LRF-837
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RESEARCH ARTICLE   Legume Research- An International Journal

Alfalfa Autotoxicity: Role of Cultivar Renovation in Mitigating Germination and Seedling Growth under Continuous Cropping Systems

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10.18805/LRF-837

ABSTRACT

Background: Alfalfa often fails in continuous cropping due to its autotoxicity effect. Rotation is considered an effective way to mitigate autotoxicity; however, crop rotation is typically practiced between alfalfa and non-leguminous crops rather than among different alfalfa varieties. Therefore, understanding the germination and seedling growth of alfalfa after continuous cropping is of great significance to reveal the possibility of continuous cropping of alfalfa.

Methods: The leaves of alfalfa (*Medicago sativa* L. cv ZhongMu No.1) were collected to determine the seed germination rate, germination potential, germination index, vigor index, seedling growth and physiological characteristics, including malondialdehyde (MDA), soluble sugar (SS), soluble protein (SP), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), in four alfalfa varieties (XunLu, GongNong No.1, ZhongMu No.1 and JuNeng 995).

Result: The germination rate, germination potential, germination index and vigor index of the four alfalfa varieties decreased with increasing concentrations of leaf extract from ZhongMu No.1. JuNeng 995 exhibited better germination conditions than the other three varieties. Similarly, JuNeng 995 demonstrated superior plant length compared to the other varieties. The alfalfa cultivar, extract concentration and their interaction significantly influenced MDA, SS, SP, CAT and POD levels. However, these effects varied inconsistently with respect to extract concentration or variety. Furthermore, while the extract inhibited germination and vigor, it promoted seedling elongation in the remaining plants. GI was found to be negatively correlated with seedling length. Conclusively, the continuous cropping barrier effect of alfalfa became more severe with increasing extract concentrations. Cultivar renovation was identified as a potentially effective strategy to reduce the autotoxicity of alfalfa in this study.

Key words: Alfalfa, Autotoxicity, Continuous cropping, Cultivar, Germination, Seedling physiology.

INTRODUCTION

Alfalfa, a highly valued perennial leguminous forage crop, is cultivated across 30 million hectares worldwide, particularly in infertile areas (Teixeira *et al.,* 2023). Simulated average alfalfa yields range from 4.5 to 28 tons of dry matter per hectare per year (Teixeira *et al.,* 2023; Glowacki *et al.,* 2023; Mohammed *et al.,* 2023). Alfalfa cultivation not only enhances soil physiochemical properties (Sainju and Lenssen, 2011) but also provides high-quality forage for local animal husbandry (Du *et al.,* 2020; Fan *et al.,* 2024). However, consecutive production of alfalfa often results in field thinning and yield reductions due to factors such as soil water depletion, plant diseases and autotoxicity (Seguin *et al.,* 2002; Li and Huang, 2008; Fan *et al.,* 2023). Among these, autotoxicity has been identified as the primary cause of reseeding failure in alfalfa (Seguin *et al.,* 2002; Zheng *et al.,* 2019).

Autotoxicity in plants refers to the harmful allelopathic effects on their own growth and development (Seguin *et al.,* 2002; Zhang *et al.,* 2020). It occurs when a plant species releases specific chemical compounds that directly or indirectly inhibit the germination or growth of the same species (Wang *et al.,* 2022; Wu *et al.,* 2023). In alfalfa, autotoxicity is caused by water-soluble compounds released from leaves, roots and stems (Yang *et al.,* 2009).

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How to cite this article: Li, H., Wang, L., Li, J., Sun, X., Lin, D. and Wang, Z. (2025). Alfalfa Autotoxicity: Role of Cultivar Renovation in Mitigating Germination and Seedling Growth under Continuous Cropping Systems. Legume Research. 1-7. doi: 10.18805/LRF-837.

Submitted: 03-10-2024 **Accepted:** 29-01-2025 **Online**: 17-03-2025

These compounds include saponins (Wyman-Simpson *et al.,* 1991), canavanine (Miersch and Jiihlke, 1992) and phenolic acids (Abdul and Habib, 1989; Hall and Henderlong, 1989). Their concentrations are influenced by factors such as stand age (Yang *et al.,* 2009), cultivar (Wyman-Simpson *et al.,* 1991), reseeding delay (Seguin *et al.,* 2002) and plant organ (Rong *et al.,* 2016), all of which impact the reseeding success of alfalfa.

To mitigate alfalfa autotoxicity, practices such as crop rotation with non-leguminous crops (Jennings and Nelson, 1998; Zhang *et al.,* 2023) and seed priming (Tanha *et al.,* 2017) have been recommended in previous studies. Moreover, Wyman-Simpson *et al.* (1991) observed that saponin concentrations varied among six alfalfa cultivars and higher saponin concentrations were associated with increased autotoxicity (Chuang and Miller, 1995). This raises the question of whether selecting alfalfa cultivars with low autotoxicity could serve as a novel and effective strategy to reduce the autotoxicity of alfalfa-a question warranting further investigation.

In this study, we evaluated the seed germination, seedling growth and physiological characteristics of alfalfa. We hypothesized that cultivar renovation could enable successful continuous cropping of alfalfa. The specific objectives were to determine: 1. How seed germination and seedling growth of four alfalfa varieties respond to different concentrations of alfalfa leaf extract; 2. The relationships between seed germination, seedling lengths and physiological characteristics of alfalfa; 3. Whether cultivar renovation could be a novel and effective approach to overcoming alfalfa autotoxicity in continuous cropping systems.

MATERIALS AND METHODS

Seed material

The four alfalfa (*Medicago sativa*) cultivars were XunLu(XL), GongNong No.1(GN,), ZhongMu No.1(ZM) and JuNeng 995(JN).

Experiment design

The experiment was carried in College of Agriculture and Forestry Science, Linyi University, China. The senesced leaves of alfalfa (ZhongMu No.1) were collected randomly in the first harvest period in 24 May, 2023. And the leaves were cut into pieces for preparation of the extract. According to the ratio of 1 g of leaves soaked in 10 ml sterile water, the leaf extract was filtered by a quantitative filter paper and the extract concentration was 0.1%. Then the 0.1 g/ml extract concentration was diluted to 0.05% and 0.01% by sterile water. The sterile water was used as the control treatment 0.

The full seeds of the four alfalfa cultivars were picked out and sterilized with 0.1% $KMnO_4$ for 20 minutes, then washed with sterile water. The seeds were soaked in sterile water for 12 h before processing. Two layers of wet filter paper were laid in the petri dishes (10 cm diameter) and 100 seeds were placed in each dish. 10 ml extract solution of different concentrations (concentration setting: 0%, 0.01%, 0.05%, 0.1%) was added slowly. Petri dishes were placed in an artificial climate chamber (PGX-38°C, Shanghai, China), which the temperature was set at 25°C and the light/dark time was 16 h/8 h. The germination parameters were counted from day 1 to day 10 and 0.5 g of whole plants (fresh weight) were weighed at day 10 to measure the physiological indexes of seedlings.

Germination test

Germination was estimated by radicle protrusion, which the appearance of a radicle ≥2 mm in length as the criterion. The germination numbers of seeds were counted from day 1 to day 10. The germination rate (%) was estimated at the day 7, *i.e.* the accumulated number of germinated seeds from day 1 to day 7 divided by 100. The germination potential (%) was estimated at the day 4, *i.e.* the accumulated number of germinated seeds from day 1 to day 4 divided by 100.

$$\text{Germination index (GI)} = \sum \frac{GT}{DT}$$

Where,

DT = Days of germination.

GT = Germinated number of seeds corresponding to DT.

$$\text{Vigor index (VI)} = GI \times W$$

Where,

W = Fresh weight of the seeds at day 7.

Measurements of seedling length and physiological index

The seedling lengths were measured by vemier calipers with accuracy of 2 mm. The malondiadehyde (MDA) concentration was measured by a modified thiobarbituric acid (Puckette *et al.,* 2007). Soluble sugar (SS) was analyzed by a modified anthrone method (Sαnchez *et al.,* 1998). Soluble protein (SP) was analyzed by a modified coomassie brilliant blue method (Georgiou *et al.,* 2008). The activity of superoxide dismutase (SOD) was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Stewart andBewley, 1980). The activity of catalase (CAT) was assayed by the modified method of Iodin titration (Kenten and Mann, 1952). The activity of peroxidase (POD) was measured as the recorded absorbance increased at 470 nm by guaiacol method (Castillo *et al.,* 1984).

Statistical analysis

The effects of cultivar, extract concentration and their interaction on the germination test and seedling physiological index were analyzed using Two-Way ANOVAs. The differences in germination test and seedling physiological index on the effects of cultivar or extract concentration were examined using One-Way ANOVAs with SPSS 17.0. The liner correlations between germination index and seedling physiological index were analyzed with the model *y=ax+b* using SPSS 26.0

RESULTS AND DISCUSSCION

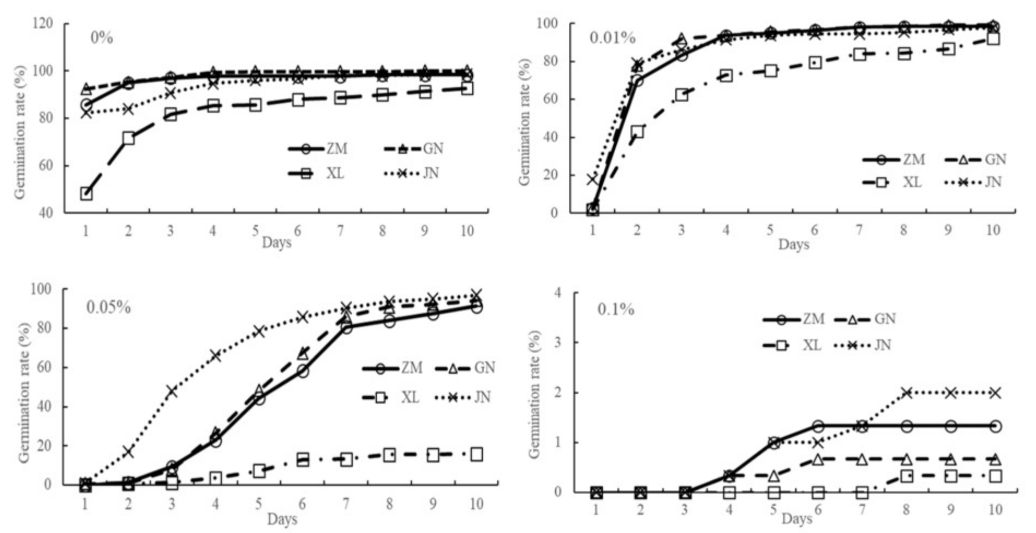
Germination rate and germination potential

Germination rate of four alfalfa cultivars increased firstly, then stabilized as germination days under the four extract concentrations (Fig 1). However, the days to stabilized germination rate were delayed with the increased extract concentrations. These indicated the higher concentration of the extract had more seriously autotoxicity on subsequent alfalfa. Even though, the 0.1% leaf extract made four alfalfa little germination rate. This might because of the phenolic acid metabolites from alfalfa which inhibit the germination of later alfalfa in the continuous cropping (Wang *et al.,* 2022).

The alfalfa cultivars, extract concentration and their interaction showed significant effects on both germination rate and germination potential (Fig 2). Seed germination of four alfalfa cultivars were significantly inhibited at 0.05% and 0.1% ($P<0.05$). Ma *et al.* (2024) found the same results which used root extracts as the treatments. Germination rate and germination potential of XunLu were lower than those of the other three alfalfa cultivars under the four extract concentrations ($P<0.05$). These indicated alfalfa germination was more inhibited in Xunlu and the 0.1% leaf extract made four alfalfa little germination rate.

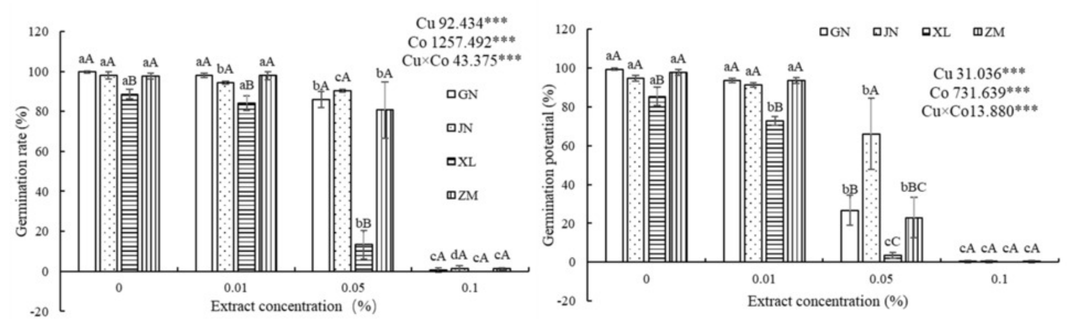
Germination index and vigor index

The alfalfa cultivars, extract concentration and their interaction showed significant effects on germination index and vigor index (Fig 3). As the increased extract concentration, the germination index and vigor index showed the significantly decreased trends ($P<0.05$). Germination index and germination index of JuNeng showed better than other cultivars ($P<0.05$). Even it still showed a high germination index and vigor index at the 0.05% extract concentration. These indicated different cultivars of alfalfa might show autotoxicity-tolerant or autotoxicity-sensitive varieties (Zhang *et al.,* 2021), JuNeng 995 would be select to renovation from the germination index and vigor index as autotoxicity-tolerant variety.



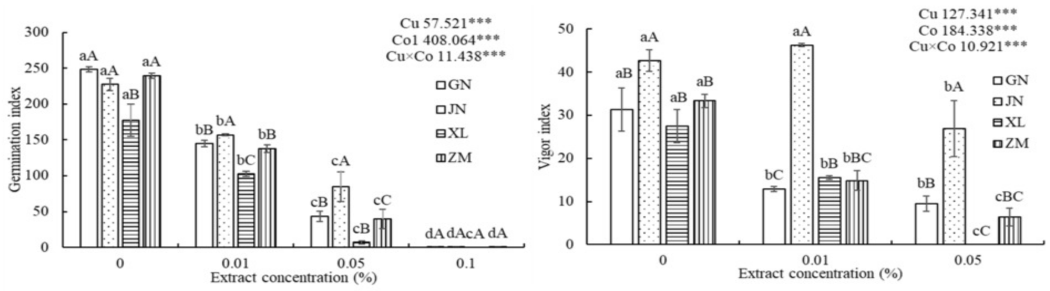
GN, JN, XL and ZM represent the four alfalfa cultivars (GongNong, JuNeng, XunLu and ZhongMu).

Fig 1: The seed germination rates of alfalfa with the germination days under the different leaf extract concentration.



GN, JN, XL and ZM represent the four alfalfa cultivars (GongNong, JuNeng, XunLu and ZhongMu). Cu, Co and Cu×Co represent the effects of cultivar, concentration and their interaction. The different lowercase letters mean the significant differences at the different extract concentrations. The capital letters mean the significant differences at the four cultivars.

Fig 2: Effects of different leaf extract concentrations on germination rates and germination potentials of four alfalfa cultivars.



GN, JN, XL and ZM represent the four alfalfa cultivars (GongNong, JuNeng, XunLu and ZhongMu). Cu, Co and Cu×Co represent the effects of cultivar, concentration and their interaction. The different lowercase letters mean the significant differences at the different extract concentrations. The capital letters mean the significant differences at the four cultivars. No statistics were made on vigor index at 0.1% extract concentration.

Fig 3: Effects of different leaf extract concentrations on germination index and vigor index of four alfalfa cultivars.

Table 1: Effects of different leaf extract concentrations on the lengths of four alfalfa cultivars.

Organs	Cultivar	Extract concentration %			P-Value
		0	0.01	0.05	
Root	GongNong	2.98 ± 0.14^{cA}	5.84 ± 0.40^{aB}	4.45 ± 0.79^{bA}	Cultivar: 0.349^{ns}
	JuNeng	3.58 ± 1.07^{aA}	6.57 ± 0.22^{aA}	4.4 ± 2.34^{aA}	Concentration: 22.831**
	XunLu	3.84 ± 1.16^{bA}	5.64 ± 0.47^{aB}	-	Cultivar× Concentration: 0.713^{ns}
	ZhongMu	2.68 ± 1.20^{bA}	5.91 ± 0.21^{aB}	4.91 ± 0.45^{aA}	
Bud	GongNong	1.54 ± 0.05^{bA}	2.82 ± 0.36^{aA}	2.78 ± 0.53^{aAB}	Cultivar: 6.559**
	JuNeng	1.58 ± 0.22^{cA}	$2.20 \pm 0.03b^{AB}$	3.12 ± 0.22^{aA}	Concentration: 20.672**
	XunLu	1.40 ± 0.46^{aA}	1.28 ± 0.79^{aB}	-	Cultivar× Concentration: 2.420^{ns}
	ZhongMu	1.28 ± 0.22^{bA}	1.79 ± 0.46^{abB}	2.30 ± 0.32^{aB}	
Whole	GongNong	4.53 ± 0.09^{cA}	8.66 ± 0.68^{aAB}	7.23 ± 0.47^{bA}	Cultivar: 1.23^{ns}
	JuNeng	5.15 ± 1.09^{bA}	8.77 ± 0.20^{aA}	7.52 ± 2.54^{abA}	Concentration: 32.156**
	XunLu	5.24 ± 0.74^{aA}	6.59 ± 0.67^{aC}	-	Cultivar× Concentration:1.155^{ns}
	ZhongMu	3.95 ± 1.36^{bA}	7.70 ± 0.41^{aBC}	7.21 ± 0.71^{aA}	

The different lowercase letters mean the significant differences at the different extract concentrations (P<0.05). The capital letters mean the significant differences at the four cultivars (P<0.05). “ns” means no significance(P>0.05); two asterisks “**” mean the significance at P<0.01.

Table 2: Effects of different leaf extract concentrations on the MDA, SS and SP concentration of four alfalfa cultivars.

Parameters	Cultivar	Extract concentration %			P-Value
		0	0.01	0.05	
MDA (μmol/g)	GongNong	17.91 ± 1.99^{bA}	20.95 ± 3.43^{abA}	26.66 ± 3.11^{aA}	Cultivar: 15.672**
	JuNeng	16.54 ± 1.44^{aA}	18.64 ± 1.58^{aA}	18.54 ± 0.15^{aB}	Concentration: 3.437*
	XunLu	13.80 ± 2.81^{aA}	12.85 ± 0.83^{aB}	-	Cultivar×Concentration:4.514**
	ZhongMu	17.08 ± 1.87^{aA}	18.57 ± 0.35^{aA}	15.32 ± 3.42^{aB}	
SS (μg/g)	GongNong	4.54 ± 2.68^{aA}	2.93 ± 1.54^{aB}	4.05 ± 1.33^{aA}	Cultivar: 5.257**
	JuNeng	4.51 ± 0.49^{aA}	3.44 ± 1.25^{abB}	2.58 ± 0.85^{bA}	Concentration: 3.456*
	XunLu	3.87 ± 0.75^{aA}	4.85 ± 0.71^{aAB}	-	Cultivar×Concentration: 1.655*
	ZhongMu	8.46 ± 3.65^{aA}	6.74 ± 1.65^{aA}	3.83 ± 0.97^{aA}	
SP(μg/g)	GongNong	6.84 ± 0.25^{aBC}	4.51 ± 0.34^{bB}	4.92 ± 1.55^{bB}	Cultivar: 7.354**
	JuNeng	5.37 ± 0.79^{bC}	6.85 ± 1.88^{bA}	10.72 ± 2.60^{aA}	Concentration: 19.537**
	XunLu	7.66 ± 0.47^{aB}	4.39 ± 0.86^{bB}	-	Cultivar×Concentration: 9.413**
	ZhongMu	10.38 ± 1.43^{aA}	4.26 ± 0.34^{bB}	9.13 ± 1.72^{aA}	

The different lowercase letters mean the significant differences at the different extract concentrations (P<0.05). The capital letters mean the significant differences at the four cultivars (P<0.05). “ns” means no significance(P>0.05); one asterisk “*” means the significance at P<0.05; two asterisks “**” mean the significance at P<0.01.

Lengths of alfalfa seedlings

The extract concentrations showed significant effects on the lengths of alfalfa seedlings (Table 1). The lengths of root and whole plants were highest in the 0.01% concentration and the lengths of bud increased with the extract concentrations. This might becaused by result of the promoting effect of allelopathy in the extract (Li *et al.,* 2021). The cultivars showed significant effects on the lengths of alfalfa bud. GongNong showed the highest lengths of bud, JuNeng showed the higher lengths of bud and JuNeng showed the highest lengths of root and whole plants in the four cultivars under the 0.01% concentration. In summary, JuNeng was selected as the optimal cultivar to cultivate for continuous cropping alfalfa from the indicators of seedling lengths in this study. Wang *et al.* (2022) found that the metabolites from alfalfa could significantly decrease the root length of alfalfa seedlings.

Malondiadehyde (MDA), soluble sugar (SS) and soluble protein (SP)

The alfalfa cultivar, extract concentration and their interaction showed significant effects on the MDA, SS and SP concentration (Table 2). MDA increased with the extract concentration in Gongnong. and it was no significant change in the four cultivars under the 0% extract concentration, the MDA of Gongnong showed the highest in the four cultivars under the 0.05% and 0.1% extract concentration. The SS decreased with the extract concentration. The SS did not change significantly with cultivars in the 0% and 0.05% extract concentrations, but it showed the highest at ZhongMu in the 0.01% extract concentration. The SP decreased at GongNong and XunLu, increased at JuNeng and decreased firstly, then increased at ZhongMu with the extract concentration. The SP of JuNeng was lowest in the 0% extract concentration and highest in the 0.01% and 0.05% extract concentration in the four cultivars. These results indicated different alfalfa varieties would regulate different osmoregulatory substances to counteract the autotoxicity from the leaf extract (Zhang *et al.,* 2021), *i.e.* JuNeng is mainly through the regulation of SP concentration.

Antioxidative systems (CAT, POD and SOD)

The alfalfa cultivar, extract concentration and their interaction showed significant effects on CAT (Table 3). The CAT was highest in 0.01% extract concentration (P<0.05). And the CAT of GongNong was highest in the four

Table 3: Effects of different leaf extract concentrations on the CAT, POD and SOD activities of four alfalfa cultivars.

Parameters (U/g)	Cultivar	Extract concentration % 0	0.01	0.05	P-Value
CAT	GongNong	$120±19b^{A}$	$182±43^{aA}$	$81±12^{bAB}$	Cultivar: 27.275**
	JuNeng	$67±24^{aBC}$	$74±10^{aB}$	$95±2^{aA}$	Concentration: 5.773**
	XunLu	$36±10^{bC}$	$71±2^{aB}$	-	Cultivar×Concentration: 9.547**
	ZhongMu	$78±14^{aB}$	$54±10^{aB}$	$66±13^{aB}$	
POD	GongNong	$1907±579^{aB}$	$1254±83^{aA}$	$1456±374^{aA}$	Cultivar: 4.094*
	JuNeng	$2053±366^{aB}$	$1567±556^{aA}$	$1904±469^{aA}$	Concentration: 7.298*
	XunLu	$2004±376^{aB}$	$1682±564^{aA}$	-	Cultivar×Concentration: 1.471^{ns}
	ZhongMu	$3063±404^{aA}$	$2034±333^{bA}$	$1672±539^{bA}$	
SOD	GongNong	$1116±29^{aA}$	$1080±32^{aAB}$	$1096±74^{aA}$	Cultivar: 0.252^{ns}
	JuNeng	$1115±77^{aA}$	$1054±67^{aB}$	$1130±42^{aA}$	Concentration: 0.594^{ns}
	XunLu	$1073±69^{aA}$	$1155±5.73^{aA}$	-	Cultivar×Concentration: 1.615^{ns}
	ZhongMu	$1067±69^{aA}$	$1120±50^{aAB}$	$1116±18^{aA}$	

The different lowercase letters mean the significant differences at the different extract concentrations (P<0.05). The capital letters mean the significant differences at the four cultivars (P<0.05). "ns" means no significance(P>0.05); one asterisk "*" means the significance at P<0.05; two asterisks "**" mean the significance at P<0.01.

Table 4: Relationship between lengths of alfalfa, physiological characters and seed germination.

Parameters		GR	GP	GI	VI
Length	Bud	-0.076	-0.406*	-0.565**	-0.297
	Root	-0.110	-0.057	-0.402*	-0.181
	Whole seedling	-0.121	-0.210	-0.555**	-0.267
Whole seedling	MDA	0.107	-0.239	-0.243	-0.205
	SS	0.266	0.251	0.332	0.045
	SP	-0.117	-0.170	0.022	0.182
	CAT	0.285	0.163	0.071	-0.167
	POD	0.132	0.242	0.430*	0.263
	SOD	-0.241	-0.115	-0.182	-0.178

One asterisk "*" means the significant correlation at P<0.05; two asterisks "**" mean the significant correlation at P<0.01.

cultivars. The alfalfa cultivar and extract concentration showed significant effects on POD (Table 3). The POD activities significantly decreased in ZhongMu (*$P<0.05$*) and changed insignificantly in the other three cultivars of alfalfa ($P>0.05$) with the increased extract concentrations. And the POD of ZhongMu was highest in the four cultivars, basically. The SOD activities were highest in XunLu on the 0.01% extract concentrations ($P<0.05$, Table 3). and there were no significant changes in other conditions ($P>0.05$). Antioxidative enzymes were also a plant's defense against adversity. These results indicated different varieties of alfalfa showed different antioxidant enzymes against autotoxicity (Zhang *et al.,* 2021).

Relationship between lengths of alfalfa, physiological characters and seed germination

There were negative correlations of alfalfa lengths with the germination parameters (Table 4) and the significant correlations were shown between alfalfa lengths with GI ($P<0.05$). But there were less correlation between the physiological characters and the germination parameters. The results indicated seed germination would negatively affect the seeding growth. This might be that weak seeds were inhibited by the leaf extract, but the remaining germinated seeds resisted the autotoxicity and promoted the seedling growth of alfalfa (Yan *et al.,* 2022).

CONCLUSION

Alfalfa autotoxicity is a generally phenomenon. The results in this study found that the alfalfa autotoxicity might be more severe as the increasing extract concentration of the leaves. Suitable cultivar renovation was found as an effective way to alleviates the alfalfa autotoxicity. And JuNeng 995 could be selected for planting after ZhongMu No.1 through the germination and seedling growth in this study.

ACKNOWLEDGEMENT

The present study was supported by the grants from the Natural Science Foundation of Shandong Province (ZR2020QC184), the National Natural Science Foundation of China (31700553), the Forage Industrial Innovation Team, Shandong Modern Agricultural Industrial and Technical System, China (SDAIT-23-8) and the key technology research project of consolidation and enhancement of carbon sequestration capacity of ecological public welfare forest in the middle mountainous area of Shandong province (SDGP370000000 2024 02000946).

Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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