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Photosynthetic Physiological Basis of Forage Mass Stability in a Progeny of Rhizome-Rooted 'Qingshui' *Medicago sativa* L.

Yun A, Shangli Shi *, Jinqing Zhang, Xiaolong Li , Fang Jing, Huihui Zhang and Ruihong Ma

Pratacultural College, Gansu Agricultural University, Lanzhou 730070, China
* Correspondence: shishl@gsau.edu.cn; Tel.: +86-139-1905-1530

Abstract: Rhizome-rooted alfalfa (*Medicago sativa* L.) is an excellent forage for establishing grazing and ecological grasslands, requiring a high and stable yield. Studying the genetic and physiological basis of stable expression of biomass traits is essential for improving production performance in rhizome-rooted alfalfa. We analyzed forage mass and photosynthetic physiological indices of the improved progenies (RSA−01, RSA−02, and RSA−03), parental "Qingshui" (CK_1), and "WL168" (CK_2) at ages one and five years and their relationships, then revealed heterotic stability. Moreover, we explored the effects of interannual dynamics and genetic differences on tested indices. The results revealed compared with the forage mass of CK_1, RSA−03 at ages one and five years increased by 22.17% and 19.72%, respectively, while RSA−01 and RSA−02 varied from 1.40% to 8.65%, indicating obvious heterosis in forage mass of RSA−03. At one year of age, G_s value, Car content and SS content of RSA−03 were higher than those of CK_1; SS content of RSA−03 were higher than those of CK_2 and RSA−02; C_i, G_s and T_r values of RSA−03 were higher than those of RSA−01. At five years of age, P_n, G_s, and WUE values, and Sta content of RSA−03 were higher than those of CK_1; C_i value and Suc content of RSA−03 were higher than those of CK_2; Car content and G_s value of RSA−03 were higher than those of other progenies. The forage mass; Chl(*a/b*) ratio; P_n, G_s, and WUE values; Suc content of RSA−03 at age five years were higher than those at age one year by 9.99%–44.24%. Through path analysis, G_s and NSC were direct factors affecting forage mass at age one year, and both P_n and SP affected forage mass indirectly through G_s; G_s and Chl(*a*+*b*) were direct factors affecting forage mass at age five years, and SS affected forage mass indirectly through G_s. Interestingly, Chl*b*, Chl(*a*/*b*), P_n, T_r, G_s, C_i, Suc, SP, and SS were more influenced by age than genetics, while the opposite was true for Car and Sta. Accordingly, RSA−03 showed obvious and stable heterosis in forage mass and photosynthetic physiology, recommending the establishment of grazing pastures and ecological vegetation.

Keywords: alfalfa; heterosis; forage mass; physiological basis; trait stability

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1. Introduction

Rhizome-rooted alfalfa (*Medicago sativa* L.) is an excellent germplasm for establishing grazing and ecological grasslands with well-developed rhizome root systems, relatively high resistance to stress and trampling as well as high renewal capacity [1,2]. In recent years, relatively less research has been conducted on grazing-type alfalfa worldwide, with Rambler, Rangelander, *Medicago falcata* "Hulunbeier", and *Medicago varia* Gannong, "No. 2" being the main creep-rooted types used [2–4]. The rhizome of rhizome-rooted alfalfa has higher renewability and greater expansion capacity, with better ground cover formation than that of creeping-rooted alfalfa [2,5,6]. *Medicago sativa* L. "Qingshui" is the only validated and registered (registration number: 412) rhizome-rooted alfalfa variety in China, warranting its exploitation owing to the high-quality grazing forage; however, "Qingshui" has inferior yield compared with the most used cultivars [5]. Karyotype analysis, enzyme profiling, DNA molecular marker identification, and tissue dissection on the "Qingshui" have been performed; moreover, leaf metagenesis patterns during shoot development from rhizomes have been examined [1,2,6,7]. However, no study on yield performance of the "Qingshui" after breeding improvement is available in the literature. Cross breeding is the most widely used and effective agricultural breeding method worldwide, creating new germplasm through artificial hybridization [8,9]. Previous studies showed that the "Qingshui" crossed with high-yield alfalfa "WL168" to produce novel rhizomatous hybrid strains (RSA−01, RSA−02, and RSA−03) [2,4,5].

More than 90% of crop biological yield comes from photosynthesis, which is the physiological basis for plant growth and development, and its intensity and frequency affect forage mass [10,11]. Photosynthetic pigments, parameters, and products are essential indicators for identifying and breeding excellent varieties [9–11] as they can reflect the photosynthetic capacity and actual yield potential of forage. Genetic factors, environmental climate, and growth and development stages influence the variation patterns and contents of photosynthetic pigments, parameters, and products [12,13]. Heterosis is the expression of superior traits in the progeny compared with the parents and is characterized by faster growth, higher biomass, and ultimately higher yields [10,12,13]. Recent studies on heterosis of light energy uptake, Chl content, and photosynthetic rate have particularly focused on maize, wheat, rice, and soybean crops [14,15]. In contrast, research on the photosynthetic characteristics of alfalfa is mainly focused on breeding varieties, and the correlation between the net photosynthetic rate and other photosynthetic factors is studied more than other aspects [11]. In addition, the establishment of grazing forage usually requires productive and stable germplasm of high and stable yield in production practice. This study aimed to improve the low production performance of the "Qingshui" variety through cross-breeding, enabling the "Qingshui" to achieve both high production and ecological values. Therefore, three hybrid strains, i.e., RSA−01, RSA−02, and RSA−03, were used as the research objects, while the parents ("Qingshui" and "WL168") were used as the control. Forage mass and photosynthetic physiological indices at ages one and five years were measured, and the stability of hybrid dominance performance was analyzed. Subsequently, the relationship between forage mass and photosynthetic physiological characteristics was investigated, and the effects of interannual dynamics and genetic differences on photosynthetic physiological indicators were discussed.

2. Materials and Methods

2.1. Plant Material

The following breeding strains (RSA−01, RSA−02, and RSA−03) of the hybrid progenies of rhizome-rooted *Medicago sativa* L. "Qingshui" (female, CK_1) and creep-rooted *M. sativa* L. "WL168" (male, CK_2) were used in this study, and the parents served as the control.

The origin and development of the hybrid strains were the same as those reported in previous studies [2,5]. The basic traits of each strain are presented in Table 1. CK_1 was provided by the Pratacultural College of Gansu Agricultural University, China, and CK_2 was provided by Beijing Zhengdao Ecological Technology Co., Ltd., Lanzhou, China.

Table 1. Morphological characteristics of parental varieties and hybrid strains.

Categories		Characteristics
Parental varieties	CK_1	Horizontal or oblique rhizomatous roots and strong extension habit; horizontal and sloping stems, slender and stiff stalks, lax plants and short plant height (62 cm); resistance to drought, cold temperatures, and trampling; hay yield (21,000 kg·h^{-1}); germination rate (87%).
	CK_2	Horizontal roots and strong extension habit; erect stems, thick stalks, high absolute plant height (71 cm); high adaptation, and high resistance to stress [16]; hay yield (27,000 kg·h^{-1}); germination rate (95%).
Hybrid strains	RSA−01	Erect stems, with an angle of 70–80° between stem and ground; high absolute plant height (64 cm); germination rate (86%).
	RSA−02	Semi-horizontal stems, with an angle of 30–69° between stem and ground; high absolute plant height (64 cm); germination rate (87%).
	RSA−03	Horizontal stems, with an angle of <30° between stem and ground; high absolute plant height (69 cm); germination rate (87%).

2.2. Growth Conditions and Treatments

All experiments were performed in June and July 2021 at the experimental base of Gansu Agricultural University (34°05′ N, 105°41′ E, 1525 m altitude), Lanzhou, China. Three hybrid strains (RSA−01, RSA−02, and RSA−03) of CK_1 and CK_2 were used as research samples, and 1- and 5-year-old alfalfa plants were selected for the experiment. These 5-year-old and 1-year-old alfalfa plants were sown on 23 April 2016 and 10 July 2020, respectively, at the same locations and under the same soil fertility conditions. The three hybrid strains were used as treatments, whereas the two parents, i.e., CK_1 and CK_2, served as controls. The plot area was 3 m × 5 m with a row spacing of 30 cm. Seeds were sown in a strip pattern at a density of 1 g/m^2. Timely field management, including drip irrigation (5 times a year), no fertilization, manual weeding, and pest control, was conducted throughout the experimental period.

Forage mass and photosynthetic physiological characteristics were measured during the early blooming phase at the first cutting (5% flowering). Leaves from the middle leaflet of the third leaf position of the main stem of 10 randomly selected plants in each plot were selected. The selected leaves were immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. Measurement Indexes

2.3.1. Determination of Forage Mass

A section (1 m × 1 m) was randomly sampled in each plot to obtain a 5 cm stubble, which was weighed immediately after mowing to calculate its weight of fresh forage. The mowed samples were dried at 110 °C for 15 min and continuously at 65 °C for 12 h in an oven, and constant weight was determined as the forage mass.

2.3.2. Determination of Photosynthetic Pigment Content

The leaf samples (0.2 g) were cut into small pieces and subsequently homogenized using 95% ethanol for 24 h at room temperature in the dark. The values of photosynthetic pigment were determined using a spectrophotometer at three wavelengths, i.e., 663, 645, and 460 nm. Referring to the method proposed by Arnon (1949) [17], Chl*a*, Chl*b*, Chl(*a*+*b*), and Car contents; and Chl(*a*/*b*) ratio were calculated.

2.3.3. Determination of Photosynthetic Parameters

The GFS-3000 Portable Gas Exchange Fluorescence system was used to measure the photosynthetic parameters of plants in the field on a clear and cloudless day (9:30–11:30 AM). The middle leaflet of the third leaf from the top to the bottom position of the main stem of each plant was randomly selected for measuring P_n (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), T_r (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$), G_s ($cm \cdot s^{-1}$), C_i ($\mu mol \cdot mol^{-1}$), and WUE ($\mu mol \cdot mmol^{-1}$). Three plants were measured for each index, and the data were recorded five times for each leaf and calculated as an average. WUE was calculated using the following formula:

$$WUE = P_n/T_r \quad (1)$$

2.3.4. Determination of the Photosynthesis Products

Sta and Suc contents were determined using the method proposed by Knudsen [18]. SS content was analyzed using anthracenone colorimetry, and SP content was analyzed via Coomassie Brilliant Blue G250 staining. NSC content is the sum of SS and Sta contents.

2.4. Statistical Analyses

Data from three independent biological replicates were analyzed using the SPSS 20.0 software, and all improvement indicator values were calculated in Excel 2010 using the formula described in Equations (2) and (3) below [5,19]. GraphPad Prism 8 was used for mapping, and statistical analyses were performed using one-way analysis and two-way analysis of variance followed by Duncan's multiple range test. Comparison between the mean values was performed using the least square difference test at a 5% probability level. Path analysis is a linear relationship between multiple independent and dependent variables. The objective of our study was to investigate the effect of photosynthetic physiological indicators on forage mass of alfalfa at different ages (1- and 5-years-old) through path analysis. Therefore, we analyzed the data as a whole with all varieties (strains) under their respective ages. We performed correlation and regression analysis by the SPSS 20.0 software to derive the correlation coefficients and direct path coefficient, and subsequently, we calculated indirect correlation coefficients in Excel 2010 using Equation (3).

(i) Mid-parent heterosis:

$$\text{Mid} - \text{parent heterosis} = \frac{\text{the mean value of the respective parameter}}{\text{the average of the mean values of the respective parameter in both parental lines}} \times 100 \quad (2)$$

(ii) Path analysis:

$$\text{Indirect path coefficient} = r_{ij} \times P_{jy} \quad (3)$$

The indirect path coefficient (r_{ij} P_{jy}) reflects the influence of the independent variable x_i on the dependent variable y through the independent variable x_j. x_i represents the direct independent variable, x_j represents the indirect independent variable, R_{ij} represents the correlation coefficient between x_i and x_j, and P_{jy} represents the direct effect of the independent variable x_j on the dependent variable y.

3. Results

3.1. Analysis of Forage Mass in Hybrid Strains and Parents

To compare the yield of the tested varieties (strains), forage mass of the hybrid strains was measured (Figure 1). At one year of age, the forage mass of RSA−01 and RSA−02 was not significantly different from that of the parental variety CK_1, whereas the forage mass of RSA−03 was significantly higher than that of CK_1 by 22.17%. At five years of age, the forage masses of RSA−01, RSA−02, and RSA−03 were significantly higher than those of CK_1 by 3.41%, 8.66%, and 19.72%, respectively.

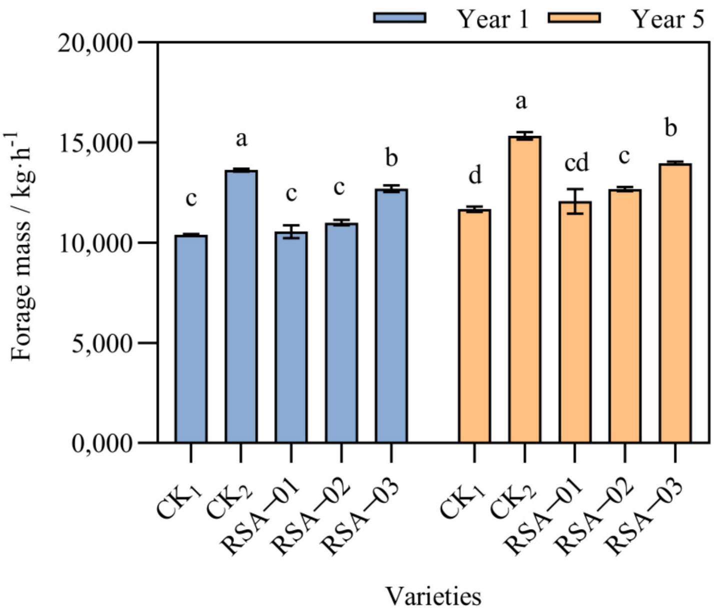

Figure 1. Forage mass of hybrid strains and parentes. Lowercase letters indicate significant differences among varieties (strains) at a p value of <0.05.

At one year of age, the forage masses of RSA−01, RSA−02, and RSA−03 were significantly lower than those of the parental variety CK_2 by 22.57%, 19.26%, and 6.80%, respectively. At five years of age, the forage masses of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by 21.28%, 17.28%, and 8.86%, respectively.

3.2. Analysis of Photosynthetic Pigment Content in Hybrid Strains and Parents

The photosynthetic pigment content was measured to compare the photosynthetic capacity of the tested varieties (strains) (Figure 2). At one year of age, the Chl*a*, Chl*b*, and Chl(*a*+*b*) contents, and Chl(*a*/*b*) ratio of RSA−01, RSA−02, and RSA−03 differed insignificantly from those of CK_1, whereas the Car content of RSA−01 and RSA−03 was significantly higher than that of CK_1 by 21.65% and 23.11%, respectively. At five years of age, the Chl*a* content, Chl*b* content, and Chl(*a*/*b*) ratio of RSA−01, RSA−02, and RSA−03 were not significantly different from those of CK_1, whereas the Chl(*a*+*b*) content of RSA−03 and Car content of RSA−01 and RSA−02 were significantly reduced by 12.20%, 21.22%, and 18.40%, respectively.

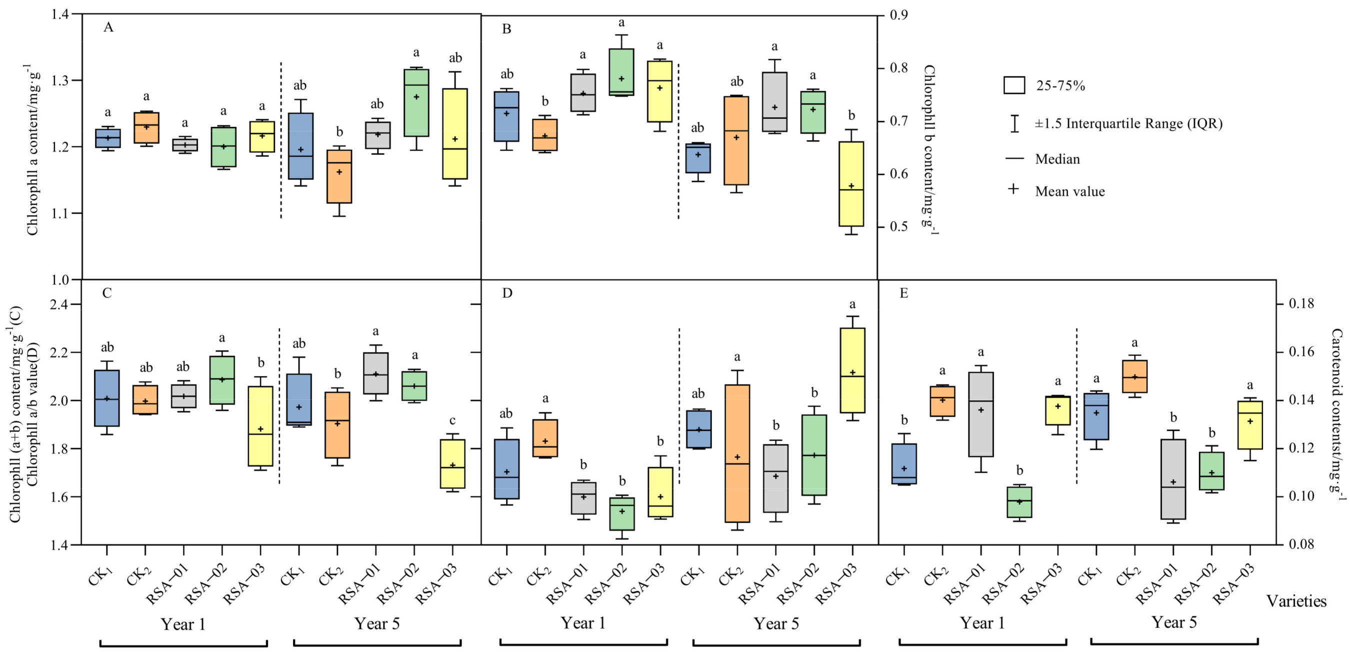

Figure 2. Photosynthetic pigment contents of hybrid strains and parents. (**A**) Chl*a*. (**B**) Chl*b*. (**C**) Chl(*a*+*b*). (**D**) Chl(*a*/*b*). (**E**) Car. Lowercase letters indicate significant differences among varieties (strains) at a *p* value of <0.05. Blue, orange, gray, green and yellow in the figure represent CK_1, CK_2, RSA−01, RSA−02, RSA−03, respectively.

At one year of age, the Chl*b* contents of RSA−01, RSA−02, and RSA−03 were significantly higher than those of CK_2 by 11.97%, 16.14%, and 17.61%, respectively, whereas the Chl(*a*/*b*) ratio of RSA−01, RSA−02, and RSA−03 was significantly lower than that of CK_2 by 12.65%, 15.90%, and 12.62%, respectively, and the Car content of RSA−02 was significantly lower than that of CK_2 by 30.10%. At five years of age, the Chl*a* content of RSA−02, the Chl(*a*+*b*) content of RSA−01, and the Chl(*a*/*b*) ratio of RSA−03 were significantly higher than those of CK_2 by 9.23%, 10.84%, and 19.89%, respectively, whereas the Chl(*a*+*b*) content of RSA−03 and Car content of RSA−01 and RSA−02 were significantly lower than those of CK_2 by 9.05%, 29.11%, and 26.57%, respectively.

3.3. Analysis of Photosynthetic Parameters in Hybrid Strains and Parents

To compare the photosynthetic parameters of the tested varieties (strains), P_n, T_r, G_s, C_i, and WUE values were measured (Figure 3). At one year of age, the T_r value of RSA−01 was significantly lower than that of CK_1 by 23.00%, whereas the G_s values of RSA−01, RSA−02, and RSA−03 were significantly higher than those of CK_1 by 17.49%, 49.84%, and 57.79%, respectively. Moreover, the C_i values of RSA−01 and RSA−02 were significantly lower than those of CK_1 by 10.66% and 6.56%, respectively, whereas the WUE values of RSA−01 and RSA−02 were significantly higher than those of CK_1 by 31.42% and 19.39%, respectively. At five years of age, the P_n values of RSA−01, RSA−02, and RSA−03 were significantly higher than those of CK_1 by 23.64%, 32.27%, and 59.01%, respectively, and the T_r value of RSA−02 was significantly higher than that of CK_1 by 38.80%. Moreover, the G_s values of RSA−02 and RSA−03 were significantly higher than those of CK_1 by 89.02% and 46.38%, respectively, and the WUE values of RSA−01 and RSA−03 were significantly higher than those of CK_1 by 33.71% and 31.92%, respectively.

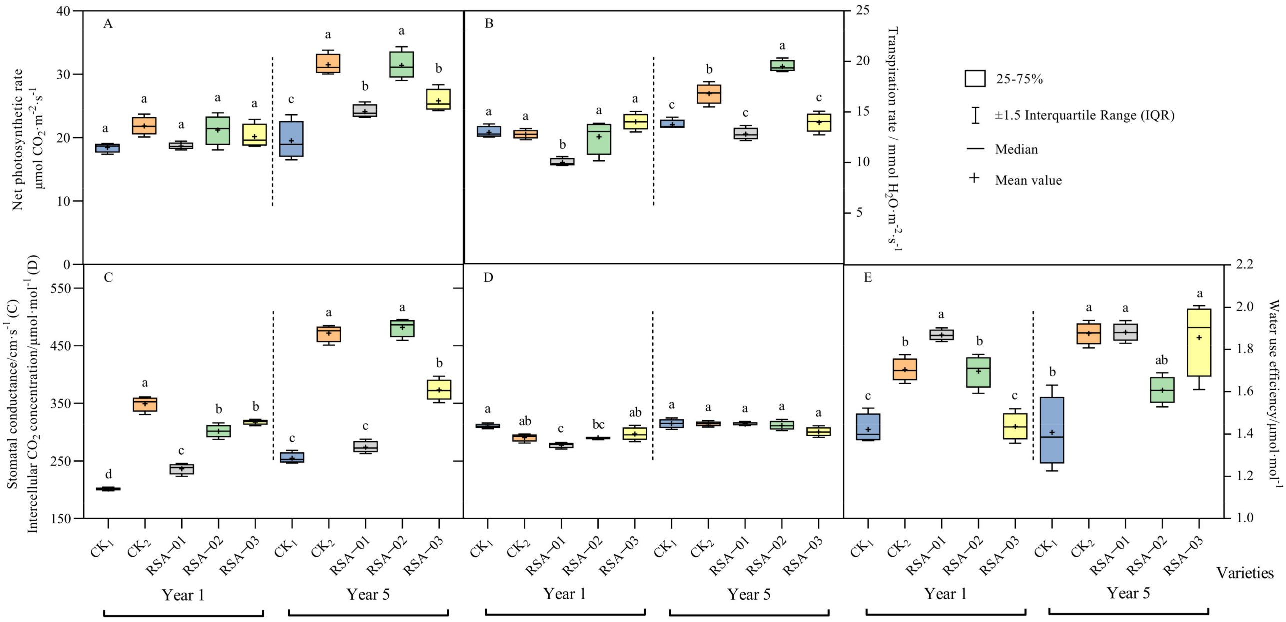

Figure 3. Photosynthetic parameters of hybrid strains and parents. (**A**) P_n. (**B**) T_r. (**C**) G_s. (**D**) C_i. (**E**) WUE. Lowercase letters indicate significant differences among varieties (strains) at a p value of <0.05. Blue, orange, gray, green and yellow in the figure represent CK_1, CK_2, RSA−01, RSA−02, RSA−03, respectively.

At one year of age, the T_r value of RSA−01 was significantly lower than that of CK_2 by 21.84%, and the G_s values of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by 9.09%, 13.64%, and 33.26%, respectively. In addition, the WUE value of RSA−01 was significantly higher than that of CK_2 by 9.64%, whereas the WUE value of RSA−03 was significantly lower than that of CK_2 by 15.76%. At five years of age, the P_n values of RSA−01 and RSA−03 were significantly lower than those of CK_2 by 23.42% and 18.06%, respectively, and the G_s values of RSA−01 and RSA−03 were significantly lower than those of CK_2 by 20.92% and 41.95%, respectively. In addition, the T_r values of RSA−01 and RSA−03 were significantly lower than those of CK_2 by 23.69% and 2.12%, respectively, and the T_r value of RSA−02 was significantly higher than that of CK_2 by 14.05%.

3.4. Analysis of Photosynthetic Products in Hybrid Strains and Parents

To compare the photosynthetic products of the tested varieties (strains), Sta, Suc, SS, NSC, and SP contents were measured (Figure 4). At one year of age, the Sta contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_1 by 6.38%, 10.91%, and 18.44%, respectively, while the SS and NSC contents of RSA−02 and the NSC content of RSA−03 were significantly lower than those of CK_1 by 6.01%, 9.26%, and 10.30%, respectively. Moreover, the SP content of RSA−01 was significantly higher than that of CK_1 by 14.78%, whereas the SP contents of RSA−02 and RSA−03 were significantly lower than those of CK_1 by 6.44% and 7.17%, respectively. At five years of age, the Sta contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_1 by 20.11%, 17.74%, and 30.93%, respectively, whereas the Suc contents of RSA−01, RSA−02, and RSA−03 were significantly higher than those of CK_1 by 41.49%, 15.38%, and 52.33%, respectively. Furthermore, the SS content of RSA−01 was significantly higher than that of CK_1 by 6.50%, whereas those of and RSA−03 were significantly reduced by 5.87% and 6.70%, respectively. In addition, the NSC contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_1 by 11.33%, 13.85%, and 23.00%, respectively, and the SP content of RSA−02 was significantly lower than that of CK_1 by 10.01%.

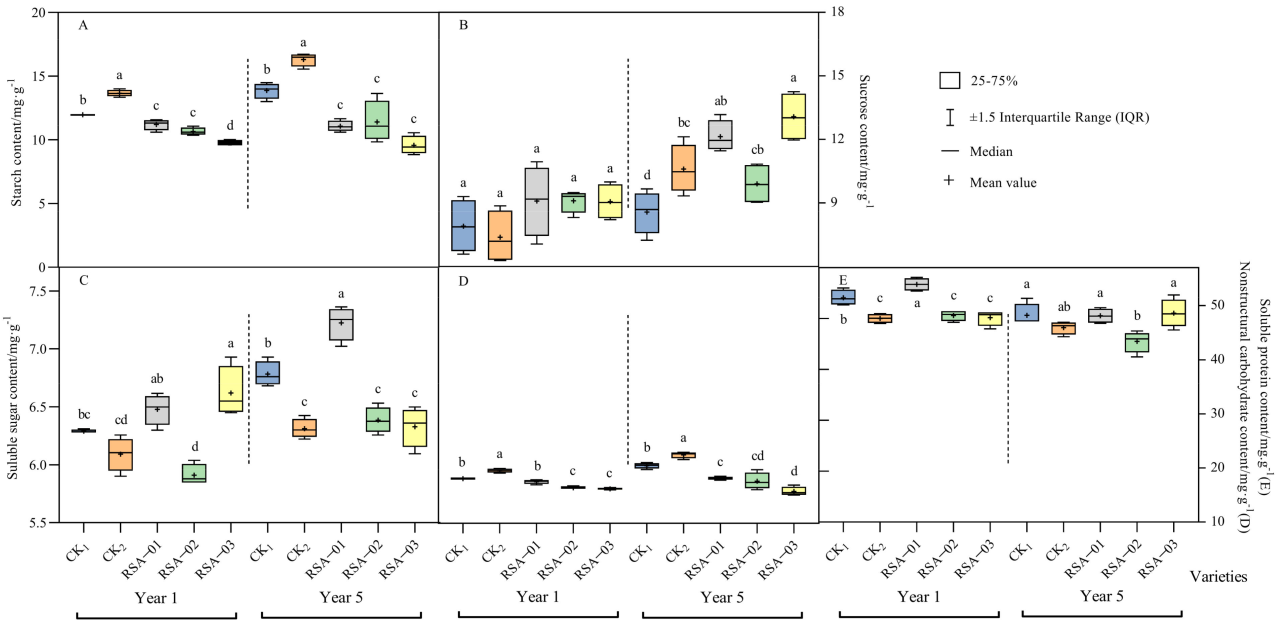

Figure 4. Photosynthetic products of hybrid strains and parents. (**A**) Sta. (**B**) Suc. (**C**) SS. (**D**) NSC. (**E**) SP. Lowercase letters indicate significant differences among varieties (strains) at a p value of <0.05. Blue, orange, gray, green and yellow in the figure represent CK_1, CK_2, RSA−01, RSA−02, RSA−03, respectively.

At one year of age, the Sta contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by17.94%, 21.96%, and 28.54%, respectively, whereas the SS contents of RSA−01 and RSA−03 were significantly higher than those of CK_2 by 6.36% and 8.67%, respectively. The NSC contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by 10.48%, 16.10%, and 17.07%, respectively, whereas the SP content of RSA−01 was significantly higher than that of CK_2 by 13.21%. At five years of age, the Sta contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by 30.10%, 32.11%, and 41.31%, respectively, whereas the Suc content of RSA−01 was significantly higher than that of CK_2 by 23.28%. The SS content of RSA−01 was significantly higher than that of CK_2 by 14.45%, whereas the NSC contents of RSA−01, RSA−02, and RSA−03 were significantly lower than those of CK_2 by 19.11%, 21.29%, and 29.72%, respectively.

3.5. Analysis of Mid-Parent Heterosis of Photosynthetic Physiological Indices in Hybrid Strains

At one year of age, RSA−01 showed obvious mid-parent heterosis in Chl$b$, Chl(a+b), and Car contents; WUE value; Suc, Sp, and SS contents (Figure 5). RSA−02 showed obvious mid-parent heterosis in Chl$b$ and Chl(a+b) contents; P_n, G_s, and WUE values; Suc content. RSA−03 showed obvious mid-parent heterosis in Chl$b$ and Car contents; P_n, T_r, and G_s values; Suc content; and forage mass.

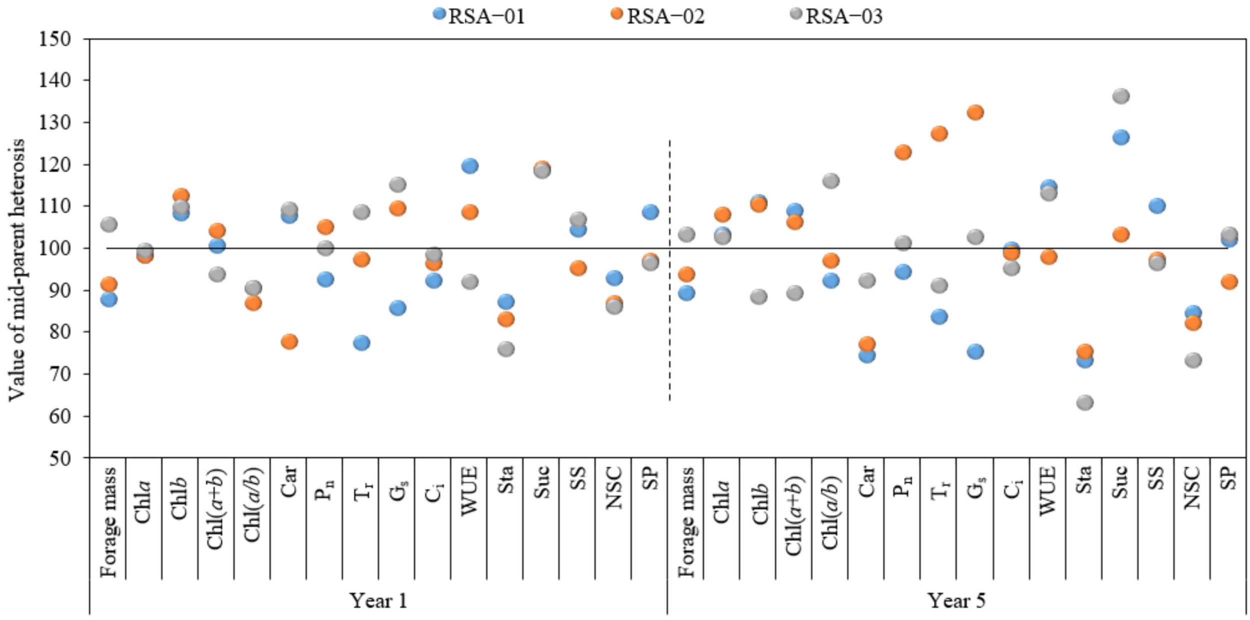

Figure 5. Mid-parent heterosis of forage mass and photosynthetic physiological indicators in hybrid strains. 1a and 5a indicates 1 year of age and 5 years of age, respectively.

At five years of age, RSA−01 showed obvious mid-parent heterosis in Chl*a*, Chl*b*, and Chl(*a*+*b*) contents; WUE value; and Suc content. RSA−02 showed obvious mid-parent heterosis in Chl*a*, Chl*b*, and Chl(*a*+*b*) contents; P_n, T_r, and G_s values; and Suc content. RSA−03 showed obvious mid-parent heterosis in forage mass; Chl*a* content, and Chl(*a*/*b*) ratio; P_n, G_s, and WUE values; and Suc, SP, and SS contents.

3.6. Interannual Stability of Forage Mass and Photosynthetic Physiological Indices in Alfalfa at One and Five Years of Age

To analyze the interannual stability of the photosynthetic capacity of alfalfa varieties (strains) (Figure 6), the rate of interannual variation in photosynthetic physiology at one and five years of age was calculated. The forage mass; P_n, T_r, G_s, and C_i values; Suc, and SS contents of RSA−01 at five years of age were significantly higher than those of RSA−01 at one year of age by 14.36%, 29.09%, 28.22%, 15.79%, 13.24%, 33.45%, and 11.51%, respectively, whereas the SP content of RSA−01 at five years of age was significantly lower than that of RSA−01 at one year of age by 10.77%. The forage mass; P_n, T_r, and G_s values; Chl*a*, and SS contents of RSA−02 at five years of age were significantly higher than those of RSA−02 at one year of age by 15.20%, 46.24%, 47.99%, 55.43%, 59.7%, and 48.02%, respectively. The forage mass; P_n, G_s, and WUE values, Chl(*a*/*b*) ratio, and Suc content of RSA−03 at five years of age were significantly higher than those of RSA−03 at one year of age by 9.99%, 32.27%, 27.91%, 17.48%, 29.28%, and 44.24%, respectively.

In practice, the selection and breeding of alfalfa varieties require germplasm with high and stable yields. The interannual variation in the forage mass of RSA−03 was lower than that in the forage mass of RSA−01 and RSA−02, whereas the forage mass of RSA−03 was significantly higher than that of CK_1 and other hybrid strains at ages one and five years (combined with Figure 1), indicating that the forage mass of RSA−03 was superior to and more stable than that of RSA−01 and RSA−02. The magnitude of interannual variation rate in P_n was ranked as follows: RSA−02 > RSA−01 > RSA−03. The difference in P_n value among varieties at one year of age was not significant, and the difference in P_n between RSA−02 and CK_2 at five years of age was not significant, but both were significantly higher than the other varieties (strains) (Figure 3A). The magnitude of interannual variation rate in G_s was ranked as follows: RSA−02 > RSA−03 > RSA−01, and the G_s of both one- and five-year-old RSA−03 was significantly higher than that of RSA−01. The magnitude of interannual variation rate in Suc content was ranked as follows: RSA−03 > RSA−01 > RSA−02, indicating a higher accumulation of Suc in RSA−03 (Figure 4B). In summary, the photosynthetic capacity of RSA−02 had a relatively higher potential in the late growth stage, while forage mass and photosynthetic physiology of RSA−03 were obvious and stable.

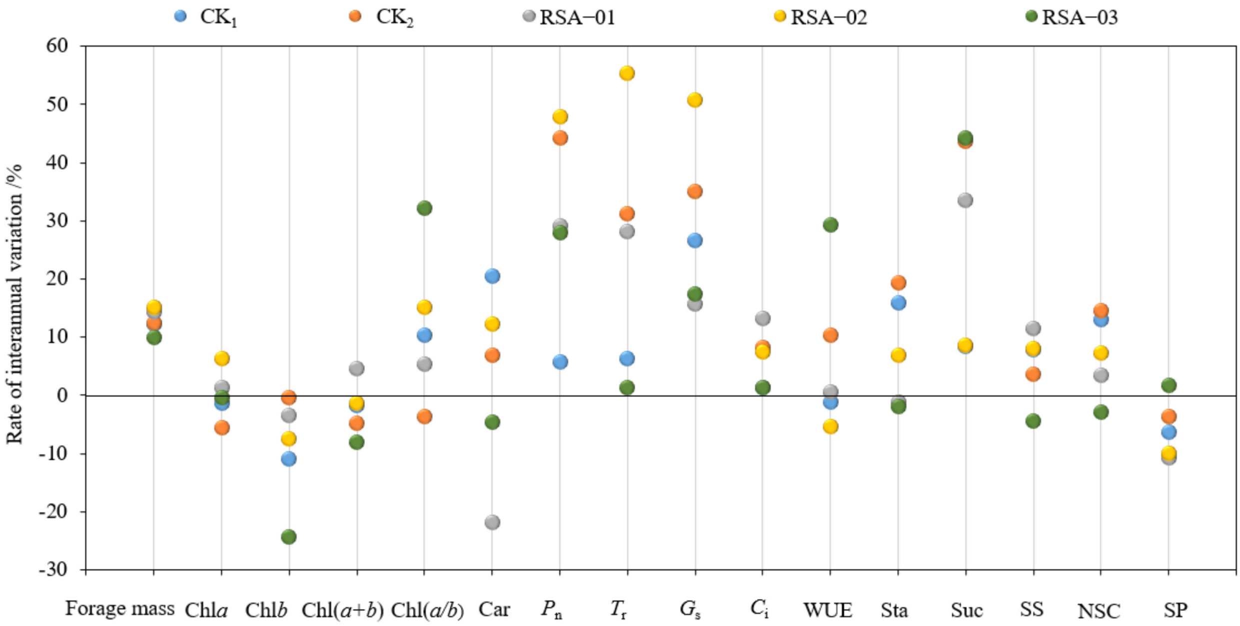

Figure 6. Interannual stability rate of forage mass and photosynthetic physiological indices of alfalfa at 1 and 5 years of age.

3.7. Path Analysis of Forage Mass Yield and Photosynthetic Physiological Indexes in Alfalfa at 1 and 5 Years of Age

To understand the effect of photosynthetic physiological indicators on the forage mass of the tested varieties, R-values, I. E., and D.E. were determined. According to path analysis, the effect of photosynthetic physiological indicators on the forage mass of alfalfa at one- and five-years-old were explored. For one-year-old alfalfa varieties (strains) (Table 2), the effect of T_r and Sta on forage mass was excluded through path analysis, and the magnitude of D.E. of other photosynthetic physiological indicators on forage mass was in the following order: G_s > NSC > SS > WUE > SP > C_i > P_n > Car > Chl(a/b) > Chl$a$ > Chl(a+b) > Suc > Chl$b$. The results showed that the G_s value and NSC content had greater positive effects on forage mass. P_n value and SP content had a greater effect on forage mass through G_s value, with positive and negative effects, respectively, while Chl$b$ and Chl(a+b) contents had greater effects on forage mass through NSC content (Figure 7A). These results indicated that G_s value and NSC content are the main direct factors affecting the forage mass of one-year-old alfalfa varieties (strains), while both P_n and SP indirectly affect forage mass through G_s value, and both Chl$b$ and Chl(a+b) contents indirectly affect forage mass through the NSC content.

For five-year-old alfalfa varieties (strains) (Table 3), the effects of the sChl(a/b), P_n, and Sta content on forage mass was excluded through path analysis, and the magnitude of D.E. of other photosynthetic physiological indicators on forage mass was in the following order: G_s > Chl(a+b) > Chl$b$ > WUE > Chl$a$ > SS > Suc > SP > Car > T_r > NSC > C_i. G_s value and Chl(a+b) content had greater positive effects on forage mass, whereas SS content had greater negative effects on forage mass through G_s value. These findings indicated that G_s valuae and Chl(a+b) content are the main direct factors affecting the forage mass of 5-year-old alfalfa varieties, while SS content indirectly affect the forage mass through G_s value (Figure 7B).

Table 2. Path analysis of photosynthetic physiological indexes and forage mass in alfalfa at 1 year of age.

Index	R	D.E.	I. E.												
			Chl*a*	**Chl*b***	**Chl(*a*+*b*)**	**Chl(*a*/*b*)**	**Car**	P_n	G_s	C_i	**WUE**	**Suc**	**SS**	**NSC**	**SP**
Chl*a*	0.465	−0.069		0.003	0.018	−0.020	−0.032	−0.010	−0.023	−0.013	0.011	0.011	−0.001	−0.028	0.024
Chl*b*	−0.276	0.022	−0.001		−0.006	0.006	0.010	0.003	0.007	0.004	−0.004	−0.003	0.001	0.009	−0.008
Chl(*a*+*b*)	−0.298	0.064	−0.017	0.007		−0.012	−0.018	0.002	−0.008	−0.004	0.038	0.007	−0.026	0.012	0.021
Chl(*a*/*b*)	0.524 *	−0.179	−0.051	0.171	0.033		−0.040	0.025	−0.010	−0.022	0.0360	0.130	0.006	−0.128	0.011
Car	0.440	−0.203	−0.095	0.034	0.058	−0.045		−0.009	−0.069	0.044	−0.031	−0.030	−0.095	−0.067	−0.003
P_n	0.378	−0.221	−0.031	−0.038	−0.007	0.031	−0.010		−0.144	−0.004	−0.039	−0.002	0.043	−0.018	0.108
G_s	0.864 **	0.978	0.321	0.005	−0.118	0.057	0.333	0.638		−0.241	0.129	0.064	−0.222	0.065	−0.765
C_i	−0.048	−0.251	−0.048	0.018	0.016	−0.030	0.055	−0.004	0.062		0.166	0.080	−0.042	−0.029	0.028
WUE	−0.083	−0.382	0.060	−0.050	−0.226	0.077	−0.058	−0.067	−0.050	0.252		−0.136	0.077	−0.084	−0.149
Suc	−0.170	0.048	−0.007	0.031	0.005	−0.035	0.007	0.001	0.003	−0.015	0.017		0.009	−0.025	0.010
SS	0.040	0.524	0.008	0.008	−0.209	−0.019	0.246	−0.102	−0.119	0.088	−0.105	0.094		−0.101	0.177
NSC	0.328	0.772	0.310	−0.499	0.148	0.550	0.252	0.062	0.051	0.090	0.171	−0.399	-0.149		0.089
SP	−0.697 **	−0.307	0.086	0.012	−0.082	0.015	−0.004	0.121	0.193	0.028	−0.097	−0.049	−0.084	−0.028	

Note: coefficients of correlation (R), direct path coefficient (D. E.), and indirect path coefficient (I. E.). (*) indicates significant correlation between forage mass and yield-related indicators at a p value of <0.05. (**) indicates significant correlation between forage mass and yield-related indicators at a p value of <0.01.

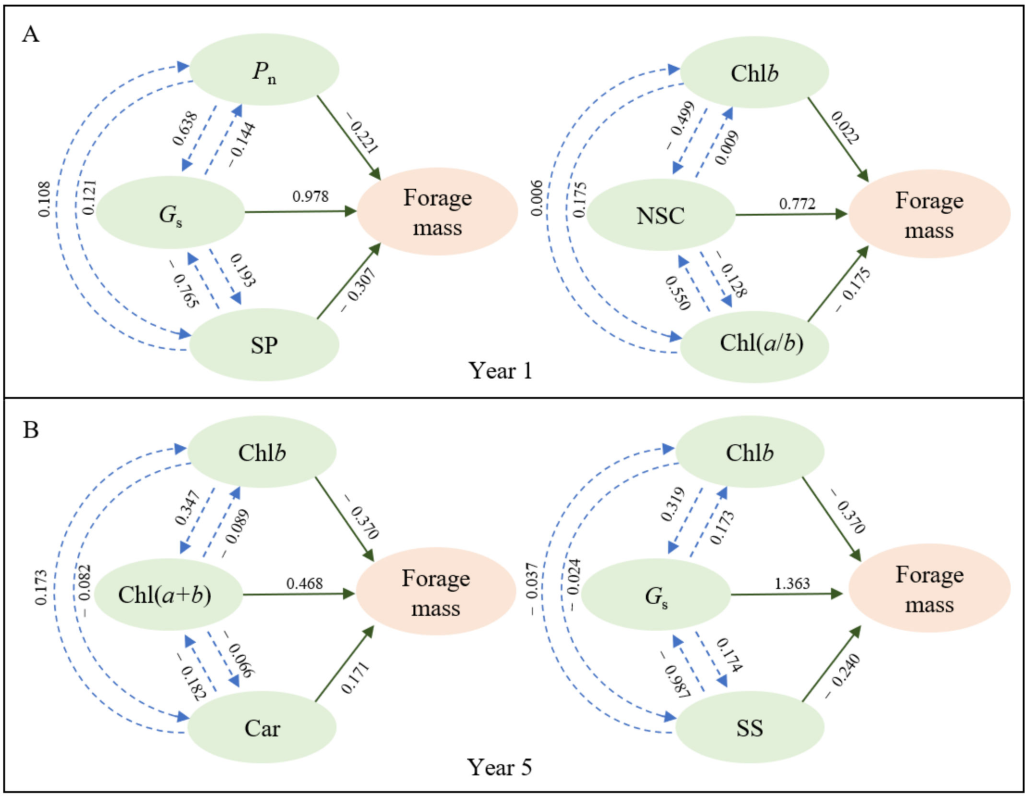

Figure 7. Path analysis of photosynthetic physiological indexes and forage mass in alfalfa at the age of 1 and 5 years. (**A**,**B**) indicates 1 year of age and 5 years of age, respectively.

Table 3. Path analysis of photosynthetic physiological indexes and forage mass in alfalfa at 5 years of age.

Index	R	D.E.	I. E.											
			Chl*a*	Chl*b*	Chl(*a*+*b*)	Car	T_r	G_s	C_i	WUE	Suc	SS	NSC	SP
Chl*a*	−0.377	−0.284		−0.019	−0.068	0.133	−0.036	−0.022	0.046	0.046	0.009	−0.028	0.071	0.103
Chl*b*	−0.141	−0.370	−0.025		−0.089	0.173	−0.047	−0.029	0.060	0.060	0.012	−0.037	0.093	0.134
Chl(*a*+*b*)	−0.395	0.468	0.112	0.347		−0.182	0.053	−0.086	0.121	−0.102	−0.161	0.165	0.082	−0.138
Car	0.489	0.171	−0.080	−0.074	−0.066		−0.007	0.020	−0.002	−0.026	−0.036	−0.076	0.095	−0.007
T_r	0.241	−0.151	−0.019	−0.072	−0.017	0.006		−0.124	−0.030	0.021	0.048	0.092	−0.013	0.119
G_s	0.660 **	1.363	0.106	0.319	−0.249	0.157	1.116		−0.098	0.333	0.027	−0.987	0.142	−0.946
C_i	−0.193	−0.112	0.018	−0.043	−0.029	0.001	−0.022	0.008		0.025	0.052	−0.017	−0.062	0.004
WUE	0.506	0.290	−0.047	0.044	−0.063	−0.044	−0.041	0.071	−0.065		0.154	−0.002	−0.022	0.082
Suc	0.239	−0.236	0.007	0.080	0.081	0.049	0.074	−0.005	0.110	−0.125		0.008	0.149	−0.088
SS	−0.673 **	−0.240	−0.024	−0.041	−0.085	0.107	0.146	0.174	−0.037	0.001	0.008		0.007	−0.091
NSC	0.257	−0.148	0.037	−0.032	−0.026	−0.082	−0.013	−0.015	−0.081	0.011	0.094	0.004		0.028
SP	−0.117	0.188	−0.068	−0.084	−0.056	−0.008	−0.149	−0.131	−0.007	0.053	0.070	0.071	−0.036	

Note: coefficients of correlation (R), direct path coefficient (D. E.), and indirect path coefficient (I. E.). (**) indicates significant correlation between forage mass and yield-related indicators at a p value of <0.01.

3.8. Effect of Interannual Variation and Genetic Differences in Photosynthetic Physiological Indexes of Alfalfa

As shown in Table 4, two-way ANOVA indicated that forage mass; Chl(a/b) ratio; Chl*b*, Suc, SP, and SS contents; P_n, T_r, G_s, and C_i values were more affected by interannual variation than genetic differences, while Car and Sta contents were more affected by genetic differences than interannual variation. Chl*a*, Car, Sta, and NSC contents were significantly affected by the interaction of interannual variation and genetic differences.

Table 4. F-values of two-way ANOVA of interannual variation and genetic differences on photosynthetic physiological indices of alfalfa.

Index	Genetic Differences	Interannual Variation	Genetic Differences × Interannual Variation
Forage mass	1.241	7.748 *	0.100
Chl*a*	2.290	0.260	6.840 *
Chl*b*	4.568 *	8.713 *	1.271
Chl (*a*+*b*)	0.049	0.902	0.143
Chl(a/b)	1.668	8.050 *	3.628
P_n	0.240	16.836 *	0.323
Car	6.141 *	0.523	4.476 *
T_r	0.114	10.969 *	0.233
G_s	0.133	8.161 *	0.002
WUE	2.470	1.530	0.054
C_i	5.607 *	20.063 *	0.710
Sta	59.064 *	7.756 *	6.027 *
Suc	16.419 *	26.973 *	0.587
SS	0.854	6.359 *	0.033
NSC	56.350 *	12.975 *	6.478 *
SP	0.001	10.164 *	0.184

Note: (*) indicates significant effect of interannual variation and genetic differences in photosynthetic physiological indicators at a p value of <0.05.

4. Discussion

4.1. Effect of Genetic Differences on the Photosynthetic Physiology of Alfalfa

The leaf is the main part of a plant where photosynthesis occurs, and Chl is an important component of the light energy absorption and electron transfer system [10]. Light is the primary factor affecting photosynthesis, providing the energy needed for assimilative force formation. In addition, light activates key enzymes involved in photosynthesis, promotes stomatal opening, and regulates the development of photosynthetic bodies [20,21]. Carbohydrates are the main products of photosynthesis, among which NSCs, particularly SS and Sta, are important substances involved in plant life metabolism [22]. Genetic characteristics are important factors influencing and governing the strength of photosynthetic capacity [23], and the positive correlation between the intercellular Chl*a*, Chl*b*, and Car contents of the hybrid rice combination (F_1) and the general combining ability (GCA) of the parent (male) was significantly high [22]. An increase in total Chl content along with a better Chl fluorescence characteristic were observed in an F_1 hybrid and were higher than in the parental inbreeds [23]. In a study by Kamphorst [19], positive values for Chl heterosis were observed in four self-incompatible maize strains. In this study, the heterosis of Chl*a*, Chl*b*, and Chl(*a*+*b*) contents of the hybrid strains was significant, and their mean values were higher than mid-parent heterosis values. Meena [23] found that the P_n, G_s, and T_r, as well as foliar carbohydrates were higher in the F_1 hybrid than in parental inbreeds at vegetative and reproductive stages. The correlation between the photosynthetic parameters of the hybrid rice combination F_1 and the GCA sum of its parents reached significant levels [24]. Zhou [25] found that the P_n and G_s of upland cotton Siza 3 demonstrated heterosis compared with its parents. Previous studies have shown that most maize hybrid strains showed positive heterosis in T_r, P_n, and WUE values, and their progeny achieved efficient photosynthesis [23–26]. In this study, the heterosis of P_n (five-year-old), T_r (five-year-old), G_s, and WUE values of the hybrid strains was obvious, and their mean values were higher than the mean values of both parents, indicating that the photosynthetic parameters of the hybrid strains had significant mid-parent heterosis, which was similar to that reported in the abovementioned studies [23–27]. Liao [28] showed a correlation between the SS and Sta contents of the female parent of the intersubspecific hybrid rice sterile strains and its corresponding hybrid combination. Li [29] found that the Sta and Suc metabolisms showed obvious gene dominant complementation in potato hybrids, and hybrids devoted more energy to primary metabolism for rapid growth. Liu [30] found that the new tetraploid rice hybrid F_1 had a large number of specific differentially expressed genes compared with its parents, among which, more than 95% of the genes were involved in yield-related Quantitative trait loci (QTLs) and were significantly enriched in the carbohydrate metabolism pathway. In this study, the heterosis of Suc, SS, and SP (age one year) of the hybrid strains at five years of age was obvious, and their mean values were higher than the mean values of the two parents, indicating that the hybrids had a super middle parent advantage in their values.

4.2. Effect of Interannual Variation on the Photosynthetic Physiology of Alfalfa

In addition to genetic influence and constraints, external ecological conditions, such as light intensity and temperature, are important factors influencing the strength of photosynthetic capacity. Cui [13] showed the Chl*a* content remained constant between years two and six, and the Car content increased and peaked in the fourth year. Hu [20] showed that the relative Chl content did not differ significantly over four, six, eight, and twelve years. In the present study, it was found that the Chl*a* content of RSA−02, Chl(*a*/*b*) ratio of CK_1, and Chl(*a*/*b*) ratio of RSA−03 at the age of five years were significantly higher than those at the age of one year, whereas the difference in pigment content among the varieties (strains) between the two years was not significant, which was not consistent with the study pattern used herein [13,20,31]. The reasons for these inconsistent results may be related to the study material, sample size, leaf age, leaf position, sampling period, environmental and other conditions, as well as to the analytical methods used, which need to be further investigated. Bai [32] found that super hybrid rice possesses a higher P_n than non-hybrid rice. Cui [13] found that the fourth-year alfalfa had the highest leaf P_n at the early flowering stage. In the present study, the P_n, T_r, and G_s values of alfalfa varieties (strains) at the age of five years were significantly higher than those at the age of one year; the C_i value of CK_2 and RSA−01 at the age of five years was significantly higher than that at the age of one year; and the WUE value of CK_2 and RSA−03 at the age of five years was significantly higher than that at the age of one year; these findings were similar to those of previous studies [13]. Wu [33] found that the SS content of leaves of *Elymus sibiricus* Linn. gradually increased with plant age, while the Sta content decreased, then increased, and then decreased. Huang [34] found significant differences in the Sta content of ginseng samples of different origins and ages, and [35] suggested that the senescence of plant leaves might be related to the excessive accumulation of sugars. In the present study, the Sta content (CK_1, CK_2), NSC (CK_1, CK_2), Suc content (CK_2, RSA−03), and SS content (CK_1, RSA−01 and RSA−02) were significantly higher in five-year-old alfalfa plants than those in one-year-old plants, while the SP content (CK_1 and RSA−01) was significantly lower in five-year-old alfalfa plants than those in one-year-old plants; these findings were similar to those of a previous study [33], suggesting that the aging of alfalfa is not caused by insufficient carbohydrate supply to meet its growth and development needs.

4.3. Relationship between Forage Mass and Photosynthetic Physiological Indicators in Alfalfa

The accumulation of forage mass is the basis of crop growth, development, and yield formation. It is an important indicator of production and economic performance, and it is also one of the main breeding objectives [36,37]. In semi-arid regions, alfalfa is at its peak yield from age one to five [38,39]. In this study, CK_2, CK_1, RSA−01, RSA−02, and RSA−03 at the age of five years were significantly higher than those at the age of one by 12.27%, 12.48%, 14.35%, 15.24%, and 9.99%, respectively. In this study, RSA−02 at age five years, RSA−03 at age one year, and RSA−03 at age five years had 3.41%, 8.66%, and 19.72% higher forage mass than CK_1, indicating that RSA−03 had the best hybrid advantage in terms of forage mass. The accumulation of forage mass is mainly governed by photosynthesis and respiration. Li [31] showed that forage yield correlated positively with P_n, T_r, and Sta, while it was inconsistently correlated with Chl, T_r, G_s, and C_i concentrations. The path coefficients are standardized bias regression coefficients, and the effects caused by the different magnitudes of variation of each indicator are eliminated, enabling an objective evaluation of the effects of photosynthetic physiological indicators on forage mass [8]. In this study, G_s and NSC were the direct factors affecting forage mass in the one-year-old hybrid strains, and P_n and SP indirectly affected forage mass through G_s. In the five-year-old hybrid strains, G_s and Chl(a+b) were the direct factors affecting forage mass, and both SS and SP content indirectly affected forage mass through G_s. The results indicated that G_s was the main direct factor affecting forage mass, while the influencing factor of G_s stomatal conductance changed with age changes, leading to differences in forage mass. About 95% of the forage mass of plants comes from CO_2 assimilated by photosynthesis, and this basic fact determines that there can only be a positive correlation between the content of P_n, Chl, and crop yield. However, this internal positive correlation may be concealed by the complex changes of other factors, resulting in the absence of this positive correlation. Among them, Chl content and P_n are related to crop senescence and programmed cell death, while yield is also affected by plant height, stem diameter, stem leaf ratio, leaf area, and other indicators [8,36,40]. It is worth noting that we select grazing alfalfa as the research object, and the breeding of new grazing varieties with high quality and yield needs to combine their agronomic traits, stress resistance, adaptability, grazing tolerance, etc., for comprehensive and systematic screening.

4.4. Comprehensive Analysis of Interannual Variation and Genetic Differences in Photosynthetic Physiological Indicators of Alfalfa

Photosynthesis of plants is affected and restricted by their biological characteristics (crop type, variety, leaf age, etc.). Additionally, light intensity, temperature, fertilizer and water management, stress, cutting method, and growth period are also important factors that affect photosynthesis, and each factor affects the other [19,24,41]. The strength of photosynthetic capacity depends on the genetic characteristics of the species to a certain extent. Still, the appropriate external ecological conditions will promote the development of its inherent photosynthetic potential [13,20]. Previous studies have shown that planting years have important effects on photosynthetic pigments [20,23], photosynthetic parameters [13,31], and photosynthetic products [20,34] of crop leaves. Chl content is a typical quantitative trait, controlled by multiple genes and easily affected by environmental conditions. QTLs that often control Chl content are detected in one environmental condition but not in another, showing genetic complexity [41]. The results showed that forage mass; Chl(a/b) ratio; P_n, T_r, G_s, and C_i values; Chl*b*, Suc, SP, and SS contents were more affected by interannual variation than by genetic differences, while Car and Sta contents were more affected by genetic differences than interannual variation. Chl*a*, Car, and NSC contents showed a significant interaction between age and genetics, which is consistent with the findings of Zhao [41]. Due to the complexity of polygene controlling genetic factors and the influence of external conditions, we had some limitations in exploring the relationships among years, inheritance, and photosynthetic physiological indicators. The molecular mechanism of their interaction and photosynthesis remains challenging for alfalfa breeding.

Breeding practice defined the main ways to increase crop yield: morphotype change, exploitation of heterosis, and increase in photosynthesis effectiveness. Establishing grazing-type grasses usually requires grazing-type materials with long life, high and stable yield, and superior nutrition [42]. CK_1 and CK_2 are ecological forages suitable for establishing grazing-type grasslands (their advantages are in Table 1). We previously studied the longevity characteristics of varieties, and concluded that long-lived (Qingshui, WL168 and RSA−03), medium-lived (RSA−02), and short-lived (RSA−01) strains corresponded to telomere length [2]. Additionally, we also determined yield performance and nutritional value and concluded that RSA−01, RSA−02, and RSA−03 performed well in yield performance, while RSA−01 and RSA−03 performed well in quality traits compared with the parental CK_1 [4,5]. In conclusion, RSA−03 is an ecological forage with a longer life span, high and stable yield, and high nutritional value, which is of great value for improving biomass and ecological conservation of grazed grassland. We started our research from two points in the future: first, planting in natural grassland or artificial grazing grass to further investigate its grazing and cold tolerance; second, trying to investigate the relationship between photosynthetic capacity and longevity from the molecular mechanism.

5. Conclusions

Compared with the rhizome-rooted Qingshui, forage mass and photosynthetic capacity have been improved by the cross of Qingshui and WL168, among which RSA−03 showed obvious and stable heterosis in forage mass and photosynthetic physiology. RSA−03 can be used as a constructive species planting in grazing and ecological grasslands. From the perspective of alfalfa species, G_s and NSC significantly affected its forage mass at age one year, and G_s and Chl(a+b) relatively affected it at age five years. Based on this study, the molecular mechanism of G_s responding to the yield of alfalfa will be explored. Interestingly, we explored the effects of interannual dynamics and genetic differences on tested indices, and the results demonstrated that forage mass, Chl(a/b), Chl*b*, P_n, T_r, G_s, C_i, Suc, SP, and SS were more influenced by age than genetics, while the opposite was true for Car and Sta.

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Abbreviations

chlorophyll (Chl); carotenoids (Car); net photosynthetic rate (P_n); transpiration rate (T_r); stomatal conductance (G_s); intercellular CO_2 concentration (C_i); water use efficiency (WUE); starch (Sta); sucrose (Suc); soluble sugars (SS); soluble protein (SP); non-structural carbohydrate (NSC)

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