



Research article

Effects of continuous monoculture on rhizosphere soil nutrients, growth, physiological characteristics, hormone metabolome of *Casuarina equisetifolia* and their interaction analysis

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ABSTRACT

Continuous planting is unavoidable in agricultural production, but continuous planting affects plant growth and physiological characteristics. In this study, we analyzed rhizosphere soil nutrients, physiological characteristics, hormone metabolome changes and their interactions of *Casuarina equisetifolia* (*C. equisetifolia*) with the increase of continuous planting number. The results found that *C. equisetifolia* root was significantly inhibited, the plant height was dwarfed and the biomass was significantly reduced as continuous planting number increased. Secondly, continuous planting caused a decrease in the rhizosphere soil nutrient transformation capacity, and a significant decrease in the total soil nutrient and available nutrient content. Analysis of physiological indexes showed that continuous planting resulted in a decrease in nitrogen, phosphorus, and potassium content, a decrease in the activity of physiological indexes of resistance, and a decrease in photosynthetic capacity of *C. equisetifolia* leaves. Hormone metabolome analysis showed that continuous planting critically affected the accumulation of five characteristic hormones in *C. equisetifolia* leaves, in which salicylic acid 2-O- β -glucoside (SAG), 2-oxindole-3-acetic acid (OxIAA), *trans*-zeatin-O-glucoside (tZOG) and gibberellin A3 (GA3) content decreased significantly while abscisic acid (ABA) content increased significantly. In conclusion, continuous planting lowered the rhizosphere soil nutrient transformation capacity of *C. equisetifolia*, lowered the soil available nutrient content, inhibited their root growth, and hindered the nutrient uptake and transportation by the root, thus led to the decrease of the nutrient accumulation capacity in the leaves of *C. equisetifolia*, and the decrease of SAG, OxIAA, and tZOG, GA3 synthesis ability decreased, ABA accumulated in large quantities, *C. equisetifolia* resistance and photosynthesis ability decreased, and their growth was impeded. This study provides insights for the effective management of continuous planting in the cultivation of *C. equisetifolia*.

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1. Introduction

Casuarina equisetifolia is an evergreen tree of Casuarinaceae that is native to Oceania, Pacific islands, and Southeast Asia. It was introduced to China in the 1950s, primarily in the coastal regions of Guangdong Province, Fujian Province, Hainan Province, and other regions along China's southern coast [1]. *C. equisetifolia* is an excellent pioneer tree species with a straight trunk reaching up to 30 m in height and a maximum diameter at breast height of 70 cm. It has a deep and extensive root system and a narrow and long conical crown. The leaves are scale-like and the flowers bloom in April–May while the fruit ripens from July to October annually. The species is primarily propagated by cuttings. It has become a vital part of soil and water conservation efforts, prevention of tidal erosion, and the establishment of agroforestry composite systems in the coastal zone [2]. Due to the limited number of tree species suitable for planting in coastal sandy areas, *C. equisetifolia* has been a primary species selected and has continued to be replanted in situ after each harvest [3]. As the number of in situ replanting increased, there were significant changes observed in *C. equisetifolia*. It is evident that the average growth rate decreased by a 1–2 grade, while the tree height, breast diameter and wood volume decreased by about 23.7%, 24.4% and 29.0%, respectively [4]. The emergence of this phenomenon has imposed severe restrictions on the sustainable development of the protection forest resources of *C. equisetifolia*'s.

At present, research on continuous planting barriers in planted timber forests has mainly focused on six tree species: *Cunninghamia lanceolata*, *Eucalyptus robusta*, *Pinus massoniana*, *Larix gmelinii*, *Pinus taeda*, *Pinus elliottii* and *Picea koraiensis* [5–8], but there is a paucity of studies dedicated to *C. equisetifolia*, and the mechanisms underlying the existence of continuous planting obstacles for this species remain under investigation. Past research has explored the alterations in microbial diversity in rhizosphere soil of *C. equisetifolia* during initial cultivation and repeated continuous planting. These studies have shown that there was an increase in the number of pathogenic microorganisms in rhizosphere soil, and a concurrent decrease in the number of probiotic bacteria. Furthermore, continuous planting results in a reduction in the number of microorganisms involved in the nutrient cycle of *C. equisetifolia* in rhizosphere soil, a decrease in the expression levels of relevant genes, and a decline in soil available nutrients, ultimately leading to reduced nutrient absorption and accumulation in *C. equisetifolia* [9–11].

With continuous planting, plants' ability to absorb and accumulate soil nutrients is negatively impacted, and directly or indirectly affecting plant hormone synthesis [12]. Secondly, the absorption and accumulation of nutrients within plants greatly influence their photosynthesis and antioxidant capacity. An increased nutrient absorption and accumulation capacity stimulates plant chlorophyll synthesis, elevates plant photosynthesis capacity, and boosts antioxidant capacity, thereby stimulating plant growth [13,14]. Plant hormones are simple small-molecule organic compounds that possess incredibly diverse and complex physiological effects. They play a significant role in regulating plant growth and development. Adversity stress, however, influences the hormone content and distribution of plants, subsequently altering plant growth [15]. During drought stress, the content of ABA and jasmonic acid in plants increases, which results in a decrease in the resistance physiological indexes of plants and an associated drop in yield [16]. Nutrient deficiencies prompt plants to synthesize jasmonic acid and its derivatives, which serve to regulate their nutrient uptake [17]. By applying exogenously gibberellin and salicylic acid to plants that are under salt stress, it is possible to boost plant resistance, thereby increasing the plant yield significantly [18]. It is evident that environmental stress significantly alters plant hormones, which can in turn alter plant physiology, ultimately affecting plant growth [19]. To date, there have been few studies on the effects of continuous planting on the growth, rhizosphere soil nutrient and hormone synthesis of *C. equisetifolia*. This study aims to uncover the impact of continuous planting on the rhizosphere soil nutrient content of *C. equisetifolia*, as well as the mechanisms behind the physiological changes triggered by these changes. The ultimate goal is to understand how these modifications contribute to the growth of *C. equisetifolia* growth.

For this study, the root soil of *C. equisetifolia* with different numbers of continuous planting was used to pot *C. equisetifolia* seedlings in this study. A year later after the experiment began, the growth indexes of *C. equisetifolia*, the physicochemical indexes of the rhizosphere soil, and the root morphology indexes were evaluated. This helped to assess the effects of continuous planting on *C. equisetifolia*'s growth. Simultaneously, the resistance physiological indexes, the photosynthetic physiological indexes and the hormone metabolome of *C. equisetifolia* leaves were also analyzed to understand how continuous planting affected plants' physiological mechanisms and hormone synthesis. On this foundation, this study further scrutinized the interactions of different indexes to elucidate the causes of the stunted growth of *C. equisetifolia* as a result of continuous planting. Additionally, it provided valuable guidance for the cultivation and management of *C. equisetifolia* in continuous planting.

2. Materials and methods

2.1. Experimental design and sampling

The rhizosphere soil of *C. equisetifolia* at different continuous planting numbers was collected and used to replant *C. equisetifolia* seedlings in pots, and the experimental indexes were measured after planting for 1 year to analyze the effect of continuous planting soil on *C. equisetifolia* growth. The rhizosphere soil of *C. equisetifolia* with different continuous planting numbers was obtained from the national protective forest farm in Chihu Town, Hui'an County, Fujian Province, China (118°55' E, 24°35' N). The total area of the forest farm is approximately 433 ha², and *C. equisetifolia* was first planted in 1987 and replanted in situ with *C. equisetifolia* in 2011 after part of the forest farm was harvested. The associated tree species and undergrowth vegetation were basically the same in different forest farms, with a small amount of *Koelreuteria elegans* and *Litsea glutinosa* as associated tree species and *Ageratum conyzoides* and *Bidens pilosa* as undergrowth vegetation. The pH of the soil was pH 5.6, its organic matter, total nitrogen, total phosphorus, total potassium, available nitrogen, available phosphorus, available potassium contents were 3.58 cmol/kg, 4.05 g/kg, 0.53 g/kg 1.28 g/kg 10.24 mg/

kg 3.98 mg/kg 68.26 mg/kg, respectively.

In March 2022, rhizosphere soil was collected from the first planting and the second continuous planting of *C. equisetifolia*, respectively, along with soil from the same area that had not been planted with *C. equisetifolia*, for a total of approximately 180 kg of each soil type. A replicate of 60 kg soil was collected from the rhizosphere of 20 randomly selected *C. equisetifolia*. To obtain this replicate, leaf litter was carefully removed, the top 10 cm of soil was scooped up, and a soil sample was collected within a radius of 10 cm from the main trunk of the plant. The soil was then thoroughly mixed between 10 and 40 cm deep. Within the unplanted *C. equisetifolia* area, twenty points were randomly selected, each located over 5 m apart. The leaf litter layer was carefully removed, the upper soil layer was scooped out about 10 cm layer by layer, and the soil within a radius of 10 cm and a depth of 10–40 cm was carefully collected. The collected soil was then thoroughly mixed to create a replicated sample weighing approximately 60 kg. Three independent replicate samples were collected from different soils.

In April 2022, the collected soil was cleaned litter and residual roots, thoroughly mixed, and then evenly divided and packed into pots of 24 cm in diameter and 25 cm in height, with each pot holding a total of 9 kg of soil. The uniform *C. equisetifolia* seedlings (plant height 28 cm) were evenly divided into pots of 2 plants each. *C. equisetifolia* transplanted to blank soil was the first planting, labeled M1; transplanted to soil that has been planted once with *C. equisetifolia* was the second continuous planting, labeled M2; and transplanted to soil that has been continuously planted twice with *C. equisetifolia* is the third continuous planting, labeled M3. The experiment consisted of 3 treatments with 12 pots of each treatment (4 pots for one replicate and 3 replicates) for a total of 36 pots. The transplanted *C. equisetifolia* seedlings were placed in an outdoor greenhouse and watered daily in the evening during planting at a rate of 200 mL per pot. In May and November 2022, compound fertilizer (N: P: K = 21:8:16) was uniformly applied to *C. equisetifolia* at a rate of 3 g per pot per application.

In April 2023, after planting *C. equisetifolia* for 1 year, plant height, photosynthetic physiological indexes of *C. equisetifolia* were determined, while *C. equisetifolia* roots, rhizosphere soil, and leaves were collected from different treatments for root scanning analysis, determination of rhizosphere soil physicochemical indexes, determination of nutrients content of leaves, leaf resistance physiological indexes and hormone metabolome of leaves. *C. equisetifolia* rhizosphere soil and leaves were sampled as follows: two pots were randomly selected in each replicate, *C. equisetifolia* seedlings were gently pulled out, and after gently shaking, the soil that remained adhered to the roots was meticulously collected and evenly mixed, which was the rhizosphere soil, with one replicate containing approximately 100 g of rhizosphere soil. At the same time, the mature fresh leaves of *C. equisetifolia* were collected and mixed well, which was about 150 g. Each treatment had three independent replicates.

2.2. Determination of morphological indexes

Morphological indexes of *C. equisetifolia* were mainly determined plant height, dry weight and root system indexes. The height of *C. equisetifolia* was measured directly from the point of separation of its stems and roots to the highest point using a tape measure. Dry weight was determined by fixing the whole *C. equisetifolia* plant at 120 °C for 15 min, drying it at 80 °C until constant weight, then determining its weight. *C. equisetifolia* root indexes were determined using a root scanner (Expression 1200XL, Epson, Suwa, Japan) [20]. The method was briefly described as, *C. equisetifolia* root was cleaned using distilled water, cleaned and arranged in transparent root trays with minimal overlap, and scanned using a root scanner with the optical resolution set at 2400 × 4800 dpi. WinRHIZO Pro 2019a software (Regent Instrument Inc, Canada) was utilized to analyze the acquired images, and from this analysis, data on *C. equisetifolia*'s total root length (cm), root surface area (cm²), root average diameter (mm), root volume (cm³), total root tips, root forks number, and root crossings number were obtained.

2.3. Determination of physicochemical indexes of soils

This study mainly measuring soil pH, cation exchange capacity (CEC), organic matter content (OM), total phosphorus (TP), total nitrogen (TN), total potassium (TK), available nitrogen (AN), available potassium (AK), and available phosphorus (AP) [21]. In brief, the collected soil samples were dried naturally in the air and then sieved through a 2 mm mesh to prepare them for the measurement of physicochemical indexes. Among that, the soil pH was measured by the water leaching potential method at a water-soil ratio of 2.5:1. Ammonium acetate exchange method was used to determine the CEC OM was measured by potassium dichromate oxidation method. TN and AN were measured by Kjeldahl method and alkali diffusion method, respectively. TP and AP were measured by molybdenum antimony anti-colorimetric method. TK and AK were measured by flame atomic absorption spectrophotometry.

2.4. Determination of photosynthetic physiological indexes of leaves

In order to evaluate the photosynthetic physiological performance of *C. equisetifolia* leaves, the primary factors taken into account were photosynthetic rate and chlorophyll content. The photosynthetic rate was measured using a LI-6400XT Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). The experiment was conducted on a sunny day between 9 a.m. and 11 a.m. The measurements showed a photon flux density of 1500 μmol/m².s. The ambient CO₂ concentration was 380 ppm. The leaf temperature was maintained at 25–26 °C, and the vapor pressure deficit in the cuvette was kept under 1 kPa. Chlorophyll was extracted by acetone and determined by UV spectrophotometer [22].

2.5. Determination of phosphorus, nitrogen, and potassium content of leaves

The phosphorus, nitrogen, and potassium content of *C. equisetifolia* leaves were determined according to "Experimental instruction in plant physiology" [23]. The leaf samples were first subjected to bio-inactivated at 105 °C for 15 min. They were then dried at 80 °C until reaching a constant weight. The dry leaves were subsequently ground, and sieved through a 60-mesh sieve. Different samples were weighed 0.3 g and transferred in a decoction tube, 5 mL of H₂SO₄ was added, decocted at 170 °C for 30 min, 300 °C for 10 min, 1 mL of H₂O₂ (30%) was added, decocted for 10 min, cooled, and fixed to 100 mL for the determination of the N, P, K content.

2.6. Determination of resistance physiological indexes of leaves

The physiological indexes of resistance in *C. equisetifolia* were mainly measured by peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) activities, content, soluble sugar and malondialdehyde (MDA) contents. Specific methods were referred to "Principles and Techniques of Plant Physiological Biochemical Experiments" [23]. Firstly, 0.5 g of fresh *C. equisetifolia* leaves were added to 5 mL of pre-cooled phosphate extraction buffer (50 mmol/L, pH 7.0, containing 1% polyvinylpyridone). The leaves were then ground in an ice bath, centrifuged at 12,000 rpm/min for 10 min at 4 °C, and the supernatant was collected for determining physiological indexes. Among that, SOD activity was measured by nitro blue tetrazolium chloride method, POD activity was measured by guaiacol colorimetric method, CAT activity was measured by potassium permanganate method, MDA content was measured by the thiobarbituric acid method, and soluble sugar content was measured by anthrone colorimetric method.

2.7. Metabolome determination and quantitative analysis of hormones

C. equisetifolia leaves with different continuous planting number were ground to powder for hormone metabolome analysis. Each sample was replicated thrice for accuracy. Firstly, 50 mg of the ground sample was accurately weighed, followed by the addition of 10 µL of the internal standard mixture with a concentration of 100 ng/mL. Next, 1 mL of methanol/water/formic acid (15:4:1, v/v/v) extractant was carefully added, and thoroughly mixed. After vortexing for 10 min at room temperature, the mixture was centrifuged at 4 °C and 12000 r/min for 5 min, and the supernatant was carefully transferred to a new centrifuge tube. The supernatant was then concentrated using a vacuum rotary evaporator, and re-dissolved in 100 µL of 80% methanol in water. The resulting solution was then passed through a 0.22 µm filter membrane and placed in the autosampler vial for LC-MS/MS analysis [24,25].

Chromatography-mass spectrometry systems utilized for data acquisition included Ultra Performance Liquid Chromatography (ExionLC™ AD, AB Sciex, Concord, Canada) and Tandem Mass Spectrometry (QTRAP® 6500+, AB Sciex, Concord, Canada). The liquid phase conditions used in this experiment are as follows [26]: column: a Waters ACQUITY UPLC HSS T3 C18 column (1.8 µm, 100 mm × 2.1 mm i.d.); mobile phase: comprised of ultrapure water (with 0.04% acetic acid) as phase A and acetonitrile (with 0.04% acetic acid) as phase B; gradient elution program: the composition of A/B changes from 95:5 (V/V) at 0 min to 95:5 (V/V) at 1.0 min, and then to 5:95 (V/V) at 8.0 min, 9.0 min, 95:5 (V/V) at 9.1 min, at 12.0 min; flow rate: a flow rate of 0.35 mL/min was used; column temperature: a column temperature of 40°C was set; injection volume: a volume of 2 µL was used for injection. The experimental conditions for the mass spectrometry analysis included [27] an electrospray ionization (ESI) source temperature of 550 °C, a mass spectrometry voltage of 5500 V in positive ion mode and -4500 V in negative ion mode, and a curtain gas (CUR) of 35 psi. Additionally, in the Q-Trap 6500+, each ion pair was scanned for detection by optimizing the declustering potential (DP) and collision energy (CE).

The standard curves for quantitative analysis of samples were established using standard solutions of different concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200, and 500 ng/mL (where L-tryptophan and SAG were 20 times of the above concentrations to account for higher sensitivity in detection, i.e. the concentration range of the standard tune was 0.2–10000 ng/mL). In accordance with the previously outlined protocol, the LC-MS/MS analysis was carried out, and the peak intensity data of the corresponding quantitative signals for each concentration standard were sequentially obtained. The standard curves of different hormones were plotted using the concentration ratio of external standard to the internal standard as the horizontal coordinate, and the peak area ratio of external standard to internal standard as the vertical coordinate (Table S1). The integrated peak area ratios of all detected samples were substituted into the linear equation of the standard curve, and the final hormone content for the actual samples were thus obtained. This calculation equation applies to determine the hormone content in ng/g, with the following formula:

$$\text{Hormone content (ng/g)} = c \cdot V / 1000 / m.$$

Here, the parameters include:

- c: The concentration value (ng/mL) obtained by substituting the integrated peak area ratio into the standard curve;
- V: The volume of the solution used for re-dissolution (µL);
- m: The mass of the sample weighed (g).

2.8. Statistical analysis

The data were categorized and mean and variance were calculated using Excel 2017 software. Data T-test and correlation analysis were performed using SPSS Statistics 21.0 software. Box plots, principal component plots, heat maps, volcano plots, and orthogonal partial least squares discriminant analysis were produced using Rstudio software (R version 4.2.3) and libraries such as ggthemes 0.1.4, ggbiplot 0.55, pheatmap 1.0.12, ggplot2 3.4.0, ropls 0.9.2. Redundancy analysis and interaction network analysis were carried out

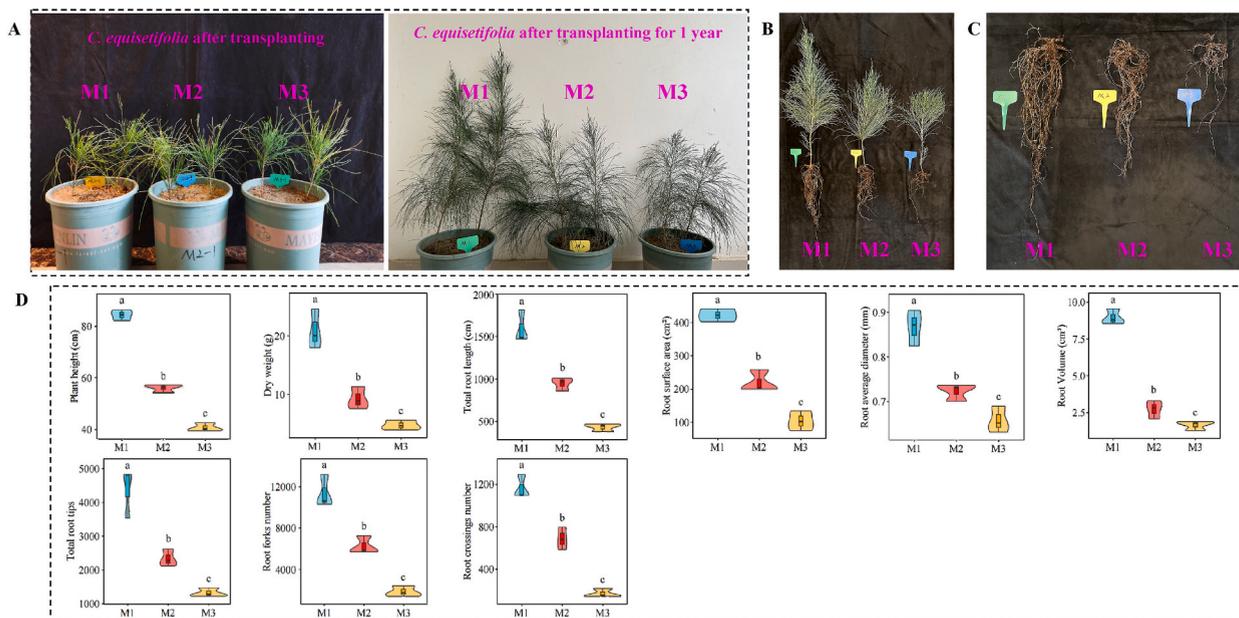


Fig. 1. Analysis of morphological indexes of *C. equisetifolia*.

Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; A: Photographs of *C. equisetifolia* before and after different treatments; B: Photographs of single plants of *C. equisetifolia* with different treatments; C: Photographs of root system of *C. equisetifolia* with different treatments; D: Analysis of plant height, dry weight and root index of *C. equisetifolia* with different continuous planting number. Different lowercase letters indicate the significant difference at $p < 0.05$ levels among different samples.

Table 1

Effects of continuous planting on the physicochemical indexes of rhizosphere soil of *C. equisetifolia*.

Index	M1	M2	M3
pH value	5.40 ± 0.03 a	5.34 ± 0.08 a	5.35 ± 0.04 a
Organic matter content (OM, g/kg)	3.83 ± 0.04 a	2.49 ± 0.04 b	1.46 ± 0.06 c
Cation exchange capacity (CEC, cmol/kg)	2.71 ± 0.09 a	1.47 ± 0.07 b	0.94 ± 0.07 c
Total nitrogen (TN, g/kg)	4.19 ± 0.13 a	3.15 ± 0.11 b	1.80 ± 0.07 c
Total phosphorus (TP, g/kg)	0.42 ± 0.04 a	0.29 ± 0.02 b	0.13 ± 0.01 c
Total potassium (TK, g/kg)	1.39 ± 0.03 a	1.02 ± 0.03 b	0.72 ± 0.03 c
Available nitrogen (AN, mg/kg)	19.32 ± 1.17 a	11.12 ± 0.79 b	5.10 ± 0.19 c
Available phosphorus (AP, mg/kg)	6.95 ± 0.08 a	4.31 ± 0.18 b	1.94 ± 0.08 c
Available potassium (AK, mg/kg)	92.66 ± 2.27 a	72.31 ± 1.71 b	47.65 ± 1.25 c

Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; Means standard error (±SE) from three replications for each site is shown; Different lowercase letters indicate the significant difference at $p < 0.05$ levels among different samples.

using Rstudio software (R version 4.2.3), and vegan 2.6.4, ggraph 2.1.0 were libraries, respectively.

3. Results

3.1. Analysis of morphological indexes of *C. equisetifolia*

Morphological analysis of *C. equisetifolia* showed (Fig. 1A) that continuous planting significantly affected the growth of *C. equisetifolia*. With the increase of continuous planting number (M1-M3), the plant height of *C. equisetifolia* was significantly reduced (Fig. 1B), the number of roots decreased, and the root length became significantly shorter (Fig. 1C). The results of morphological indexes of *C. equisetifolia* (Fig. 1D) showed that with the increase of continuous planting number (M1-M3), plant height decreased from 84.26 to 40.74 cm, the dry weight from 20.88 to 4.68g, and the root morphological indexes, i.e., total root length, root average diameter, root surface area, total root tips, root volume, root crossings number, and root forks number decreased from 1590.09 to 430.28 cm, from 0.87 to 0.66 mm, from 422.11 to 103.97 cm², from 4388.33 to 1325.00, from 8.96 to 1.65 cm³, from 1161.00 to 171.33 and from 11368.67 to 186.33, respectively. It is evident that continuous planting significantly inhibited *C. equisetifolia* growth and the effect was greater with the increase of continuous planting number.

Table 2
Effect of continuous planting on the physiological indexes of *C. equisetifolia* leaves.

Index		M1	M2	M3
Photosynthetic physiological indexes	Net photosynthetic rate ($\mu\text{mol}/\text{m}^2\cdot\text{s}$)	3.290 \pm 0.104 a	2.740 \pm 0.070 b	1.970 \pm 0.106 c
	Chlorophyll (mg/g)	0.133 \pm 0.001 a	0.124 \pm 0.001 b	0.103 \pm 0.002 c
Nutrient content	Total nitrogen (Total N, mg/g)	0.581 \pm 0.013 a	0.524 \pm 0.010 b	0.495 \pm 0.006 c
	Total phosphorus (Total P, mg/g)	1.391 \pm 0.021 a	0.541 \pm 0.013 b	0.506 \pm 0.012 c
	Total potassium (Total K, mg/g)	4.341 \pm 0.137 a	3.377 \pm 0.178 b	1.025 \pm 0.083 c
Resistance physiological indexes	Superoxide dismutase (SOD, U/mg)	19.878 \pm 0.282 a	6.626 \pm 0.519 b	4.511 \pm 0.099 c
	Peroxidase (POD, U/mg)	0.029 \pm 0.005 a	0.010 \pm 0.001 b	0.009 \pm 0.001 b
	Catalase (CAT, U/mg)	0.248 \pm 0.022 a	0.156 \pm 0.008 b	0.098 \pm 0.012 c
	Soluble sugar (%)	0.274 \pm 0.003 a	0.166 \pm 0.001 b	0.098 \pm 0.001 c
	Malondialdehyde (MDA, $\mu\text{mol}/\text{g}$)	4.412 \pm 0.190 c	6.439 \pm 0.983 b	9.356 \pm 0.614 a

Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; Means standard error (\pm SE) from three replications for each site is shown; Different lowercase letters indicate the significant difference at $p < 0.05$ levels among different samples.

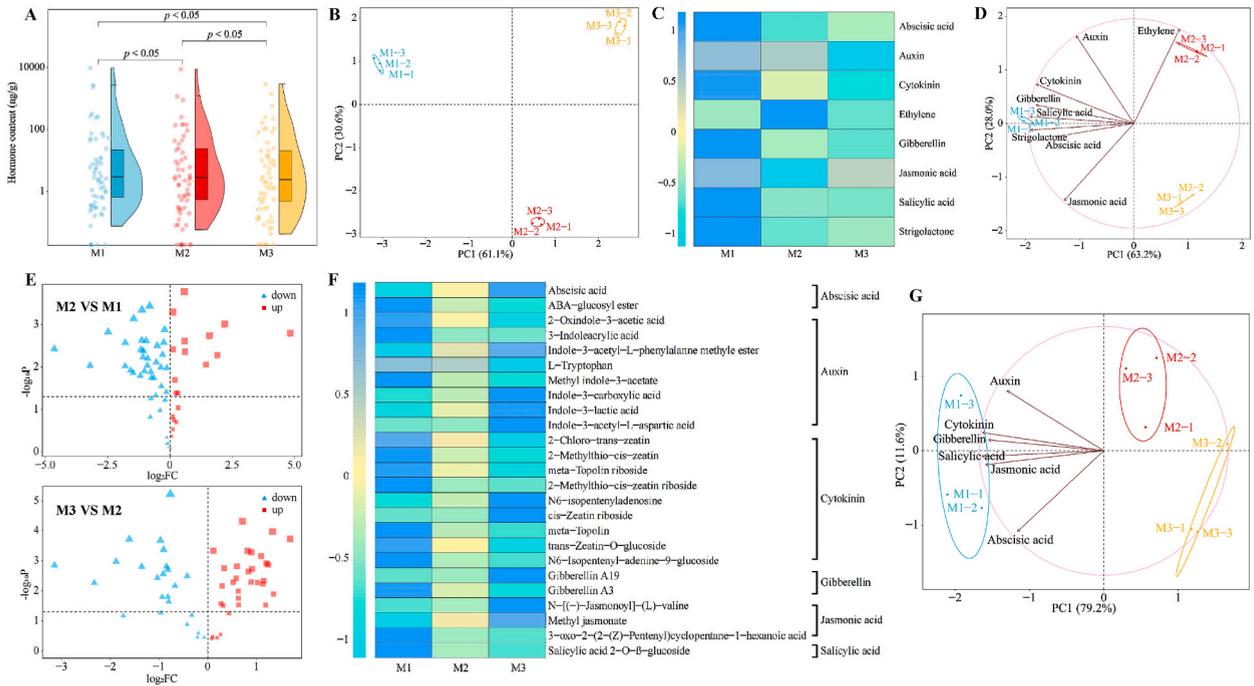


Fig. 2. Effect of continuous planting on the hormone content of *C. equisetifolia*.

Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; A: Overall analysis of hormone content of *C. equisetifolia*; B: PCA analysis of hormone content of *C. equisetifolia*; C: Content analysis of different categories of *C. equisetifolia* hormones; D: PCA analysis of different categories of *C. equisetifolia* hormone content; E: Volcano plot screening of hormones with differences in content due to continuous planting; F: Heat map analysis of hormones with differences in content; G: PCA analysis of differential hormones after classification.

3.2. Analysis of soil physicochemical indexes of *C. equisetifolia*

The soil physicochemical indexes in the rhizosphere *C. equisetifolia* with different continuous planting numbers showed (Table 1) that with the increase of continuous planting number (M1 - M3), soil pH value did not vary significantly, and OM, CEC, TN, TP, TK, AN, AP, AK contents decreased from 3.83 to 1.46 g/kg, from 2.71 to 0.94 cmol/kg, from 4.19 to 1.80 g/kg, from 0.42 to 0.13 g/kg, from 1.39 to 1.02 g/kg, from 19.32 to 5.10 mg/kg, from 6.95 to 1.94 mg/kg, and from 92.66 to 47.65 mg/kg, respectively. It is evident that due to continuous planting, soil nutrient conversion capacity and nutrient content of *C. equisetifolia* significantly declined, and the degree of decline was greater as the planting number of *C. equisetifolia* increased.

3.3. Analysis of physiological indexes of *C. equisetifolia*

Table 2 showed that photosynthetic physiological indexes (chlorophyll content and photosynthetic rate) of *C. equisetifolia* were significantly reduced with the increase of continuous planting number. Secondly, the total nitrogen, total potassium, and total phosphorus content of *C. equisetifolia* leaves also showed a significant decrease ($p < 0.05$) as continuous planting number increased.

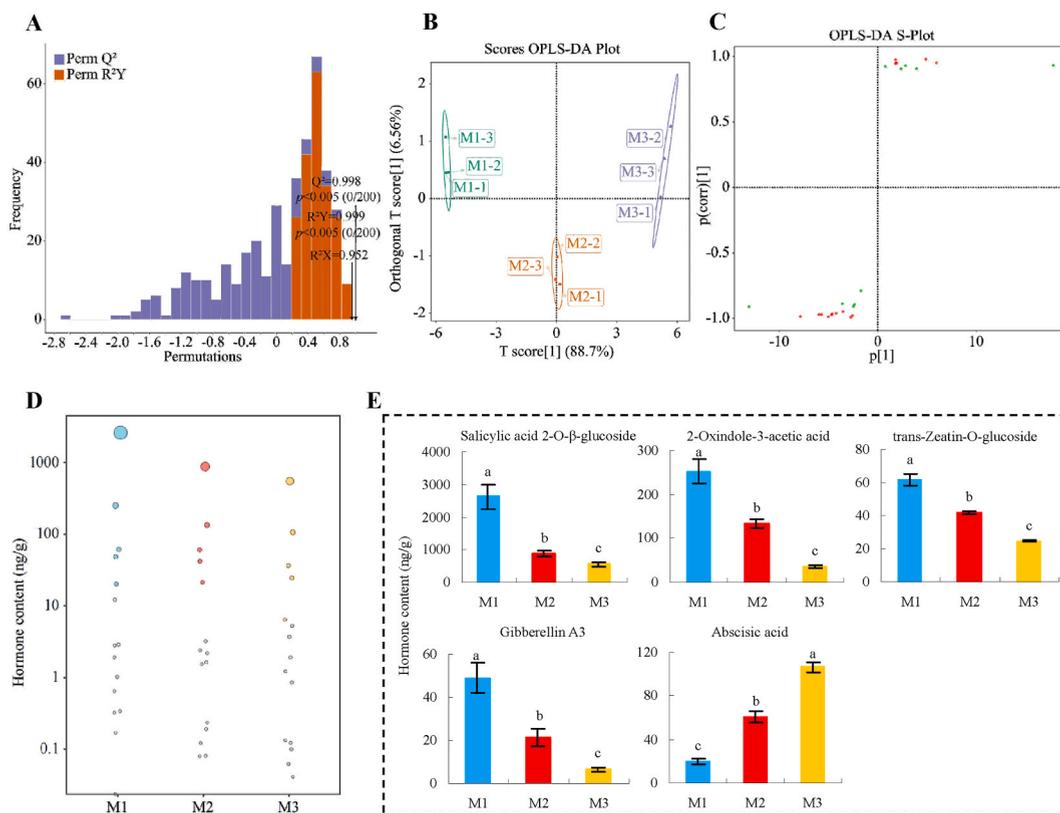


Fig. 3. Screening of key hormones with significant changes in content in *C. equisetifolia* leaves with different continuous planting times. Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; A: OPLS-DA model analysis of hormone contents in *C. equisetifolia* leaves with different continuous number; B: Score chart analysis of OPLS-DA model; C: S-plot analysis of screening key hormones by OPLS-DA model; D: Screening of characteristic hormones from key hormones by bubble characteristic plot; E: Content analysis of characteristic hormones; Different lowercase letters indicate the significant difference at $p < 0.05$ levels among different samples.

Furthermore, in terms of resistance physiological index of *C. equisetifolia* leaves, catalase, superoxide dismutase activities, and soluble sugar content still had a significant decreasing trend as continuous planting number increased ($p < 0.05$), while peroxidase activity was significantly greater in M1 than in M2 and M3, and malondialdehyde content had a significant increasing trend. It is evident that continuous planting decreased the physiological resistance and photosynthetic capacity of *C. equisetifolia* and its nutrient accumulation capacity, and continuous planting was not conducive to the growth of *C. equisetifolia*.

3.4. Hormone metabolome analysis in *C. equisetifolia* leaves

Firstly, this study analyzed the content of 61 hormones in *C. equisetifolia* leaves with different continuous planting number. As demonstrated by the M1, M2, and M3, a significant decrease in hormone contents was observed with the increase of continuous planting number. Specifically, the contents of hormones detected in the leaves were 15.95, 11.91, and 6.06 mg/g, respectively (Fig. 2A). As shown in Fig. 2B, the two principal components were able to effectively distinguished M1, M2, and M3 in different regions. The total contribution of the two principal components was 91.7%.

Further study revealed that the 61 hormones were categorizable into eight groups, of which auxins, cytokinins, gibberellins, salicylic acid, and ABA hormones contents in *C. equisetifolia* leaves showed a significant decreasing trend with the increase of continuous planting number, while ethylene, jasmonic acid, and dictamnolide hormones contents showed fluctuating changes (Fig. 2C). PCA analysis showed (Fig. 2D) that M1 was significantly correlated with auxins, cytokinins, gibberellins, salicylic acid, and ABA hormones. This study aimed to investigate the impact of continuous planting on the hormone content of *C. equisetifolia*. A volcano plot (Fig. 2E and F) was utilized to depict the significant changes in the content of 25 hormones, with 15 displaying a decrease and 10 showing an increase. The analysis revealed a correlation between continuous planting number and hormone changes. PCA analysis revealed (Fig. 2G) that 25 hormones that changed significantly were mainly divided into six categories, and the content of these six categories was significantly correlated with M1. Continuous planting had a significant impact on the hormone content of *C. equisetifolia* leaves.

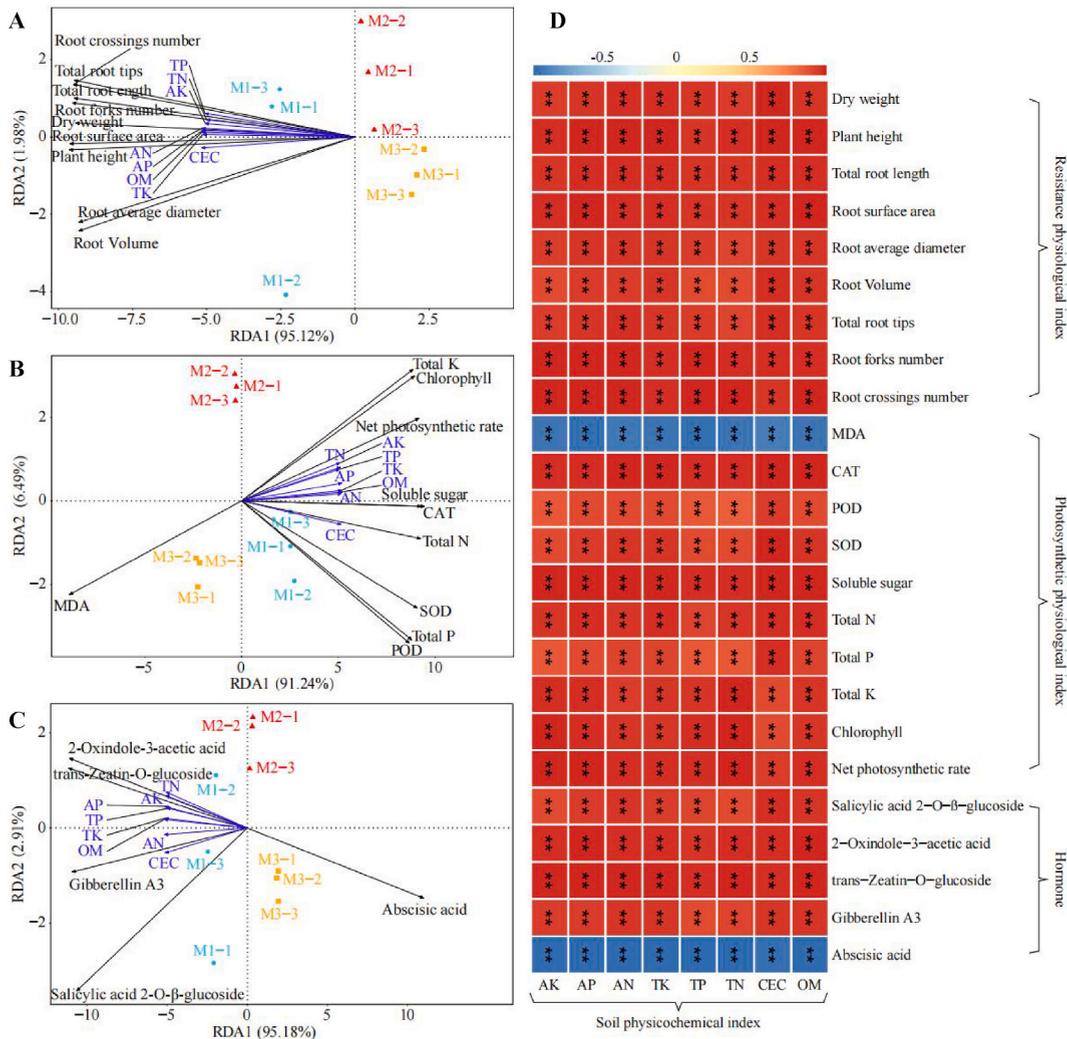


Fig. 4. Interaction analysis between different indexes.

Note: M1: First planting; M2: Second continuous planting; M3: Third continuous planting; A: Redundancy analysis of rhizosphere soil physicochemical indexes and growth indexes of *C. equisetifolia*; B: Redundancy analysis of rhizosphere soil physicochemical indexes and physiological indexes of *C. equisetifolia*; C: Redundancy analysis of rhizosphere soil physicochemical indexes and characteristic hormones of *C. equisetifolia*; D: Correlation analysis of rhizosphere soil physicochemical indexes and growth indexes, physiological indexes and characteristic hormones of *C. equisetifolia*; ** indicates that the correlation between different indexes reaches the $p < 0.01$ level.

3.5. Screening of characteristic key hormones and their content analysis

Upon completion of the above analysis, this study employed the orthogonal partial least squares discriminant analysis (OPLS-DA) model to screen hormones that underwent key changes in *C. equisetifolia* leaves after continuous planting. The OPLS-DA model fit analysis of hormone content in *C. equisetifolia* leaves with different continuous planting number (Fig. 3A) found that the R^2Y value of the model fit was 0.999, which had an extremely significant level ($p < 0.005$), and the Q^2 value of predictability was 0.998, which also reached an extremely significant level ($p < 0.005$). It is evident that the OPLS-DA model displayed a significant and reliable fit to the data with high confidence, which provided strong evidence of its ability to accurately distinguish between different samples, making it a useful tool for further analysis. The results of the score plot analysis of the OPLS-DA model showed (Fig. 3B) that the model can effectively distinguish different samples in different coordinate regions, with 88.7% difference between groups. It is evident that the hormone content of *C. equisetifolia* varied significantly after continuous planting, while significant differences existed between the numbers of continuous planting. Results from the S-plot analysis of the OPLS-DA model found (Fig. 3C) that there were 15 hormones with key differences in *C. equisetifolia* leaves with different continuous planting number. Further bubble characteristic plot analysis of 15 key hormones was performed, and a total of five characteristic hormones with significant changes were obtained, and the content of SAG, OxIAA, tZOG, and GA₃ had a significant decreasing trend, however ABA content had a significant increasing trend as continuous planting number of *C. equisetifolia* increased (Fig. 3D and E). It was apparent that continuous planting of *C. equisetifolia* significantly

impacted the hormone levels within the leaves, and most critically, continuous planting reduced the content of SAG, OxIAA, tZOG and GA₃, and increased the content of ABA in *C. equisetifolia* leaves.

3.6. Analysis of the interactions between different indexes

Building on the above studies, this study explored the relationships among rhizosphere soil physicochemical indexes and growth indexes, leaf physiological indexes and hormone contents of *C. equisetifolia* with different continuous planting number. Redundancy analysis of soil physicochemical and growth indexes of *C. equisetifolia* (Fig. 4A) revealed that the soil physicochemical indexes associated with M1 were OM, TN, CEC, TP, AN, AP, TK, and AK, while the growth indexes associated with M1 were plant height, dry weight, total root length, root average diameter, root surface area, root volume, total root tips, root forks number, and root crossings number. Correlation analysis displayed (Fig. 4D) a significant positive correlation between soil physicochemical indexes and growth indexes. Redundancy analysis of soil physicochemical and leaf physiological indexes of *C. equisetifolia* (Fig. 4B) showed that the soil physicochemical indexes associated with M1 were OM, TN, CEC, TP, AN, TK, AP, and AK, while the physiological indexes associated with M1 were photosynthetic rate, chlorophyll content, SOD, POD, CAT, soluble sugar, Total N, Total K, Total P, while the indexes related to M3 was MDA. Correlation analysis also displayed (Fig. 4D) a significant positive correlation between soil physicochemical and physiological indexes, except for MDA. Redundancy analysis of soil physicochemical indexes and characteristic hormones of *C. equisetifolia* showed (Fig. 4C) that the soil physicochemical indexes associated with M1 were OM, TN, CEC, TP, AN, AP, TK, and AK, while the characteristic hormones associated with M1 were AG, OxIAA, tZOG, and GA₃, while the index associated with M3 was ABA. Correlation analysis displayed again (Fig. 4D) a significant positive correlation between soil physicochemical indexes and characteristic hormones, except for ABA. It is evident that continuous planting altered soil nutrient content, which in turn affected root growth and nutrient uptake, regulated hormone synthesis capacity, reduced the resistance and photosynthesis capacity, and impeded *C. equisetifolia* growth.

4. Discussion

Continuous planting was very likely to cause alterations in soil physicochemical indexes, thus affected plant growth [28,29]. In this study, it was found that the plant height and biomass of *C. equisetifolia* decreased significantly with the increase of continuous planting number, and the root growth of *C. equisetifolia* was significantly inhibited. It is evident that continuous planting inhibited *C. equisetifolia* growth. Secondly, this study found that OM, CEC, total nutrient and available nutrient contents in the soil of *C. equisetifolia* decreased significantly after continuous planting. N, P, and K are the main elements necessary for plant growth, and their content in the soil, especially AN, AP and AK contents, directly affects plant growth [30]. OM and CEC are important indexes for evaluating soil fertility, and OM is beneficial to soil improvement, while CEC is beneficial to nutrient transformation in the soil, increasing available nutrient levels in the soil and maintaining soil fertility, thus promotes plant growth [31,32]. It becomes apparent that as continuous planting number increased, the nutrient transformation capacities of the rhizosphere soil decreased, resulting in a decrease in the available nutrient content of the soil. This subsequently hampers root growth, ultimately affecting nutrient uptake and utilization by *C. equisetifolia*. The plant consequently experiences stunted growth and reduced biomass.

The root, an essential carrier for the connection between plants and soil, directly influences plant's ability to absorb and transport nutrients, which subsequently influences their physiological functions [33,34]. This study found that the leaf nitrogen, phosphorus and potassium contents in the leaves of *C. equisetifolia* exhibited a significant decrease as continuous planting number increased, along with significant declines in the leaves' photosynthetic physiological indexes and resistance physiological indexes. Nitrogen, potassium, and phosphorus are closely related to plant photosynthesis and resistance, and the overall plant growth is significantly inhibited when plants are in scarce supply of nitrogen, phosphorus and potassium [35,36]. Secondly, the reduced photosynthetic capacity also reduces nutrient uptake by the plant and reduces the plant's ability to resist the external environment, thus hinders plant growth [37,38]. It is evident that continuous planting reduced the nutrient transformation capacities of the soil, which led to the impediment of its root growth, and thus affected the abilities of the root system to absorb and transport nutrients. Decreased nutrient uptake and translocation capacity of *C. equisetifolia* reduced nutrient levels in the leaves, thus led to reduced resistance, reduced photosynthetic capacity, and severely stunted growth of *C. equisetifolia*.

Although plants may have low levels of hormones, they serve as pivotal regulators in growth and development. It has been reported that under environmental stress, the hormone balance in plants was imbalanced, the hormone content was reduced, and the photosynthetic capacity and physiological resistance of plants were reduced [39–41]. Shikha et al. [42] discovered that nutrient stress adjust their nutrient uptake by regulating the synthesis of jasmonic acid hormones. Ahmad et al. [43] discovered that under salt stress, plants primarily employ the tactics of escalating gibberellin and salicylic acid content to boost their salt resistance and photosynthetic capacity, thereby ensuring plant yield. In this study, it was found that the total amount of hormones in *C. equisetifolia* leaves tended to decrease significantly with the increase of continuous planting number. It is evident that continuous planting significantly affected the hormone synthesis of *C. equisetifolia* leaves, thus affected its growth. Second, this study found that there were five main characteristic hormones that led to key changes in *C. equisetifolia* leaves due to continuous planting, among which the contents of SAG, OxIAA, tZOG and GA₃ had a decreasing trend with continuous planting number, while the content of ABA presented an increasing trend. Salicylic acid is a plant hormone, which is mainly stored as SAG in plants, and its increased content is beneficial to improve plant growth and enhance its defense and disease resistance [44,45]. OxIAA is an indoleacetic acid oxide, and at its proper concentration, can improve plant growth and photosynthesis [46]. tZOG is one of the plant cytokinins, which is beneficial to improve the proliferation capacity of plant cells, as well as to improve the photosynthetic capacity of plants [47]. GA₃ can enhance plant resistance to external

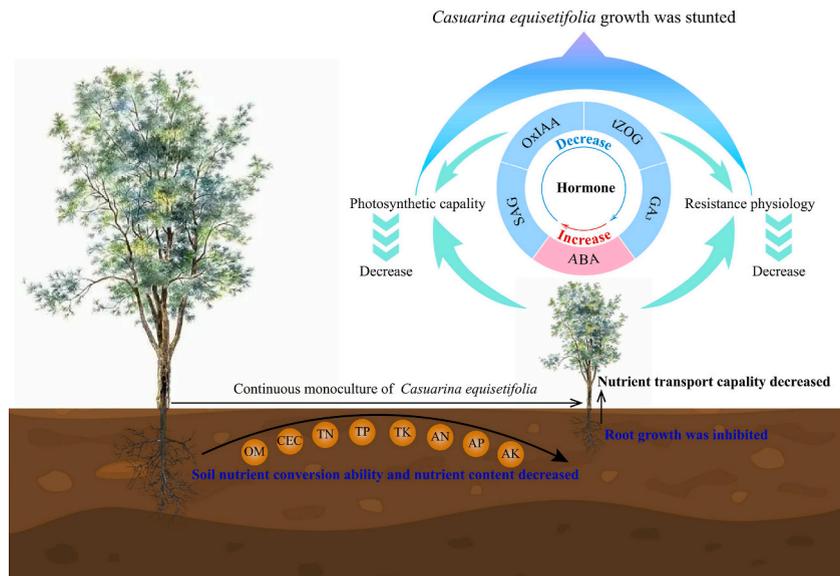


Fig. 5. Physiological mechanism analysis of effects of continuous planting on *C. equisetifolia* growth.

environmental stress, reduce ABA content in plants, promote plant growth, and increase plant yield [48,49]. The level of abscisic acid is a response of plants to adversity stress, and under long-term adversity stress, ABA accumulation in plants increase, plant resistance decrease, and plants gradually age [50]. It is evident that continuous planting resulted a decrease in the SAG content and an increase in the ABA content in *C. equisetifolia* leaves, and a decrease in the resistance of *C. equisetifolia* to the external environment. Second, continuous planting reduced OxIAA, tZOG and GA₃ content in *C. equisetifolia* leaves, thus led to a decrease in *C. equisetifolia* growth capacity.

In addition, this study revealed that the rhizosphere soil's physicochemical indexes of *C. equisetifolia* were significantly and positively related to its leaf photosynthetic physiological indexes, nutrient contents, activities of SOD, POD, CAT, and soluble sugars, SAG, OxIAA, tZOG, GA₃ contents. However, the rhizosphere soil's indexes were significantly and negatively correlated with the MDA, ABA contents. It is evident that continuous planting lowered the synthesis of SAG, OxIAA, tZOG, GA₃, and improved the accumulation of ABA in *C. equisetifolia* leaves, which reduced the ability of *C. equisetifolia* to resist the environmental change, inhibited their photosynthetic ability, and then hindered their growth.

5. Conclusions

This study revealed that continuous planting could weaken the nutrient transformation capacity of *C. equisetifolia* rhizosphere soil nutrients, thus inhibited the root growth of *C. equisetifolia* and diminished the nutrient absorbing and accumulating abilities of *C. equisetifolia* leaves. This caused a significant decrease in the content of SAG, OxIAA, tZOG, and GA₃, as well as an extensive ABA accumulation. These factors collectively resulted in reduced the activity of physiological indexes of resistance, reduced resistance, and reduced photosynthetic capacity of *C. equisetifolia* (Fig. 5). And, these factors resulted in dwarf plants and a significant reduction in the biomass of *C. equisetifolia*. It is evident that continuous planting significantly inhibited *C. equisetifolia* Growth. In this study, we found that the key reason for the stunted *C. equisetifolia* growth during continuous planting was the reduced nutrient transformation capacity of the soil. This study provided an important reference for the cultivation management and fertilization of continuously planting *C. equisetifolia*. However, what relationship exists between the reduction in soil nutrient conversion capacity after continuous planting of *C. equisetifolia* and changes in soil enzyme activities and microbial communities, and how soil microorganisms affect nutrient conversion, in-depth analysis and exploration of these aspect are essential.

Data availability statement

The data presented in this study are available as Supplementary Files.

CRedit authorship contribution statement

Yuhua Wang: Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Yuchao Wang:** Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Jianjuan Li:** Writing – original draft, Formal analysis, Data curation. **Yuhong Cai:** Writing – original draft, Formal analysis. **Mingyue Hu:** Writing – original draft, Project administration, Formal analysis. **Wenxiong Lin:** Writing – review & editing, Project

administration, Conceptualization. **Zeyan Wu:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e26078>.

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