of competition may be explained by the relative differences in *P. edulis* and *C. illinoensis* morphology. While *P. edulis* has a small crown and poor vertical light interception capacity, *C. illinoensis* has a relatively larger crown. Consequently, the mixture of *P. edulis* with *C. illinoensis* would reduce self-shading and ensure that the lower leaves can compete for light. Fair competition among *C. illinoensis* individuals favors light capture by the upper canopy. The geometric parameter measurements showed that the leaves of *C. illinoensis* were comparatively rounder under MF treatment. Yield may increase with leaf roundness in certain plants [71].

In certain prior studies on the relationship between anatomical structure and photosynthesis, emphasis was placed on leaf thickness, intercellular space, and stomatal morphology [67, 75, 76]. Some of these indices were evaluated herein. There were relatively more empty spaces in the palisade layers of tree leaves in the MF and DF plots than in CK trees, possibly because of competition among chloroplasts in the palisade mesophyll. Certain stress factors may contribute to this condition [31, 67]. On the other hand, we observed no significant changes or differences in leaf, palisade layer, or spongy layer thickness among cultivation models. We did, however, detect differences among treatments in terms of photosynthetic capacity and leaf size. Moreover, relative differences in leaf anatomy were reflected mainly in the palisade layer widths. The palisade layer was comparatively denser under MF than it was under DF or CK treatments. Hence, there were relatively more chloroplasts on the light-receiving surfaces of the palisade layer of trees leaves in MF plots. This configuration compensated for the empty spaces between chloroplasts. The petiole is a vital photosynthate transport channel [70]. However, there were few differences among cultivation models in terms of the petioles. The xylem and phloem areas in the vascular tissues were effectively the same across treatments. In contrast, the midvein vascular-tissue areas differed among cultivation models. trees in DF plots had relatively more prominent leaf veins with comparatively larger vascular tissue, phloem fiber, phloem, and xylem areas. Therefore, the leaf blades had more robust translocation and mechanical support capacities under DF than under MF or CK treatment [77, 78]. Leaf vein reinforcement might improve competitiveness in poor environments. Several reports have claimed that plants increase their leaf vein areas and densities to acclimated to the environment [79, 80].

Relationships among photosynthesis, fluorescence dynamics, leaf physiology, and leaf shape

Morphology is the basis of plant function, while antioxidant enzymes indicate the oxidative status of plants [32, 33, 81]. However, not all indices are strongly correlated. Thus, clarifying the correlations among indices should facilitate screening for them in future research endeavors. This study conducted PCA on various leaves. The principal component scores for the physiological indices were significantly and strongly correlated with those for the other three types of indices. In contrast, this and some previous studies demonstrated that the correlation between leaf structure and photosynthetic capacity was not obvious. Under some circumstances, leaf physiology seemingly expresses photosynthetic capacity more effectively than leaf anatomy. However, anatomical changes are not necessarily synchronized with changes in photosynthetic capacity [75, 76, 82].

Leaf physiology indices may be significantly correlated with various exchange parameters when the indices are subdivided. Particularly, under PAR levels below LCP, most leaf physiological indices were significantly correlated with Pn. In contrast, these correlations weakened and became non-significant at PAR levels above LCP.

Chlorophyll fluorescence parameters were seldom significantly correlated with Pn. However, this finding does not necessarily imply that chlorophyll fluorescence parameters have no influence on surface photosynthesis. In fact, several chlorophyll fluorescence parameters were significantly correlated with other gas exchange parameters besides Pn. However, some of these associations differed below and above LCP.

Conclusions

Our research proved that competition increased and *C. illinoensis* photosynthetic capacity decreased under both MF and DF relative to the control treatment, to varying degrees. Changes in *C. illinoensis* leaf morphology differed between MF and DF in response to the specific conditions of competition in each environment. In competitive environments, leaf veins developed to a greater extent than leaves or petioles. Hence, leaf vein anatomical structure should be the focus of future investigations into plant morphology in complex environments. The decline observed in photosynthetic capacity under a competitive mixed forest system might have resulted from the inhibition of PQ reduction in the photosystem. Leaf physiology indices were more closely associated with Pn than fluorescent dynamic or leaf morphology indices. Leaf physiology mainly affected LCP formation, while leaf macromorphology primarily influenced LSP. The relationships among fluorescent dynamic parameters, anatomical structure, and Pn were not synchronous. Fluorescent dynamic parameters may also be associated with stomatal movement and the palisade layer. The geometrical configurations of leaf vein vascular tissue and the petiole linked anatomical structure and photosynthetic capacity. Leaf macromorphology and anatomical structure were closely related to reaction center density. Leaf nitrogen content had a significant impact on

the photosystem. *C. illinoensis* trees were subjected to stress in both MF and DF forest models. Nevertheless, the productivity declined and the morphology changed to a lesser extent under the former than under the latter. Thus, *C. illinoensis* tolerated stress relatively more effectively under MF conditions. Therefore, *C. illinoensis*-*P. edulis* co-plantation may be a feasible afforestation strategy involving a trade-off highly advantageous for local rural economies.

Declarations

Acknowledgments:

We thank Huang Xuan and Zhou Lizhen for their great help to the field work of this experiment.

Author Contributions

Methodology, Wang Anke, Du Xuhua and Bi Yufang.; investigation, Lu Qifan, Zhang Xu, Liu Shenghui, Zhou Zhou, Wang Zijie, Zhong Hao and Ning Yilin; resources, Yu Qunfen; data curation, Lu Qifan, Zhang Xu, Liu Shenghui, Zhou Zhou, Wang Zijie, Zhong Hao and Ning Yilin; writing—original draft preparation, Lu Qifan and Zhang Xu; writing—review and editing, Lan Lan.

Conflicts of Interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Funding

This study is funded by the (1) Fundamental Research Funds for the Central Non-profit Research Institution of Chinese Academy of Forestry (Grant No. CAFYBB2019MB005). (2) the People's Government of Zhejiang Province-Chinese Academy of Forestry cooperative project (Grant No. 2019SY01) (3) the key research and development program of Zhejiang Province (Grant No. 2020C02008) (4) the National Natural Science Foundation of China (Grant No. 31600448).

Data Availability Statement

Not applicable

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Figures

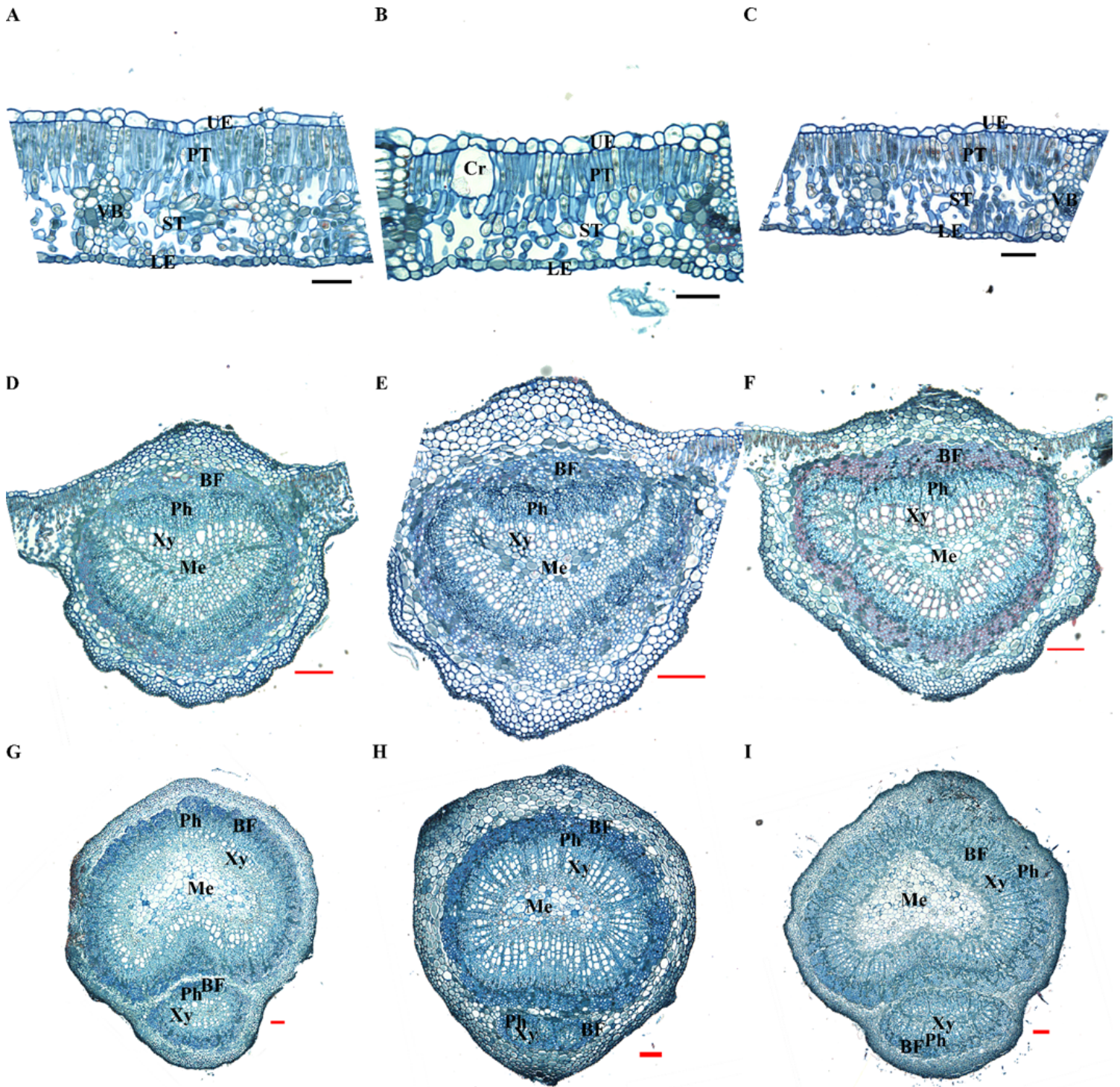


Figure 1

Anatomical structure of different parts

A is the leaf anatomical structure of control group (CK); **B** is the leaf anatomical structure of mixed forest (MF); **C** is the leaf anatomical structure of densely planted forest (DF); **D** is the anatomical structure of the central leaf vein of CK; **E** is the anatomical structure of the central leaf vein of MF; **F** is the anatomical structure of DF's central leaf vein; **G** is the petiole anatomical structure of CK; **H** is the petiole anatomical

structure of MF; I is the petiole anatomical structure of DF. UE refers to the upper epidermis, LE refers to the lower epidermis, PT refers to palisade tissue, ST refers to spongy tissue, VB refers to the vascular bundle, and ME refers to the central medulla; Xy refers to the xylem, Ph refers to the phloem, and BF refers to phloem fiber. The length of the black line is equal to 20 μm . The size of the red line is equivalent to 50 μm .

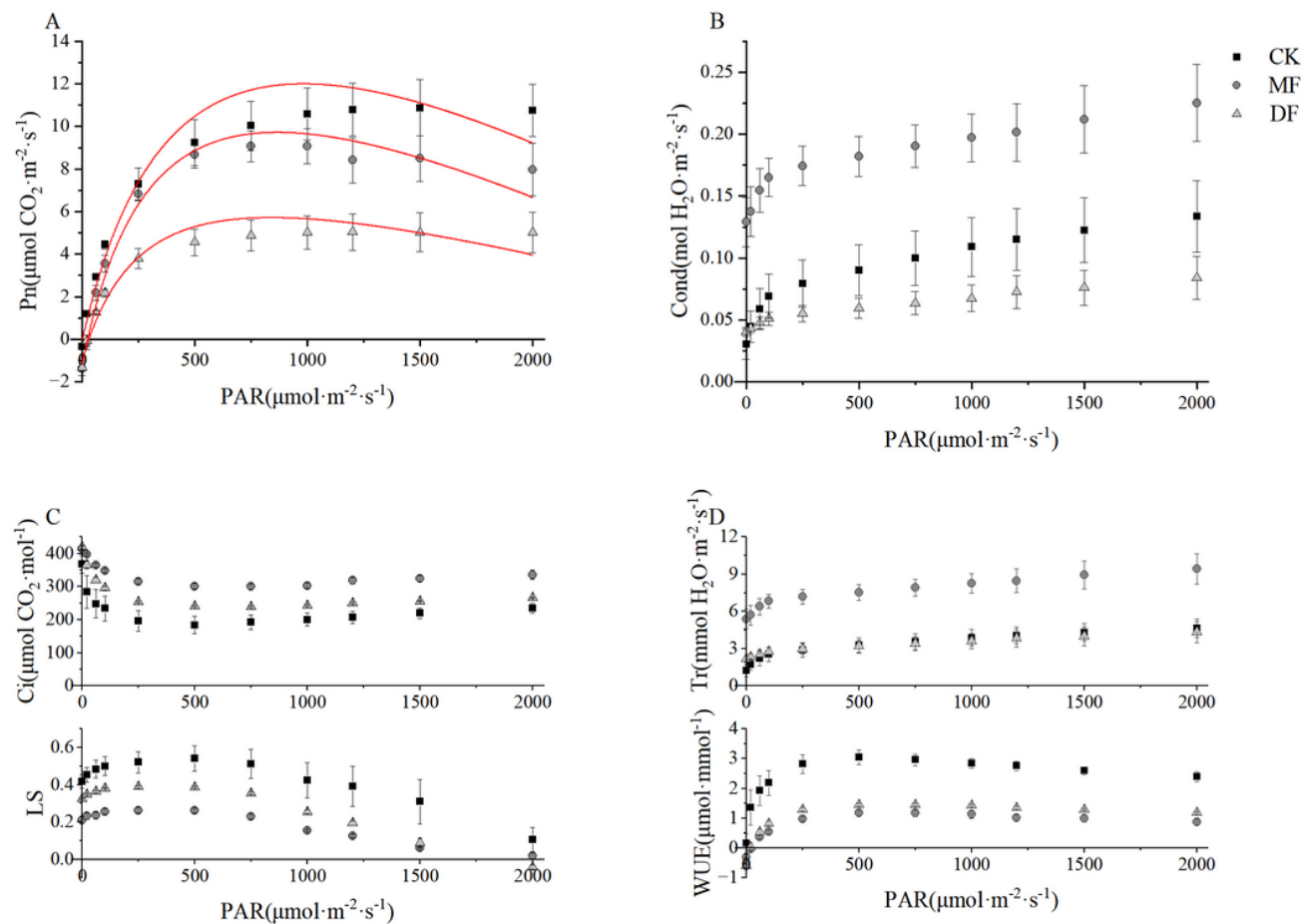


Figure 2. Comparison of photosynthetic characteristics under different cultivation modes

A is the net photosynthetic rate(Pn) under different PAR, The red line in the figure is the light response curve fitted by the formula; **B** is the stomatal conductance(Cond) under other PAR; The figure in the upper part of **C** shows the intercellular carbon dioxide concentration(Ci) under different PAR, and the figure in the lower part shows the stomatal limitation($LS=1-\frac{C_i}{C_a}$, C_a means the concentration of carbon dioxide in the atmosphere)[52]; The figure in the upper part of **D** shows the trmmol(Tr) under different PAR, and the figure in the lower part shows the water use efficiency($WUE=\frac{P_n}{T_r}$) [53]. The error bar in the figure is SE. The same is below.

Figure 2

See image above for figure legend

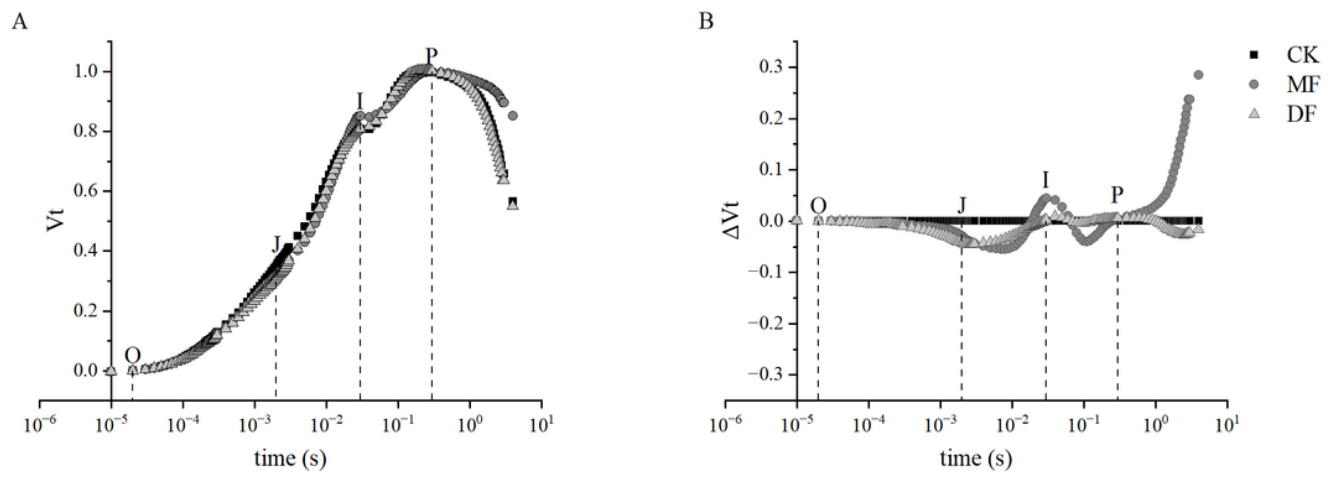


Figure 3

Standardized OJIP curves and relative variable fluorescence under different cultivation Patterns

A is the standardized OJIP curve; **B** is the relative variable fluorescence based on CK.

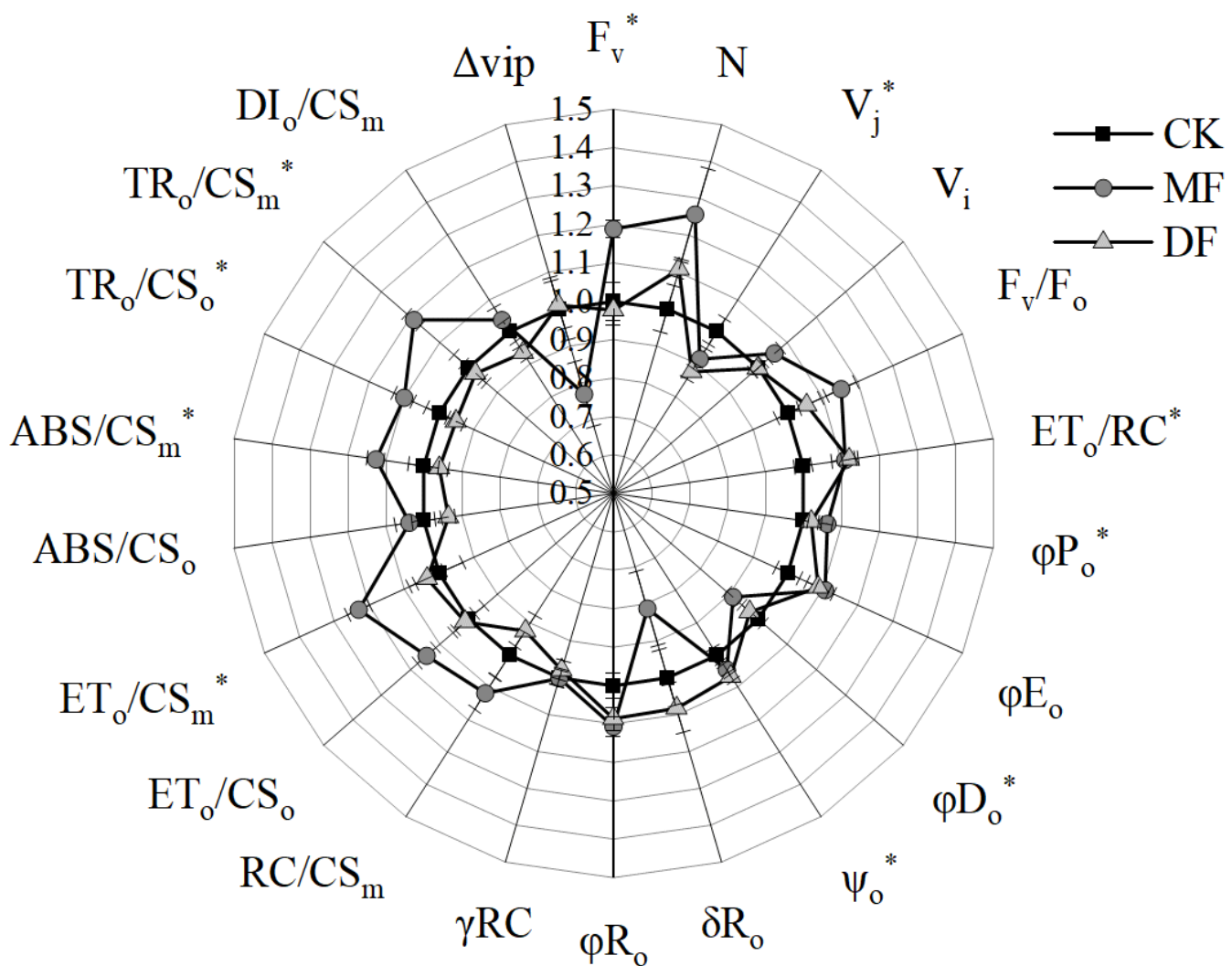


Figure 4

Radar map of chlorophyll fluorescence parameters under different cultivation modes

The above are the main chlorophyll fluorescence parameters with differences at the level of 0.1. All values have been relatively processed based on CK. * represents that there are differences at the level of 0.05.

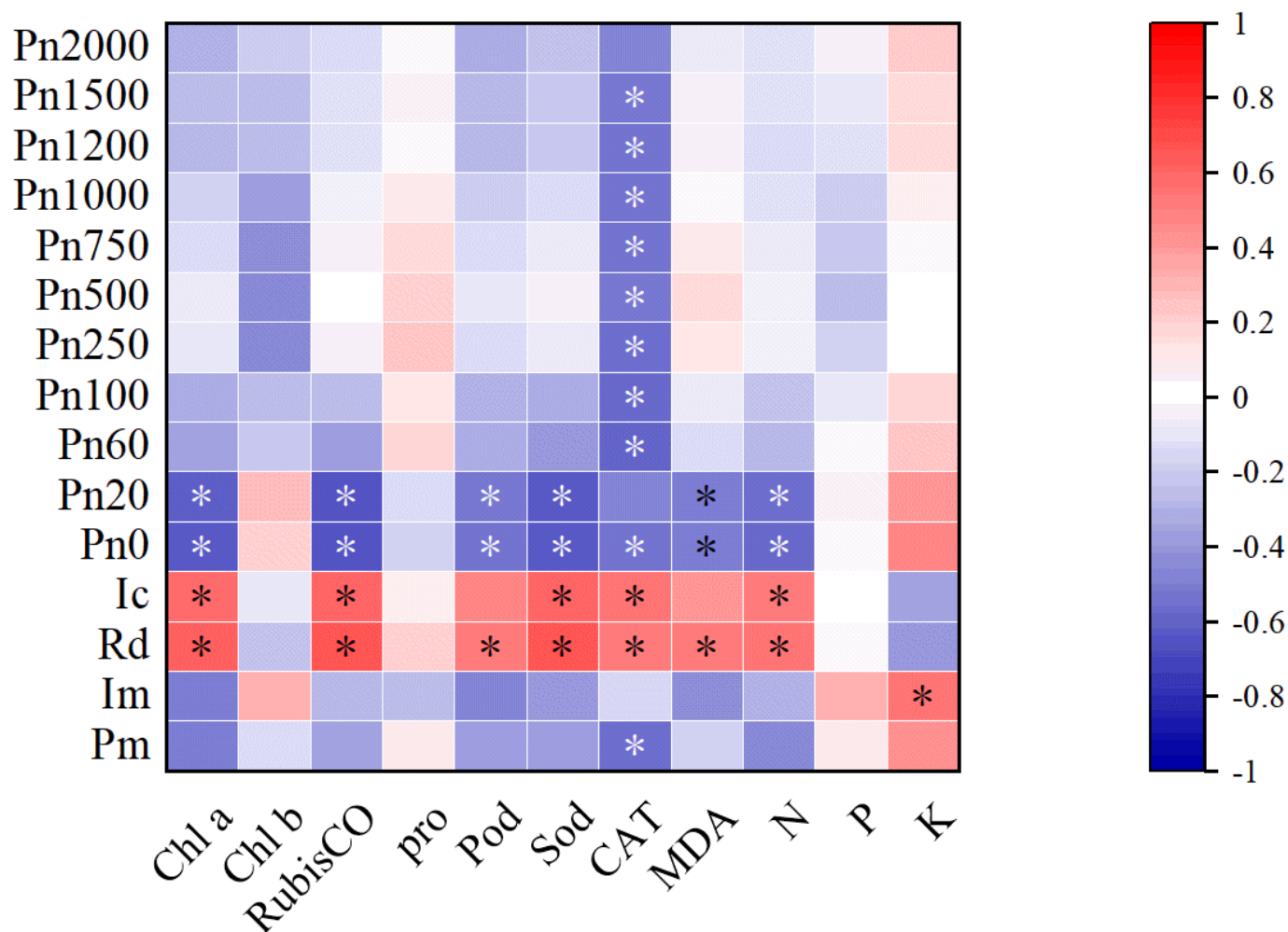


Figure 5

Heat diagram of a correlation between leaf physiology and Pn

The number after Pn represents the corresponding PAR. * represents that there are differences at the level of 0.05. Red represents a positive correlation, blue represents a negative correlation, and the darker the color, the greater the absolute value of the correlation coefficient.

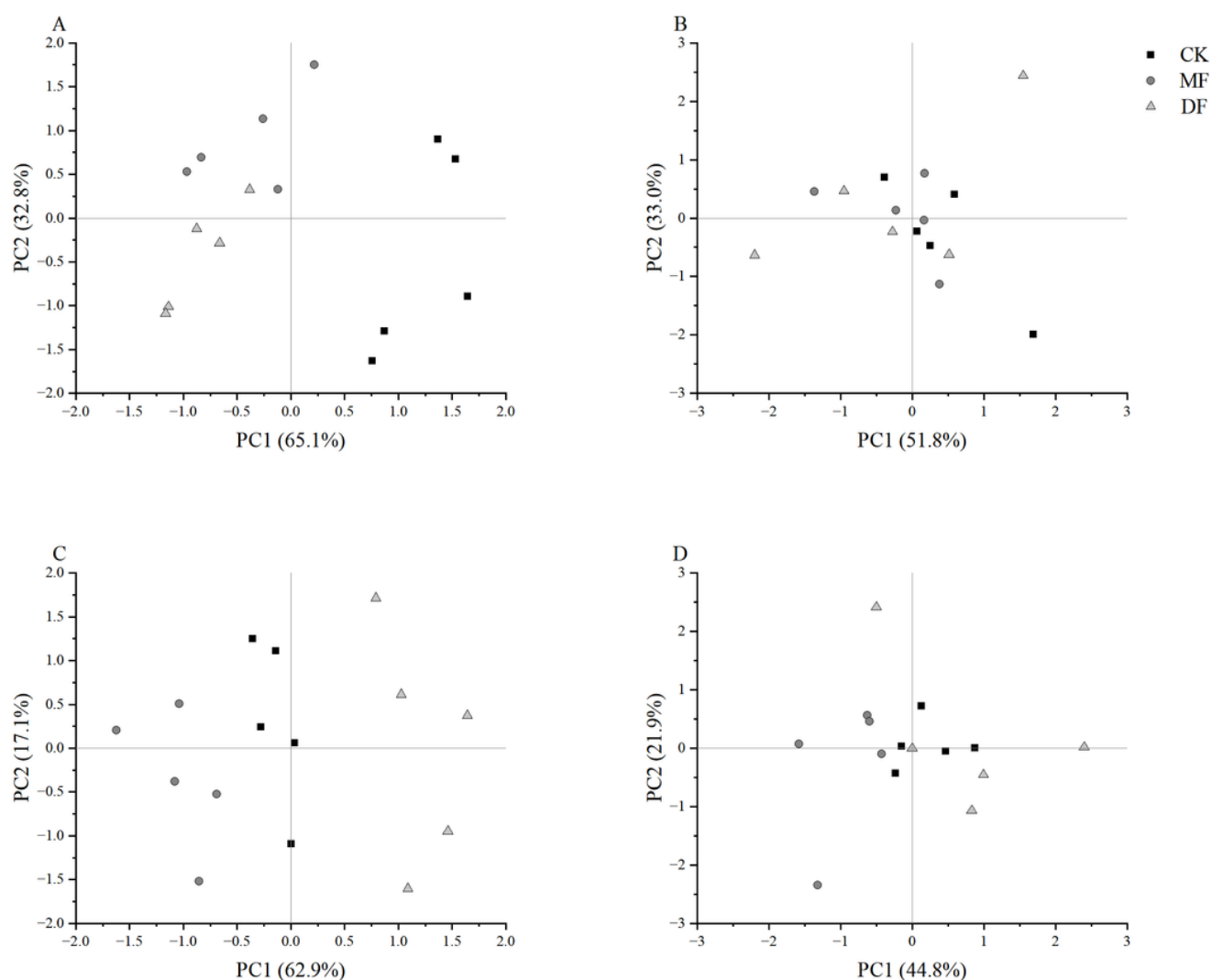


Figure 6

PCA diagram

A is the principal component analysis diagram of the apparent photosynthesis parameter; B is the principal component analysis diagram of the first two principal components of chlorophyll fluorescence parameters; C is the principal component analysis diagram of the first two principal components of photosynthetic physiological substances; D is the principal component analysis diagram composed of the first two principal components of leaf morphological parameters.