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Effects of Autotoxicity on Alfalfa (*Medicago sativa*): Seed Germination, Oxidative Damage and Lipid Peroxidation of Seedlings

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Abstract: Alfalfa (*Medicago sativa*) is a highly valuable perennial forage legume that suffers from autotoxicity, which decreases plant resistance, reduces soil fertility, causes serious soil-borne diseases, and promotes ecological imbalance. We evaluated the effects of autotoxicity on the seed germination of 22 alfalfa varieties, and then elucidated the oxidative damage and lipid peroxidation in two alfalfa varieties with contrasting autotoxicity tolerances. The technique for order of preference by similarity to ideal solution (TOPSIS) method was used to rank the germination of the 22 alfalfa varieties when exposed to six autotoxic concentrations (0, 0.025, 0.075, 0.125, 0.175, and 0.225 g·mL^{-1}). We found WL656HQ and 3105C to be autotoxicity-tolerant and autotoxicity-sensitive varieties, respectively. The germination index mainly affects the comprehensive allelopathic index of WL656HQ and 3105C, which were the simple vigor index and radicle length according to the random forest model, respectively. 3105C eliminates reactive oxygen species (ROS) via antioxidant enzymes and antioxidants under T1 (0.025 g·mL^{-1}), but the oxidative stress system and the oxidative scavenging system cannot maintain the balance under T2 (0.125 g·mL^{-1}), causing oxidative bursts. In comparison, WL656HQ used its oxidative scavenging system (peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR)) to maintain its redox dynamic balance by removing excess ROS at all concentrations. In conclusion, the positive and negative indicators of autotoxicity for the two varieties were ascorbate (ASA) and hydroxyl free radicals (OH$^{\bullet}$), and proline (Pro) and dehydroascrobate (DHA), respectively. The most sensitive autotoxic concentrations of 3105C and WL656HQ were T2 (0.125 g·mL^{-1}) and T1 (0.025 g·mL^{-1}), respectively.

Keywords: alfalfa; autotoxin; germplasm; abiotic stress; autotoxicity effect

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1. Introduction

Legumes comprise one of the largest plant families on Earth [1]. Alfalfa (*Medicago sativa*) is a perennial forage legume with high nutritive values, high yield, and strong adaptability and is widely used as a high-quality livestock feed. Alfalfa also protects soil and reduces wind erosion. Due to its biological ability to fix nitrogen, it also plays an extremely important role in the ecosystem's nitrogen cycle, giving it high economic and ecological value [2]. As the quality of life in China has improved with rapid economic development, the demand for high-protein foods has increased in recent years. This has promoted the rapid development of high-quality forage, and a sharp increase in alfalfa demand [3]. The production of alfalfa in China cannot meet this higher demand, and thus China depends on alfalfa imports more than any other country, and ranks first globally in alfalfa imports. In

addition, domestic alfalfa cultivation areas are mainly concentrated in low-yield fields and the farming-pastoral transition zone, where conditions are relatively harsh [4]. The primary method to cultivate alfalfa is via single, intensive production. This large-scale continuous cropping method has led to increasingly difficult production obstacles. The greatest of these are autotoxic effects, which is a major problem in sustainable animal husbandry development [5].

Autotoxicity in crops can be best traced in alfalfa, where it has been demonstrated convincingly by various workers. It relates to the poor reestablishment of alfalfa in the soil. Autotoxicity has been shown to be due to the presence of water-soluble toxic compounds that are released into the outer environment [6]. Autotoxicity occurs when a plant releases toxic chemical substances into the environment that inhibit germination and growth of same plant species [7]. The main components of autotoxic chemicals are some secondary metabolites originating from self-secretion, aboveground leaching and residue decomposition [8]. Autotoxicity can occur especially obvious in large-scale continuous cropping [9]. Autotoxic effects shorten the continuous cropping period and reduce land utilization rates, resulting in a decline in plant resistance, yield, and quality. These lead to a series of problems, including reduced soil fertility, serious soil-borne diseases, and ecological imbalances [10,11]. The autotoxicity will continue to increase with the extension of the alfalfa planting period. Usually after destroying an old alfalfa stand, the delay required to replant alfalfa is up to 2 years [9]. Currently, crop rotation or fallow is traditionally used methods to alleviate autotoxic effects. Although these traditional methods can effectively alleviate autotoxic effects, they also have disadvantages, including prolonged production cycles, reduced land utilization efficiency, increased production costs, and reduced production benefits [12]. In contrast, breeding autotoxicity-tolerant alfalfa varieties could be the most direct, effective, and economical method to alleviate autotoxic effects with limited land resources. Studies have shown that autotoxicity is a type of abiotic stress that affects cell membranes and plant hormone activities, causes DNA damage, and impairs photosynthesis and mitochondrial function. Autotoxic effects inhibit the growth of other plants of the same species by releasing allelochemicals that act on cell membrane target sites to induce oxidative stress and cause the excessive accumulation of reactive oxygen species (ROS) [13]. The excessive accumulation of ROS poses a threat to cells and results in membrane lipid peroxidation and cell death [14]. Thus, there is an urgent need to study the toxic mechanism of autotoxic effects to provide a theoretical basis for toxicity prevention and control, and for the breeding of tolerant and sensitive varieties. Currently, most studies focus on the effect of a single autotoxic substance by exogenous addition in plants. Chon et al. showed that mixtures of autotoxic substances are more phytotoxic than their components [9], and that alfalfa leaf extract has a greater autotoxic effect on seedlings than on other tissues [15]. Chung et al. ranked the autotoxic effects of water extracts of plant parts of alfalfa > leaf > seed > root > flower > and > stem [16]. Under laboratory conditions, the water extract of the aboveground part (especially the leaf) usually has a greater autotoxic effect on the seedlings than the water extract of the root [15], and the extract in the reproductive stage has a more inhibitory effect than the extract in the vegetative stage [16].

This study aimed (a) to screen for sensitive and tolerant varieties of alfalfa by adding leaf extracts from 22-cultivars of alfalfa to the flowering stage to simulate autotoxic effects; (b) to explore the autotoxicity differences in response to the oxidative stress system of sensitive and tolerant varieties from the aspects of growth change, ROS production, osmotic regulation, and antioxidant systems; and (c) to compare and analyze the critical concentration and sensitive indicators in response to autotoxic effects. This work provides a novel idea that different varieties of alfalfa should systematically respond to autotoxic effects at the physiological level to provide a theoretical basis for the breeding of autotoxic-tolerant alfalfa varieties.

2. Materials and Methods

2.1. Plant Materials

Medicago sativa L. cv. Longdong was chosen for this study based on our previous studies [17]. Seeds from different alfalfa varieties (Sanduli, Debao, Jiasheng, Canon 429, SG-601, SG-501, SG-401, SG-201, 329 Wonder, 3105C, 218TR, 416WET, and 420Ya) were obtained from the Key Laboratory of Grassland Ecosystems of the Ministry of Education (Lanzhou, China). The seeds from the remaining alfalfa varieties (WL-298HQ, WL-319, WL-354HQ, WL-656HQ, WL-525, WL363HQ, WL343HQ, and WL168HQ) were purchased from the Beijing Zhengdao Seed Industry Co., Ltd. (Beijing, China) [18] (Table 1).

Table 1. Alfalfa varieties and serial numbers.

Number	Alfalfa Varieties	Number	Alfalfa Varieties
1	WL-298HQ	12	SG-201
2	WL-319	13	Debao
3	WL-354HQ	14	Longdong
4	WL-656HQ	15	Jiasheng
5	WL-525	16	Canon 429
6	WL343HQ	17	329 Wonder
7	WL-363HQ	18	Sanduli
8	WL-168HQ	19	3105C
9	SG-601	20	218TR
10	SG-501	21	416WET
11	SG-401	22	420Ya

2.2. Experimental Conditions

The alfalfa varieties were cultivated using the mixed method with nutritious soil, vermiculite, and garden soil (without planted alfalfa). Flowerpot size was 25 cm wide × 20 cm height. The pots were loaded with nutritious soil: vermiculite (3:1). Full and uniform alfalfa seeds were disinfected for 5 min with 0.1% $HgCl_2$ solution, then rinsed with distilled water, and dried with absorbent paper before being sowed 30 seeds evenly into the flowerpots. The flowerpots were subsequently buried in the field (geographical coordinates are 105°41′ E and 34°05′ N) at the Herbage Training Station (Lanzhou, China) with the mouth of the pot flush to the ground. Daily quantitative watering (500 ML) evenly into every flowerpot ensured normal germination and emergence.

2.3. Sampling and Preparation of Extracts

The autotoxic substance content in alfalfa leaves is relatively high. The aboveground portions of the 22 alfalfa cultivars were harvested at an initial flowering stage from the field in the first year after planting [16]. After the stems and leaves were separated, the leaves (leaves and petioles) were oven-dried at 60 °C for 5 d [19]. Next, the leaves were ground with a grinder to pass a 1 mm screen. The ground samples (0.25, 0.75, 1.25, 1.75, and 2.25 g) were weighed into a clean triangular bottle and soaked in 100 mL distilled water. The bottle was sealed and shaken for 24 h at 180 rpm and 25 °C. Afterwards, vacuum filtration was used to obtain the extract in concentrations of 0.025 g·mL^{-1} (S1), 0.075 g·mL^{-1} (S2), 0.125 g·mL^{-1} (S3), 0.175 g·mL^{-1} (S4), and 0.225 g·mL^{-1} (S5). The samples were then stored at 4 °C. Distilled water was used as the control (CK).

2.4. Effects of Extracts on Seed Germination and Seedling Growth

In Stage 1, the 22 alfalfa seed cultivars were exposed to the different extract concentrations. First, two layers of filter paper were placed at the bottom of petri dishes with 9 cm diameter, and each treatment was performed in triplicate. Next, 30 sterilized alfalfa seeds were placed evenly in each petri dish, and 3.5 mL leaf extract from the 22 alfalfa cultivars was added to each petri dish in different concentrations. The control was added to distilled water. The seeds were allowed to germinate in an artificial climate box with the

photoperiod set at 25 °C for 12 h and the dark cycle set at 20 °C for 12 h. The standard for seed germination was when the radicle broke through the seed coat at 1–2 mm. The number of germinated seeds was recorded daily. On the 7th day, the germination number and seedling weights were recorded to calculate the germination percentage (Gp), germination potential (GP), simple vigor index (SVI), and dry–fresh ratio. In addition, the radicle length, germ length, and seedling height were measured according to Equations (1)–(3) (ISTA, 2012) [20]:

$$\mathrm{Gp} = n_7 / n_{total} \times 100\% \quad (1)$$

$$\mathrm{GP} = n_3 / n_{total} \times 100\% \quad (2)$$

$$\mathrm{SVI} = \mathrm{Gp} \times \text{Seeding height} \times 100\% \quad (3)$$

where n_7 is the number of normal germinated alfalfa seeds in 7 d; n_{total} is the number of total tested alfalfa seeds; and n_3 is the number of normal germinated alfalfa seeds in 3 d.

In addition, alfalfa seedling height, radicle length and germ length were measured on the 7th day, and the response index (RI) values were calculated using the method of Williamson and Richardson (1988) [21]:

$$\mathrm{RI} = 1 - \mathrm{C}/\mathrm{T} \quad (4)$$

where RI represents the allelopathic effect index; C is the control value; and T is the treatment value. When RI $\geq$ 0, it shows stimulatory effects and RI $\leq$ 0, it shows inhibitory effects. The absolute value of RI indicates the intensity of allelopathy. The comprehensive allelopathic index (SE) is the arithmetic mean of the allelopathic sensitivity index (RI) of the donor to multiple test items of the same recipient, indicating the comprehensive effect of allelopathic autotoxicity.

Based on the results of Stage 1, the seedlings were exposed to different concentrations of extract in Stage 2. The extract treatments determined the seedlings morphological and physio-biochemical responses to autotoxicity. The autotoxicity-sensitive varieties (3105C) and autotoxicity-tolerant varieties (WL656HQ) were cultured using the sand culture method, based on our previous studies [17]. First, the fine sand was washed with tap water, then loaded into a nutrition bowl (diameter 9 cm) and placed in a plastic square basin (25 cm length × 15 cm width × 10 cm height). Then, the full and uniform 3105C and WL656HQ alfalfa seeds were disinfected for 5 min with 0.1% $HgCl_2$ solution, and rinsed with distilled water. Next, the seeds were dried with absorbent paper, then sown evenly into cultivar-specific culture bowls, and placed in a light culture chamber (light: 14 h; luminous flux density: 400 μmol m^{-2} s^{-1}; day and night temperature 25 ± 1 °C and 20 ± 1 °C; relative humidity about 60%). The seedlings were watered daily to ensure normal germination and emergence. After emergence, 3105C and WL656HQ seedlings with the same growth were kept in each bowl, and 100 mL Hoagland nutrient solution was added daily for 7 d [22]. On the 45th day, extract concentrations of 0.025, 0.125, and 0.225 g·mL^{-1} were sprayed evenly in the nutrition bowls, while the control group received the same amount of distilled water. T1, T2, and T3 represented the three treatment concentrations, and the distilled water treatment was named CK. After treatment, different concentrations of 100 mL extract were irrigated every other day to maintain the solution concentration. After 21 d of continuous extract treatment, the leaves of the alfalfa seedlings were stored at −80 °C for determination of the physiological indexes.

2.5. Measurement of Seedlings' Physiological Parameters

2.5.1. Measurement of Soluble Protein, Soluble Sugar and Free Proline Content

The soluble protein content was assayed using bovine serum albumin [23]. The soluble sugar content was determined by anthrone colorimetry [24]. Free proline content was estimated according to the acid-ninhydrin method [25]. The soluble protein, soluble sugar, and free proline contents were calculated via a standard curve and all estimated on a fresh weight basis.

2.5.2. Measurement of MDA and ROS (H_2O_2, $OH^•$ and $O_2^{•-}$) Levels

The malondialdehyde (MDA) content was determined using the thiobarbituric acid method [26]. The hydrogen peroxide (H_2O_2) content was determined using the KI colorimetric method [27]. The hydroxyl radical ($OH^•$) concentration was determined using the 2-deoxy-D-ribose colorimetric method [28]. The superoxide anion radical ($O_2^{•-}$) production rate was determined using the p-aminobenzenesulfonic acid method [29], The methods for determining enzyme activity and antioxidant levels were shown in Table 2.

Table 2. Methods used for determining enzyme activities and antioxidant levels.

Enzymes	Buffer (PBS)	Reaction System	Measuring Wavelength	Reference
SOD	0.1 mM EDTA 1% PVP pH7.8	50 mM PBS; 100 μL enzyme solution; 100 μL riboflavin	560 nm	Giannopolitis et al., 1977 [30]
POD	0.1 mM EDTA 1% PVP pH7.8	100 mM PBS; 20 mM guaiacol; 40 mM H_2O_2	470 nm	Chance et al., 1955 [31]
CAT	0.1 mM EDTA 1% PVP pH7.8	50 mM PBS; 50 μL enzyme solution; 19 mM H_2O_2	240 nm	Havir et al., 1987 [32]
APX	-	50 mM PBS; 0.25 mM AsA; 0.1 mM EDTA; 5 mM H_2O_2	290 nm	Murshed et al., 2008 [33]
GR	-	50 mM PBS; 0.5 mM EDTA; 0.25 mM NADPH; 0.5 mM GSSG	340 nm	Murshed et al., 2008 [30]
ASA plus DHA	150 mM EDTA; 10 mM DTT; 0.5% (*w*/*v*) N-ethylmaleimide	10% TCA; 44% orthophosphoric acid; 0.5%	525 nm	Murshed et al., 2013 [34]
ASA	150 mM EDTA; 0.3 mL water	BP-ethanol; 0.3% (*w*/*v*) FeCl3	525 nm	Murshed et al., 2013 [34]

2.6. Statistical Analysis

All data were analyzed with R Software (Version 4.0.2). (http://cran.rstudio.com/index.html; accessed on 14 March 2021). The plyr package in technique for order of preference by similarity to ideal solution (TOPSIS) analysis was used to rank the 22 alfalfa varieties using the entropy-positive function to calculate the weights. The TOPSIS is a multi-index decision analysis and evaluation method [35]. In the random forest analysis, the random forest package ranks the relative importance of the seed germination index, which is calculated using the random forest function. The differences in aboveground biomass, plant height, antioxidant enzymes, antioxidants, osmotic regulators, ROS, and MDA, between the treatment groups and the control group were compared using one-way ANOVA (significance level of $p < 0.05$). The avo function in the agricolae package was used for the calculations. Multiple comparison analyses were calculated using the duncan.test function in the agricolae package. The positive and negative contribution indicators and sensitive concentrations of the two varieties were conducted with the PCAtools package in R. The principal component analysis (PCA) function in the PCAtools package was used for the calculations. The figures and graphs were created with the ggplot2 and ggpubr package in R.

3. Results

3.1. Effect of Autotoxicity on Seed Germination

3.1.1. Technique for Order Preference by Similarity to an Ideal Solution (TOPSIS Variety Selection)

The TOPSIS comprehensive evaluation method was used to rank seven indices (seedling height, SVI, radicle length, germ length, dry fresh ratio, GP, germination percent-

age under six concentrations (Figure 1a), and the ranking score (Figure 1b)). According to the rank score of the 22 varieties (alfalfa varieties and serial numbers were shown in Table 1), the most sensitive and most tolerant varieties to autotoxicity were 3105C and WL656HQ, respectively (Figure 1b).

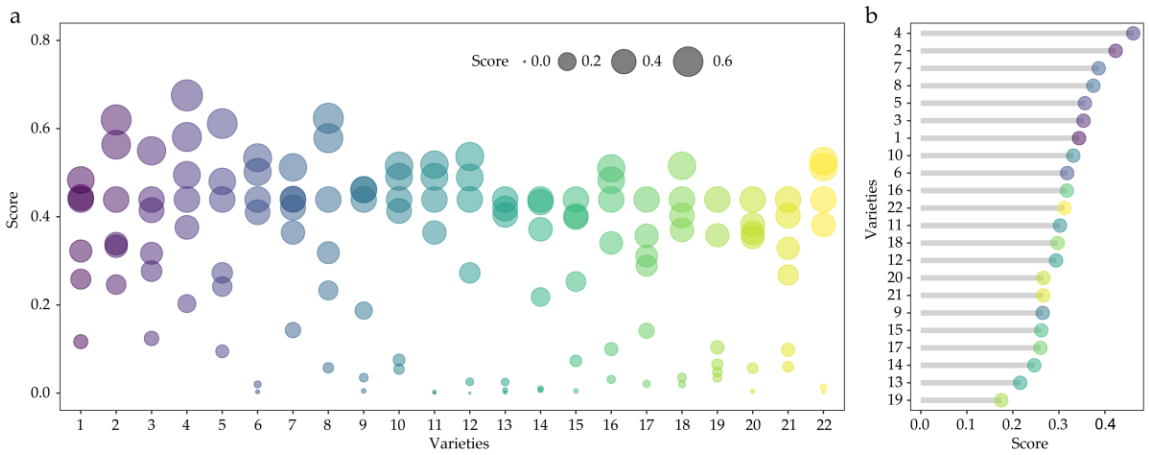

Figure 1. Comprehensive evaluation of the autotoxic effect of 22 alfalfa varieties exposed to six leaf extract concentrations. (**a**) Under six concentrations, the seven germination indexes of the 22 varieties are relative to the comprehensive score of CK. 1–22 represent twenty-two alfalfa varieties, and each circle represents each treatment concentration. (**b**) Comprehensive score ranking of 22 varieties.

3.1.2. Effect of Autotoxicity on Allelopathic Comprehensive Effect Index of Alfalfa

In this study, the random forest model was used to evaluate variable importance. The prediction accuracy of the allelopathic comprehensive effect index for 3105C and WL656HQ were 96.9% and 65.2%, respectively (Figure 2). The ranks of variables affecting the comprehensive allelopathic index of WL656HQ were SVI, seedling height, radicle length, GP, germination percentage, dry fresh ratio, and germ length. The SVI was the main influence index with an importance of 9.5% (Figure 2a). The ranks for 3105C were radicle length, SVI, seedling height, germ length, germination percentage, dry fresh ratio, GP and radicle length was the main influence index, and the degree of importance was 10.8% (Figure 2b).

There was a significant difference in the allelopathic comprehensive effect indices (SE) between WL656HQ and 3105C under the CK, S1, S2 and S3 concentrations (Figure 3h). For WL656HQ, it was indicating that the extract had a stimulatory effect on seed germination at S1 and S2, ($SE \geq 0$); however, it was indicating that the extract had an inhibitory effect on seed germination at S3, S4, and S5, ($SE \leq 0$). The SE values for 3105C showed that autoallelopathy significantly inhibited seed germination after S2, S4 and S5 (Figure 3a,b).

The allelopathy index (radicle length, seedling height, SVI) and SE of WL656HQ showed stimulatory effects at low concentrations (S1 and S2), and inhibitory effects at high concentrations (S3, S4, and S5; Figure 3). In contrast, the allelopathy index (germination percentage, GP, radicle length, germ length, seedling height and SVI) and SE of 3105C at all concentrations showed inhibitory effects (Figure 3c–h).

3.2. Effect of Autotoxicity on Aboveground Biomass and Alfalfa Height

The aboveground biomass of WL656HQ was significantly higher in both T2 ($p = 0.01$) and T3 ($p = 0.03$) compared to 3105C (Figure 4a). WL656HQ had a significantly lower height value compared to 3105C under CK ($p = 0.012$); however, 3105C had a significantly lower value at T2 ($p = 0.016$; Figure 4b). The WL656HQ levels of aboveground biomass and height increased under T1, T2, and T3 compared to CK. On the contrary, the 3105C levels of aboveground biomass and height decreased under T1, T2, and T3 compared to CK (Figure 4).

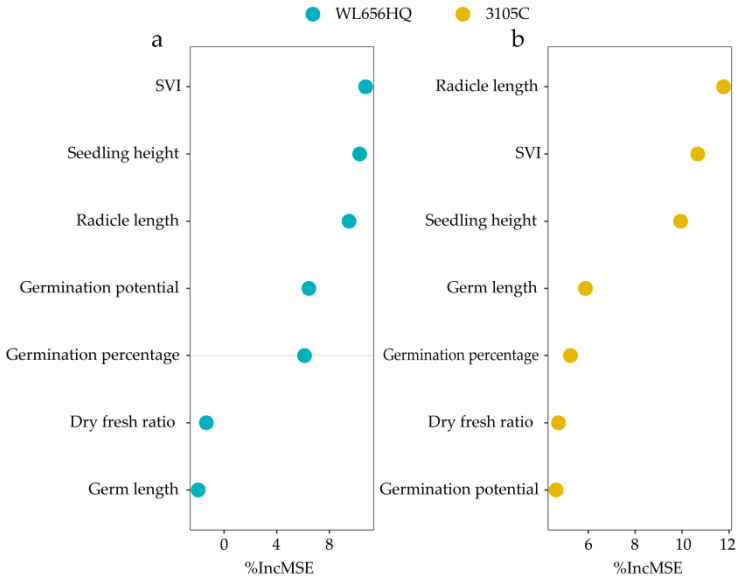

Figure 2. The relative importance of variables affecting the comprehensive allelopathic index of sensitive and tolerant alfalfa varieties. (**a**) Blue indicates the index ranking of the autotoxicity-tolerant variety WL656HQ affecting the comprehensive allelopathy index. (**b**) Yellow indicates the index ranking of the autotoxicity-tolerant variety 3105C affecting the comprehensive allelopathy index.

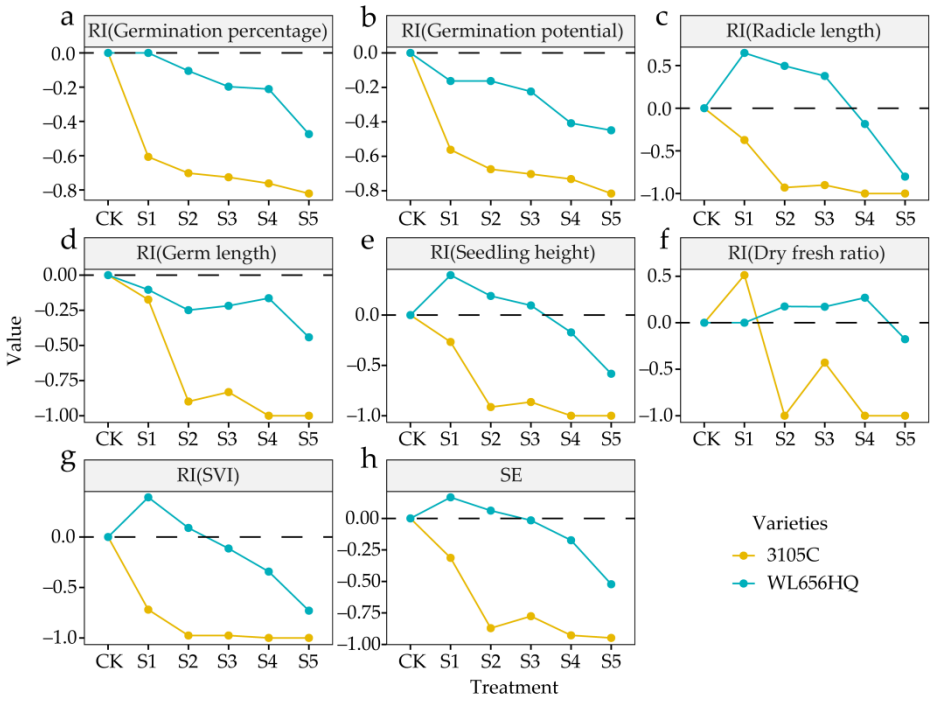

Figure 3. Effects of autotoxicity on comprehensive allelopathic coefficient of alfalfa. RI represents the allelopathic effect index (**a**) germination percentage; (**b**) germination potential; (**c**) radicle length; (**d**) germ length; (**e**) seedling height; (**f**) dry fresh ratio; (**g**) simple vigor index; (**h**) comprehensive allelopathic index of two alfalfa varieties under six concentrations of leaf extracts. When RI $\geq$ 0, it shows stimulatory effects, and when RI $\leq$ 0, it shows inhibitory effects. Blue represents WL656HQ. Yellow represents 3105C.

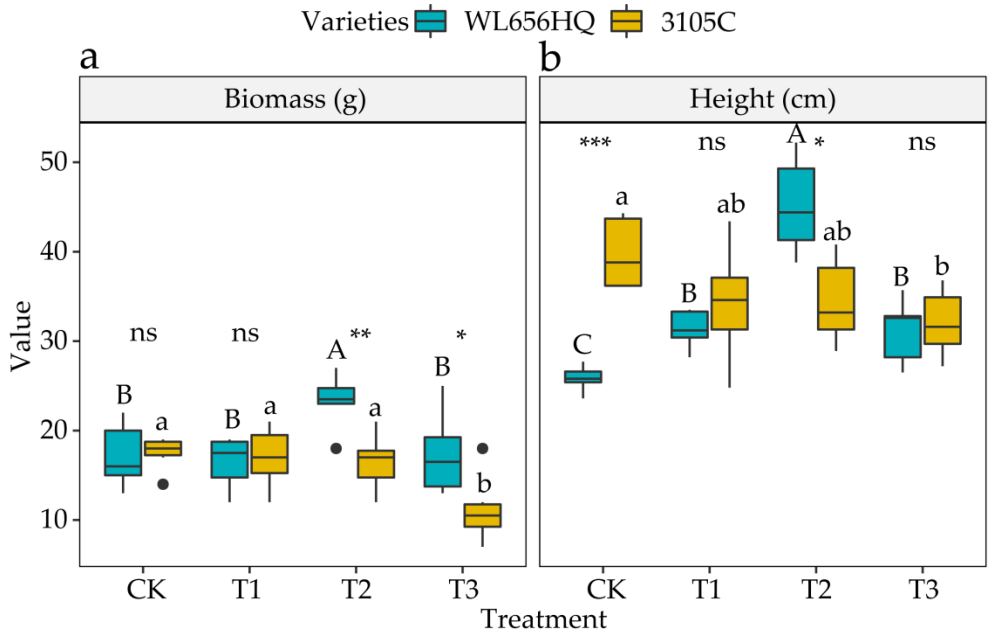

Figure 4. Effect of autotoxicity on aboveground biomass and height of alfalfa. The blue in the box plot represents the variety WL656HQ; the red represents the variety 3105C. The different lowercase and uppercase letters represent the significance of 3105C and WL656HQ at different concentrations. * Indicates the difference between the two varieties. The value $p \leq 0.05$ is marked as *. The value $p \leq 0.01$ is marked as **. The value $p \leq 0.001$ is marked as ***. The value $p > 0.05$ is marked as ns. (**a**) biomass. Filled circles represent outlier value. (**b**) Height.

3.3. Change of Osmotic Adjustment Substance of Alfalfa Leaves

The autotoxic extract increased the soluble sugar and soluble protein contents of WL656HQ and 3105C. For the proline content, WL656HQ exhibited a significantly higher value compared to 3105C at T2 (Figure 5a). The soluble sugar content of WL656HQ and 3105C showed an increasing trend with increasing extract concentrations (Figure 5b). The soluble protein content of 3105C tended to first increase, then decrease under T3 (Figure 5c).

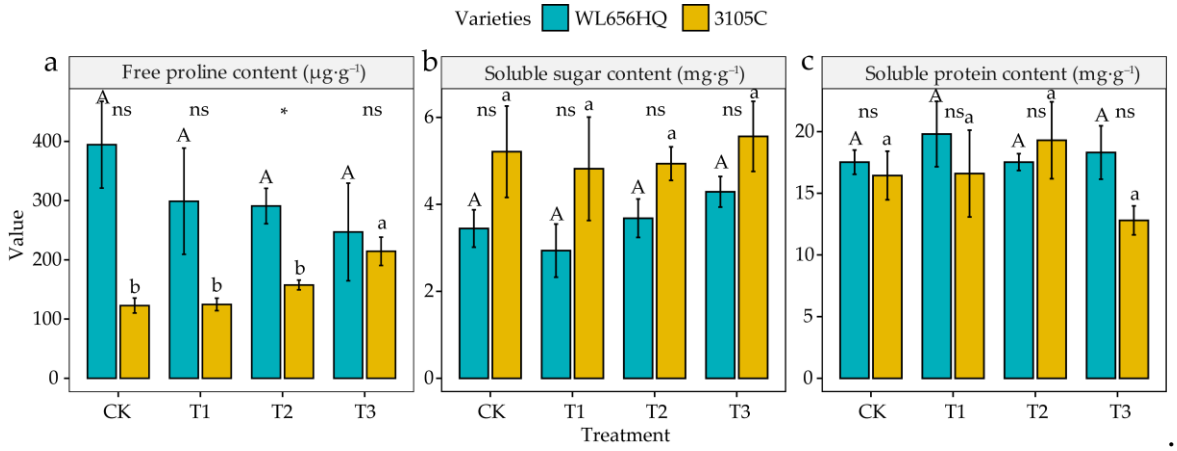

Figure 5. Effects of autotoxicity on proline, soluble sugar and soluble protein content of alfalfa. The blue in the box plot represents the variety WL656HQ; the yellow represents the variety 3105C. The different lowercase and uppercase letters represent the significance of 3105C and WL656HQ at different concentrations. * Indicates the difference between the two varieties. The value $p \leq 0.05$ is marked as *. The value $p \geq 0.05$ is marked as ns. (**a**) Proline content; (**b**) soluble sugar content; (**c**) soluble protein content.

3.4. Effect of Leaf Extract Autotoxicity on Lipid Peroxidation of Alfalfa

The H_2O_2 content of 3105C was significantly higher than that of WL656HQ at T2. With extract treatment, the H_2O_2 contents of WL656HQ deceased slightly compared to CK, but 3105C had a significantly higher value compared to WL656HQ at T2. In addition, the H_2O_2 content of 3105C at T2 was significantly higher than CK (Figure 6a). The $OH^{\bullet}$ content was higher for 3105C than WL656HQ at T1 and T3. WL656HQ showed the highest $OH^{\bullet}$ content for the T1 treatment, and the lowest $OH^{\bullet}$ value with the T3 treatment. The $OH^{\bullet}$ content of 3105C first decreased, then increased for T3 (Figure 6b). The $O_2^{\bullet-}$ content of 3105C was higher than that of WL656HQ at T2, but not significant. For WL656HQ, the levels of $O_2^{\bullet-}$ increased significantly under T1 and T2 compared to CK, (Figure 6c). The MDA content of 3105C was significantly higher than that of WL656HQ at T2. For 3105C, we observed a significant peak value of MDA content at T2 compared to CK (Figure 6d).

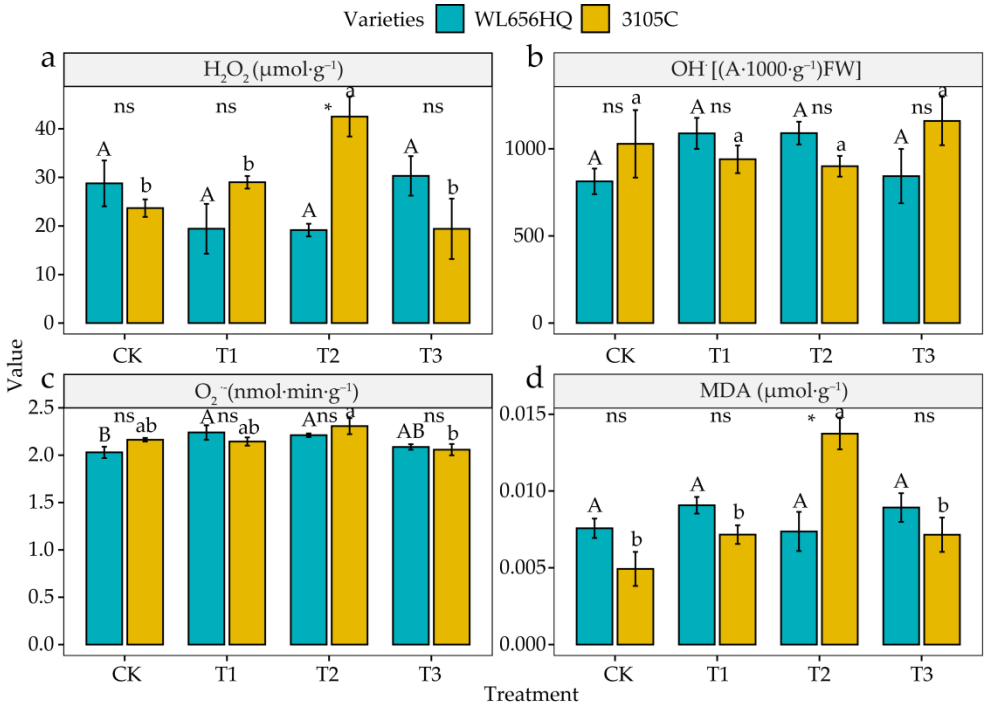

Figure 6. Effects of autotoxicity on hydrogen peroxide, hydrogen radical, superoxide anion radical and malondialdehyde content of alfalfa. The blue in the box plot represents the variety WL656HQ; the yellow represents the variety 3105C. The different lowercase and uppercase letters represent the significance of 3105C and WL656HQ at different concentrations. * Indicates the difference between the two varieties. The value $p \leq 0.05$ is marked as *. The value $p > 0.05$ is marked as ns. (**a**) H_2O_2 content; (**b**) $OH^{\bullet}$ value; (**c**) $O_2^{\bullet-}$ content; (**d**) MDA content.

3.5. Alterations in Activities of Antioxidant Enzymes and Contents of Antioxidant

There were significant differences in POD and CAT of WL656HQ and 3105C at T1 and T2, respectively. Increasing treatment concentrations caused increases in the levels of SOD, POD, and CAT in the leaves from WL656HQ and 3105C (Figure 7). Among them, the 3105C at T2 had significantly higher SOD activity compared to those at CK (Figure 7a). WL656HQ at T1 had a significantly higher POD activity compared to those at CK (Figure 7b). 3105C exhibited significantly higher CAT activity than that of WL656HQ at T2 (Figure 7c). 3105C exhibited significantly higher APX activity than that of WL656HQ at T1. The APX activities of WL656HQ increased under T1, T2, and T3 compared to CK with increasing extract concentrations. The APX level of 3105C increased at first, then decreased with increasing extract concentrations, but there was significant difference between CK, T1, T2, and T3 (Figure 7d). In addition, there was no significant difference in GR between WL656HQ and

3105C under the four treatments. The GR level of 3105C decreased at first then increased with increasing treatment concentrations (Figure 7e).

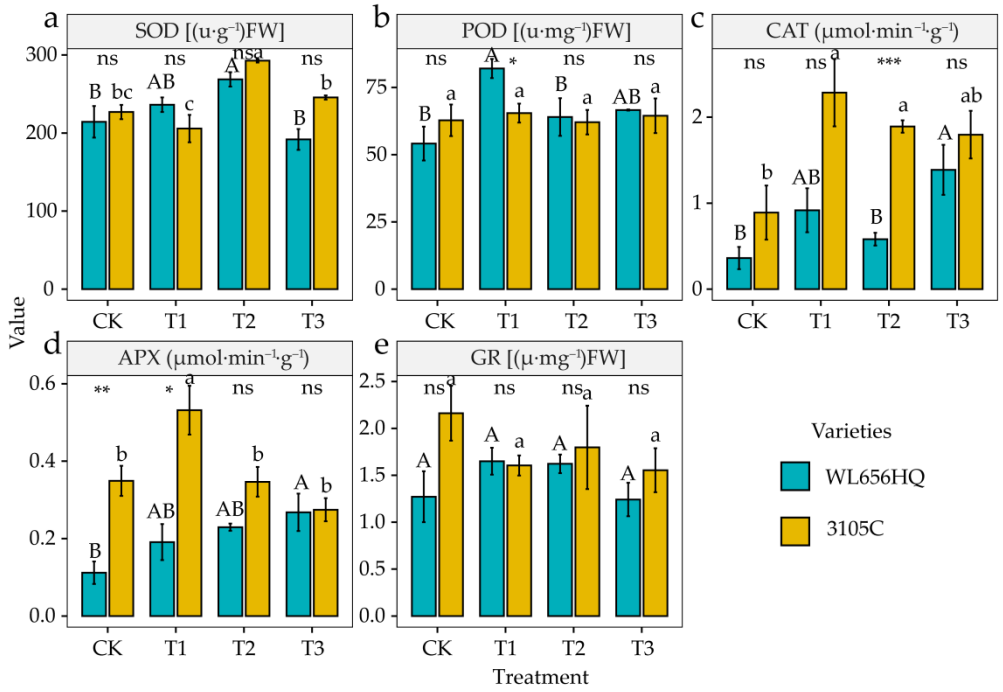

Figure 7. Effect of autotoxicity on antioxidant enzyme activities and antioxidant contents of alfalfa. The blue in the box plot represents the variety WL656HQ; the yellow represents the variety 3105C. The different lowercase and uppercase letters represent the significance of 3105C and WL656HQ at different concentrations. * Indicates the difference between the two varieties. The value $p \leq 0.05$ is marked as *. The value $p \leq 0.01$ is marked as **. The value $p \leq 0.001$ is marked as ***. The value $p > 0.05$ is marked as ns. (**a**) SOD; (**b**) POD; (**c**) CAT; (**d**) APX; (**e**) GR.

The ASA content of 3105C was significantly higher than WL656HQ at T1, T2 and T3. With increasing concentrations, the ASA level in the leaves of WL656HQ at T2 decreased significantly, which was significantly lower than that of CK and T1 (Figure 8a). The DHA content of WL656HQ at T2 was significantly higher than that of CK (Figure 8b). Among them, the ASA content and the DHA content of 3105C first increased then decreased with increasing extract concentrations. The AsA contents of WL656HQ and 3105C showed a declining trend, while the DHA content showed a gradually increasing trend. For WL656HQ and 3105C, the ratios of ASA/DHA significantly decreased under T1 and T3 compared to CK, but the AsA/DHA ratio of 3105C was higher than WL656HQ during T1 and T3 (Figure 8c).

3.6. Principal Component Analysis of the Positive and Negative Contribution Indicators of the Two Varieties

The PCA model was used to compare the treatment and control group, and to test which factors contribute to the observable differences caused by the different treatment concentrations (Figure 9). For WL656HQ, the PCA plot showed that PC1 could separate CK, T1, T2, and T3 well, with the explanation degree accounting for 76% of the total variation (Figure 9a). The indicators with the largest positive and negative contribution under PC1 were Pro and DHA, respectively (Figure 9b). For 3105C, the PCA plot showed that the four groups were well separated by PC1, which explained 66% of the total variation (Figure 9c). The indicator with the largest positive contribution under PC1 was ASA, and the indicator with the largest negative contribution was $OH^{\bullet}$ (Figure 9d).

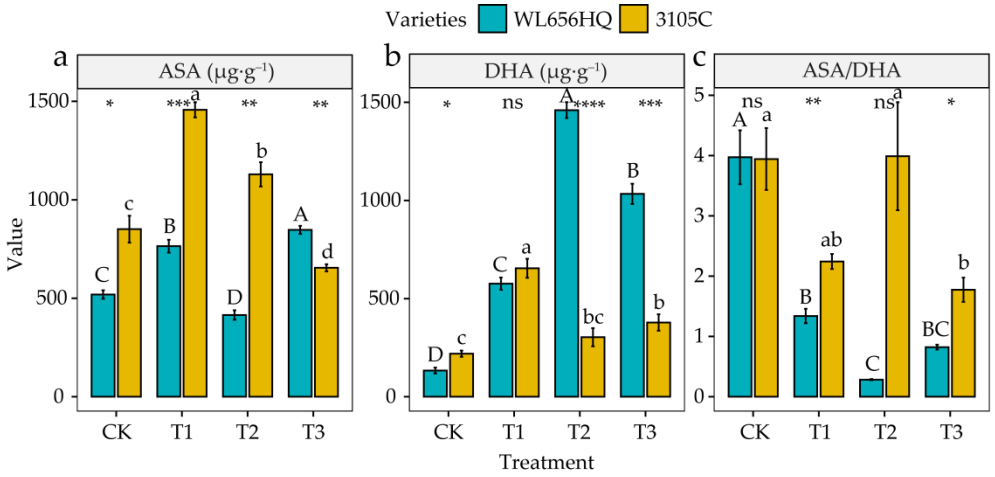

Figure 8. The effects of autotoxic extract on ASA, DHA Content, and AsA/DHA of alfalfa. The blue in the box plot represents the variety WL656HQ; the yellow represents the variety 3105C. The different lowercase and uppercase letters represent the significance of 3105C and WL656HQ at different concentrations. * Indicates the difference between the two varieties. The value $p \leq 0.05$ is marked as *. The value $p \leq 0.01$ is marked as **. The value $p \leq 0.001$ is marked as ***. The value $p \leq 0.0001$ is marked as ****. The value $p > 0.05$ is marked as ns. (**a**) ASA; (**b**) DHA; (**c**) AsA/DHA.

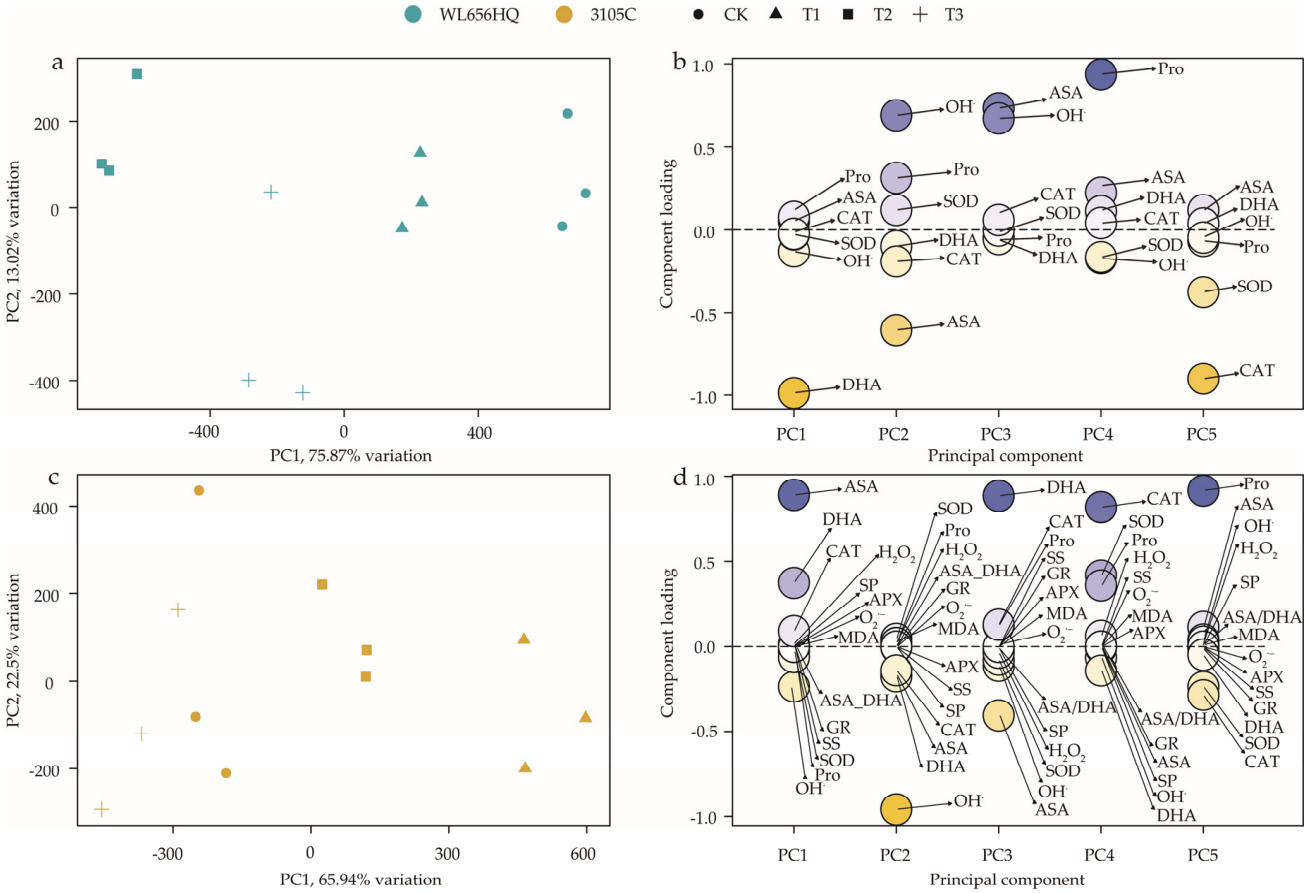

Figure 9. Scores plot (PC1 vs. PC2) of principal component analysis (PCA). (**a**). PCA of WL656HQ. (**b**). Each indicator with component loading in WL656HQ. (**c**). PCA of 3105C. (**d**). Each indicator with component loading in 3105C.

4. Discussion

4.1. Effects of Autotoxicity on Seed Germination

Alfalfa displays severe autotoxicity in artificial ecosystems, making it vitally important to select for varieties with lower autotoxicity and to clarify the response mechanism of alfalfa to autotoxins. The autotoxins are mainly some secondary metabolites. It mainly including organic acids, aldehyde aromatic acids, coumarins, quinones, alkaloids and terpenoids, among which phenols and terpenoids are the main autotoxic substances in plants [18]. A previous study demonstrated that autotoxicity inhibits seed germination and early root growth by causing deep damage to seedling growth [5], which results in a low seed germination rate, poor seedling growth, and a sharp decline in yield and quality [36]. In this study, we found that the autotoxic effects of 22 alfalfa varieties affected seed germination differently. Based on these stage 1 results, we selected the most sensitive (3105C) and the most tolerant (WL656HQ) varieties to autotoxicity. These results indicate that not all alfalfa varieties exhibit autotoxic effects. The significant differences in autotoxicity were due to the different autotoxic substances content released by different varieties. The sensitive varieties may be due to high levels of the primary autotoxic substances in the extract. We also found that two varieties (3105C and WL656HQ) had different sensitivity thresholds (S1 and S3) to autotoxicity. The minimum concentration of the same allelochemicals on different plants is different. Namely, the inhibition threshold of allelochemicals is different [37].

TOPSIS analysis demonstrated that the 22 alfalfa varieties had different ranks of autotoxicity. The autotoxicity-sensitive variety (3105C) and the tolerant variety (WL656HQ) were ranked highest and lowest, respectively. The previous study reported that all fig extracts displayed significant phytotoxic impact, and the degree of inhibition was appeared to be dependent on the cultivars and target species [38]. Moreover, according to the random forest model evaluation of the importance of SE to WL656HQ and 3105C, the germination indices were the SVI and radicle length, respectively. A previous study reported that autotoxicity primarily affects seed germination and early root growth, with the latter more sensitive than the former [9]. The root growth of the alfalfa seedlings of 3105C was mainly affected by the inhibition of radicle length, but the root growth of WL656HQ was only inhibited at high concentrations (S3, S4 and S5). Alfalfa plant extracts significantly affected root growth and morphological differentiation of susceptible plants [39].

In further analysis of the two varieties, the seed germination of WL656HQ was promoted at low (S1 and S2) concentrations, but was inhibited at high (S3, S4 and S5) concentrations. In contrast, the seed germination of 3105C was inhibited at all concentrations. These results show that different varieties cause different concentration-effects. A previous study found that root growth was stimulated at low leaf extract concentrations, but was inhibited at high leaf extract concentrations [9]. We showed that different alfalfa varieties display different degrees of autotoxicity, as shown by the inhibition of root growth in 3105C at low concentrations (S1 and S2). In addition, the occurrence of autotoxic effects is dependent on the concentration of the autotoxic compounds. Similarly, the sensitivity thresholds of autotoxicity in different alfalfa varieties were also different. Thus, the threshold of autotoxicity in WL656HQ was higher than in 3105C. Consequently, growth was promoted in WL656HQ below the threshold, but was slowed above a certain threshold (S3, S4 and S5).

4.2. Effect of Autotoxicity on Aboveground Biomass and Height

Alfalfa continuously releases certain secondary metabolites, which enters the soil via self-secretion, aboveground leaching, and residue decomposition. These metabolites directly or indirectly inhibit seed germination, seedling radicle germination, and seedling growth [11], resulting in low seed germination rates, poor seedling growth, and sharp declines in yield and quality in the presence of either autotoxic or allelopathic compounds [39]. A previous study showed that alfalfa plant extracts significantly affected root growth and the morphological differentiation of susceptible plants, resulting in reductions in biomass.

This study showed that the T1 and T2 leaf extract concentrations promoted the aboveground biomass and plant height of WL656HQ, but significantly inhibited the growth of 3105C at T2 and T3. Autotoxicity has also been observed in other studies focused on reducing the yield and quality of plants: ginsenosides served as the autotoxins responsible for the replanting failure of Sanchi (*Notoginseng Radix* et Rhizoma) [40], and ferulic acid inhibited the growth of rice (*Oryza sativa*) seedlings [41].

4.3. Change of Osmotic Adjustment Substance

Autotoxic substances can damage the membrane system and impact the normal growth and development of plants [10]. Osmotic substances (soluble sugar, soluble protein, and proline) can stabilize the membrane system. Proline is an important penetrant and effective hydroxyl radical scavenger, and can protect plants from oxidative stress [42]. In this study, WL656HQ maintained membrane stability primarily by increasing the soluble sugar and soluble protein contents. The response of proline was significantly higher in WL656HQ than in 3105C under T2. The proline and soluble protein contents in 3105C were also increased, but not significantly, which suggests that alterations in the proline and soluble protein contents could not maintain the stability of its membranes. These results revealed that WL656HQ exhibited higher osmotic adjustment capacity than 3105C in response to autotoxicity. The change in osmotic substance content inhibited the permeability of the cell membrane and the energy of the related conversion process.

4.4. Effect of Autotoxicity on Lipid Peroxidation

After autotoxic substances act on the plasma membrane, stress information is transmitted to the cells via target sites on the plasma membrane, which transmits the signal downstream and causes the production of secondary messengers, including Ca^{2+}, ROS, and inositol phosphate [43]. MDA content can be used to measure the stability of plasma membranes under stress [44]. If excessive ROS is produced, then superoxide anions produce hydrogen peroxide and singlet oxygen through disproportionation, which allows hydrogen peroxide to penetrate the cell membrane and form hydroxyl radicals [45]. To determine if this autotoxic effect occurs due to oxidative stress, we analyzed the levels involved in the detoxification and balance of ROS (H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet -}$), as well as membrane damage via lipid peroxidation. Our results revealed that autotoxicity induced mitochondrial oxidative stress, as characterized by increased ROS. We found a significant difference in the ROS response between 3105C and WL656HQ. The rapid response of ROS (H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet -}$) in 3105C to autotoxicity differed from WL656HQ, which accumulated ROS at T2. Allelochemicals can induce the production of ROS and Ca^{2+} signals, which alters gene expression, and eventually leads to programmed cell death [14]. In addition, autotoxicity caused the over-accumulation of MDA in the leaves of alfalfa 3105C resulting in serious oxidative damage at T2. The MDA content of 3105C was higher than that of WL656HQ. MDA had little effect on WL656HQ, which was able to maintain the relative integrity of its membrane. Previous studies have shown that different concentrations of benzoic acid and cinnamic acid promoted MDA synthesis in seedling roots, with the promotion effect enhanced with the extension of treatment time [46]. The promoting ability of high concentrations of benzoic acid was significantly higher than that of cinnamic acid, but the opposite was found at low concentrations [47]. After treating soybean (*Glycine max* (L.) Merr.) seedlings with benzoic acid and cinnamic acid, it was found that these two substances induced lipid peroxidation, which was mainly due to the formation of free radicals in the plasma membranes, the inhibition of catalase and peroxidase activities, and the consumption of sulfhydryl groups [48]. These studies imply that autotoxicity caused the accumulation of ROS, which leads to membrane lipid peroxidation. We found that 3105C and WL656HQ had two different strategies: 3105C responds to oxidative stress and produces too much ROS and MDA at T2 and T3 and cannot maintain oxidative balance, causing oxidative damage; while WL656HQ used its oxidative scavenging system

(POD, APX and GR) to maintain its redox dynamic balance by removing excess ROS at all concentrations.

4.5. Alterations in Antioxidant Enzyme Activities and Antioxidant Contents

In order to maintain intracellular balance, there are a series of antioxidant enzymes (SOD, POD, CAT, APX and GR) and antioxidants (ASA and GSH) found in plants. SOD transforms $O_2^{\bullet -}$ to H_2O_2, while CAT, POD, and ASC-GSH cycle-related enzymes reduce H_2O_2 [49]. In this study, the membrane stability in WL656HQ was maintained despite increasing extract concentrations primarily through the increase of antioxidant enzyme (POD, APX, and GR) activities. 3105C responded to abiotic stress by increasing alfalfa leaf protective enzyme (SOD, POD and CAT) activities and antioxidant enzyme (APX, GR, ASA and DHA) activities under T1. At T2 and T3, 3105C increased the amount of reactive oxygen free radicals, aggravating the degree of membrane lipid peroxidation and causing membrane damage. Ginsenoside Rg1, an autotoxic substance of Panax ginseng (*Panax ginseng* C. A. Mey), can lead to the accumulation of ROS in root cells and inhibits APX and the ASC-GSH cycle. The activities of SOD, POD and CAT were all promoted at low extract concentrations for short time periods [13]. Phthalic acid induces oxidative stress in *M. prunifolia* roots through the generation of ROS and decreases plant growth, despite the concomitant increase in antioxidant enzymes [50].

Autotoxins act on the target site of cell membranes and affect photosynthesis, mitochondrial function (respiration, oxidative stress, and signal transduction), and hormone levels. They induce ROS and MDA production in plant cells and disrupt the integrity of the cell membrane system [13]. In Stage 2, ROS over-accumulation was induced in 3105C leaves, which inhibited the antioxidant enzyme and ASC-GSH cycles and subsequently caused membrane lipid peroxidation to ultimately disrupt the integrity of the cell membrane. The SOD and CAT activities in 3105C leaves were significantly reduced, so that they could not effectively remove the oxygen free radicals generated by autotoxicity. Thus, the generation rate of $O_2^{\bullet -}$ was greater than the scavenging rate by the antioxidant enzymes. The accumulation of $O_2^{\bullet -}$ causes increased damage in 3105C, which inactivates the antioxidant enzymes and results in a series of chain effects. $O_2^{\bullet -}$ can also destroy intercellular sulfhydryl groups by oxidatively decomposing the unsaturated fatty acid chains in the phospholipid bilayer of the plasma membrane, causing the destruction of the plasma membrane structure, oxidative damage to the cell membrane system (the product is MDA), and the further destruction of the internal structure of the cell. In comparison, autotoxicity had little effect on WL656HQ, because it maintains the relative integrity of its membrane by activating protective mechanisms that increases the activity of antioxidant enzymes, which stabilizes $O_2^{\bullet -}$. This results in a dynamic balance between the generation and elimination of oxygen free radicals in the cell. Since MDA did not accumulate in large amounts, the cell membrane stability was preserved.

4.6. PCA Analysis of the Positive and Negative Contribution Indicators

PCA analysis showed that WL656HQ and 3105C responded to oxidative stress caused by autotoxicity differently, which may be due to differences in their sensitivity to autotoxicity. The most sensitive autotoxin concentrations for 3105C and WL656HQ were T2 (0.125 $g \cdot mL^{-1}$) and T1 (0.025 $g \cdot mL^{-1}$), respectively. This difference is due to the different maximum positive and negative indicators 3105C and WL656HQ displayed in response to autotoxicity. For WL656HQ, the indicators with the largest positive and negative contribution under PC1 were Pro and DHA, respectively. For 3105C, the indicator with the largest positive contribution under PC1 was ASA, and the indicator with the largest negative contribution was $OH^{\bullet}$. These results indicate that 3105C cannot maintain its oxidative stress system and that the oxidative scavenging system was affected by autotoxicity. WL656HQ maintained the redox dynamic balance by removing excess ROS with the oxidative scavenging system (POD, APX and GR).

5. Conclusions

In this study, we found that the autotoxicity of 22 alfalfa varieties had significantly different effects on seed germination. We used TOPSIS analysis to determine that WL656HQ and 3105C were the most tolerant and sensitive varieties to autotoxicity, respectively. We conducted a comparative analysis of WL656HQ and 3105C based on their seed germination and seedling's physiological changes. However, more long-term research is needed to evaluate the autotoxicity of different varieties of alfalfa in field conditions

In Stage 1, we determined that autotoxicity has three main effects. First, we found that the 22 alfalfa varieties generate variations in the strength of their autotoxic effects on seed germination. Second, we found that 3105C and WL656HQ had different trigger concentrations and reaction thresholds to autotoxicity. Third, we found that the SVI and radicle length were mainly affected by SE in both WL656HQ and 3105C. In Stage 2, our results showed that oxidative stress caused an imbalance between ROS production and the capacity of the antioxidant defense system. Autotoxicity caused different oxidative stress strategies for the two alfalfa varieties. WL656HQ responded to autotoxicity by increasing antioxidant enzyme (POD, APX and GR) activities and the proline content–an osmotic adjustment substance–to maintain membrane stability and oxidation scavenging. In 3105C, ROS production was greater than the capacity of the antioxidant defense system, resulting in oxidative damage. In conclusion, the positive and negative indicators to autotoxicity in the two alfalfa varieties were ASA and $OH^{\bullet}$, and Pro and DHA, respectively.

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Abbreviations

APX, ascorbate peroxidase; ASA, ascorbate; ASA/DHA, ratio of reduced to oxidized ascorbate; ASC-GSH, Mitochondria ascorbate-glutathione; CAT, catalase; DHA, dehydroascrobate (oxidized ascorbate); DTT, dithiothreitol solution; Gp, germination percentage; GP, germination potential; GPX, glutathione peroxide; GR, glutathione reductase; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitroblue tetrazolium; $O_2^{\bullet -}$, superoxide anion free radical; $OH^{\bullet}$, hydroxyl free radical; PBS, phosphate buffer saline; POD, peroxidase; PVP, polyvinylpyrrolidon; ROS, reactive oxygen species; SE, allelopathic comprehensive effect indexes; SVI, simple vigor index; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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