LRF-885
[1-10]

RESEARCH ARTICLE **Legume Research- An International Journal**

Effect of Exogenous Hormones on the Growth of Alfalfa Seedlings under Phosphate Starvation Stress

Na Guo[1], Jiarong Li[1], Mingjiu Wang[1,2], Xiaowei Huo[1], Lin Bian[1], Zhenyi Li[1], Zhiqiang Zhang[1] **10.18805/LRF-885**

ABSTRACT

Background: Alfalfa (*Medicago sativa*) is a high-quality legume forage. Phosphorus (P) is essential for plant productivity. Approximately half of global arable soils exhibit available-P deficiency, constraining plants growth and yield. This study aimed to explore how hormones mediate the responses of alfalfa to phosphate starvation.

Methods: We studied the morphological and physiological changes alfalfa (Zhongmu No.3) seedlings treated with auxin, ethylene and gibberellin under conditions of phosphate deficiency. 20-day-old alfalfa seedlings were exposed to varying concentrations of hormones in Hoagland solution. Changes in root morphology, photosynthesis, physiological characters and the levels of *phosphate starvation induced* (*PSI*) genes expression were monitored.

Result: IAA stimulated lateral-root growth (1.16-fold vs. control), up-regulated the *organic phosphate transporter* (*PHT1-4*) and *purple acid phosphatase* (*PAP23*) and inhibited primary-root elongation. Ethylene promoted the growth of root hairs and 10 µM aminocyclopropane-1-carboxylic acid (ACC) increased the elongation of roots and their surface area.1 µM ACC boosted shoot and root acid phosphatase activity and up-regulated *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*), whereas 5 µM gibberellin A3 (GA_3) promoted stem and leaf growth, increasing stem diameter by 16.10% and leaf area by 36.90%, while elevating s*ulfoquinovosyl diacylglycerol* (*SQD2*) content and *DELLA* expression. There was a strong negative correlation (-0.79) between the plant height and root-to-shoot ratio and a positive correlation (0.62) between the number of lateral roots and the activity of acid phosphatase (ACP) in the roots. The most effective concentrations of hormones was 1 µM ACC, which enhanced the plant height, stem diameter, lateral root formation, chlorophyll biosynthesis and nitrogen content and induced the levels of expression of *PSI* and key genes in hormone signaling.

Key words: Alfalfa seedling, Auxin, Ethylene, Gibberellin, Phosphate starvation response.

INTRODUCTION

Phosphorus (P) is a necessary macronutrient for plants and it is involved in processes, such as photosynthesis and enzyme regulation (Sulieman *et al*. 2013; Prakash *et al*., 2025; Journals *et al*., 2024). However, nearly half of the global arable land is deficient in available phosphate (Hynšt *et al*., 2024; Yang *et al*., 2024). This widespread deficiency in available phosphate significantly hinders plant growth, development and yield due to stunted development, including reduced height and leaf area (Wang *et al*., 2023; Puga *et al*., 2024; Nussaume *et al*., 2024; Balboa *et al*., 2024). The root system is essential for nutrient and water uptake and root architecture strongly governs inorganic phosphate (Pi) acquisition (Comte *et al*., 2013; Ren *et al*., 2023). Plants absorb Pi through their roots and respond to Pi deficiency by remodeling root morphology and physiology (Kumar *et al*., 2019; Nahar *et al*., 2022; Chiou *et al*., 2011).

Exogenous auxin, ethylene and gibberellins collectively enhance plant growth and phosphorus-use efficiency (PUE) under low-phosphate (LP) stress by modulating root architecture, Pi transporters and the phosphate-starvation response (Xu *et al*., 2020; Han *et al*., 2022; Mimura *et al*., 2024; Anfang *et al*., 2021; Zhao *et al*., 2021). Auxin adjusts the root architecture and distribution of dry matter under low available phosphate stress, which supports the development of lateral roots (Nacry *et al*., 2005; Jain *et al*. 2007; Xia *et al.,* Wong *et al*., 2023). Auxin reallocates dry matter and stimulates lateral-root formation, mitigating P deficiency (Di *et al*., 2018). Under LP stress, it triggers ethylene production and citric-acid exudation in *Medicago truncatula* roots, enhancing Pi uptake (Chen *et al*., 2023). In *Cunninghamia lanceolata* seedlings, ethylene boosts root growth and Pi absorption. Through its receptor ethylene response sensor 1 (ERS1) and the transcription factor ethylene insensitive 3 (EIN3), ethylene up-regulates root-

[1]College of Grassland Science, Inner Mongolia Agricultural University, Technology Engineering Center of Drought and Cold-Resistant Grass Breeding in North of the National Forestry and Grassland Administration, Key Laboratory of Grassland Resources of the Ministry of Education, Hohhot 010010, China.
[2]National Center of Pratacultural Technology Innovation (under preparation), Hohhot 010010, China.

Corresponding Author: Zhenyi Li, Zhiqiang Zhang; College of Grassland Science, Inner Mongolia Agricultural University, Technology Engineering Center of Drought and Cold-Resistant Grass Breeding in North of the National Forestry and Grassland Administration, Key Laboratory of Grassland Resources of the Ministry of Education, Hohhot 010010, China.
Email: lizhenyily@163.com; zhangzq1989@imau.edu.cn

How to cite this article: Guo, N., Li, J., Wang, M., Huo, X., Bian, L., Li, Z. and Zhang, Z. (2025). Effect of Exogenous Hormones on the Growth of Alfalfa Seedlings under Phosphate Starvation Stress. *Legume Research.* 1-10. doi: 10.18805/LRF-885.

Submitted: 03-07-2025 **Accepted:** 15-08-2025 **Online**: 20-09-2025

hair genes, promoting hair formation and improving phosphorus-acquisition efficiency (Zhang *et al*., 2021). Gibberellins enhance plant-specific photosynthetic and nitrogen-metabolizing enzyme activities, increasing plant height and leaf area (Fonouni-Farde *et al*., 2019; Brini *et al*., 2022). Phosphate deficiency triggers complex crosstalk among gene expression, root architecture and hormonal signaling (Nasr Esfahani and Sonnewald 2024). In gibberellin-treated *Arabidopsis thaliana*, overexpression of specific genes represses phosphorus-starvation-induced genes, modifies root morphology, reduces Pi uptake and acid phosphatase (ACP) activity and consequently lowers shoot phosphorus content (Gong *et al*., 2024; Jiang *et al*., 2007).

Alfalfa (*Medicago sativa*) requires 10-15 mg kg^{-1} of available phosphate (Pi) for optimal growth, yet soil Pi frequently falls below this threshold, resulting in chronic deficiency. The hormonal mechanisms underlying alfalfa's response to LP remain unresolved. We therefore applied graded concentrations of auxin, ethylene and gibberellin to Pi-limited alfalfa to elucidate the associated physiological and morphological changes and to evaluate the potential of phytohormone supplementation for alleviating Pi deficiency.

MATERIALS AND METHODS

Plant material

This study was carried out at the Key Laboratory of Grassland Resources of the Ministry of Education, located at Inner Mongolia Agricultural University in Hohhot, China, from December 2023 to December 2024. Alfalfa Zhongmu No. 3 seeds were germinated for 8 days and then grown in Hoagland solution at pH 6.0 for 12 days (Xia *et al*. 2024). Control groups received 1,000 μM KH_2PO_4 (normal Pi) or 10 μM KH_2PO_4 (LP). The treated groups were established under LP conditions with auxin (0.1, 1 and 10 μM IAA), ethylene (1, 10 and 50 μM ACC), or gibberellin (0.1, 5 and 10 μM GA_3). To eliminate differences in K^+ concentration between treatments, K^+ in the LP treatment was provided by 1 mmol L^{-1} K_2SO_4 (Zhu *et al*., 2022). The plants were maintained in an artificial climate chamber at 22°C with a 16 h/8 h light/dark cycle. The Hoagland solution was refreshed every 3 days (Chen *et al*., 2017).

Measurement of the morphological characters

Height, stem diameter (3, 6, 12 d), leaf area (YaXin, Beijing, China) and the dry weight of the shoots and roots was determined. The roots were separated, imaged by an image scanner and analyzed for their length, surface area and numbers of lateral roots using WinRHIZO software (v.5.0, Regent Instruments, Quebec City, Canada). Root hairs from the seedlings were observed using a microscope (BX41, Olympus, Japan). Three biological repetitions that contained four technical repetitions per repetition, respectively, were established in this study (Ma *et al*., 2023).

Agronomic and physiological character measurements

The net photosynthetic rate (Pn) and transpiration rate (Tr) were measured at 3, 6, 12 d (LI-6400XT; LI-COR, Lincoln, NE, USA). The gas flow was set at 500 μmol·s^{-1} and the leaf chamber was illuminated by a 3 cm × 2 cm red and blue LED. Chlorophyll and nitrogen were quantified with a portable chlorophyll meter (n = 5). Leaf enzymes were assayed with *p*-nitrophenyl phosphate (pNPP) as substrate to generate *p*-nitrophenol, (PNP) on 405 nm; ACP activities in shoots and roots were quantified with an ACP Activity Assay Kit (ACP-1-W, Comin, Nanjing, China).

Analysis of the expression of the PSR genes

Total RNA was extracted from the shoots and roots using a FastPure Universal Plant Total RNA Isolation Kit (RC411-01, Vazyme Biotech Co., Ltd., Nanjing, China). quantified with NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), reverse transcription to complementary DNA (cDNA) using a HiScript II kit (Vazyme) and stored at -20°C. Gene-specific primers were designed with Primer Premier 5.0 and Primer-BLAST (NCBI) to ensure specificity. In addition, the primer pair of actin has been reported in previous studies (Zhou *et al*., 2019). The amplification program was as follows: 95°C for 30 s, followed by 95°C for 5 s, 60°C for 30 s, and 95°C for 15 s, repeated for 40 cycles. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Table 1).

Statistical analysis

The data was organized using Microsoft Excel 2020 (Redmond, WA, USA). Statistical analysis appears to have been performed with appropriate tools (SPSS, PCA,Origin) (IBM, Inc, Armonk, NY, USA and OriginLab, Northampton, MA, USA)

The affiliation function is as follows:

$$U(X) = \frac{(X - X_{min})}{(X_{max} - X_{min})} \quad \text{... (1)}$$

The formula for weighting is as follows:.

$$W_j = P_j / \sum_{n=1}^{j} P_j \quad \text{... (2)}$$

Finally, the combined evaluation value (d) is calculated using the following formula (d) value:

$$D = \sum_{n=1}^{j} [U(X) \times W_j] \quad \text{... (3)}$$

Where,

$U(X)$ = Affiliation function value of an evaluation indicator.

X = denotes the measured value of that indicator.

The symbols X_{max} and X_{min} indicate the maximum and minimum values for each respective indicator. j = 1,2,…,n. W_j denotes the weighting formula and P_j denotes the contribution of the j^{th} principal component.

RESULTS AND DISCUSSION

Influence of the hormones on agronomic traits

Pi deficiency differentially modulated hormone mediated growth of alfalfa seedlings at 3, 6 and 12 d, resulting in reduced height, leaf area, stem diameter and shoot

biomass (Fig 1). IAA (0.1 and 1 μM) alleviated Pi-deficiency growth inhibition, elevating height, stem diameter and shoot biomass within 3-12 d. (Fig 1a, c, d). 0.1 μM IAA boosted height by 25% and 1 μM IAA increased stem diameter by 27% at 6 d and leaf area by 55% at 12 d relative to LP. 10 μM IAA suppressed leaf growth. ACC inhibited height except for 1 μM at 12 d versus control, whereas 1 and 10 μM ACC significantly increased height and stem diameter at 6 and 12 d. (Fig 1b, c). Treatment with 50 μM ACC inhibited the biomass but increased the root-to-shoot ratio (P<0.05) (Fig 1e). An increase in plant height would normally be expected with GA_3 application. Instead, a reduction in leaf area and dry weight is observed with higher GA_3 concentrations (Fig 1a). The stem diameter at 12 d after treatment with 5 and 10 μM GA_3 and the aboveground weight from 6 d to 12 d treated with 0.1 and 5 μM GA_3 were significantly enhanced (Fig 1c, d). At 6 d, 0.1 and 5 μM GA_3 elevated shoot biomass above +Pi levels and 5 μM GA_3 further thickened stems by 16.10% and expanded leaf area by 36.90% (Fig 1d).

Influence of hormones on the morphological characteristics of the roots

Among the three hormones, auxin and ethylene enlarged the root system by increasing root surface area, lateral root number and total root length, with 1 μM IAA and 10 μM ACC providing the greatest enhancement. 1 μM ACC resulted in the most significant increase in dry weight of underground plant (Fig 2a). Treatment with 1 μM IAA increased alfalfa root surface area 1.25-fold, lateral root number 2.16-fold and total root length 0.36-fold under phosphorus deficiency, whereas 10 μM IAA suppressed root weight, surface area and total root length (Fig 2b, d). Treatment with 1 and 10 μM ACC promoted the growth of root hairs and lateral roots, while 50 μM ACC inhibited the growth of lateral roots (Fig 2c, e).10 μM ACC increased root surface area by 28% and maximized cumulative root elongation. Among GA_3 treatments, 0.1 μM GA_3 raised root dry weight to 1.29-fold of the +P control at day 3, whereas 5 μM GA_3 significantly extended total root length without affecting lateral-root density. ACC promoted root growth in a concentration-dependent manner over 3, 6 and 12 days (Fig 2e).

Effects of hormones on the photosynthetic traits

IAA reduced the Pn of the alfalfa seedlings and 0.1 μM yielded the lowest Pn. It also significantly increased the Tr, which peaked on day 6 at 3.4-fold the normal levels of phosphorus. Conversely, 10 μM IAA suppressed the Tr (Fig 3a). The Pn peaked at 10 μM ACC but was hindered by 1 and 50 ìM ACC. The addition of 1 μM ACC significantly increased the early-stage Tr in phosphorus-stressed seedlings but later decreased. Treatment with 1 μM ACC increased the biosynthesis of chlorophyll by 12.80% and the uptake of nitrogen by 13.60%. Treatment with 0.1 μM GA_3 resulted in the highest Pn, which was 1.82-fold that of the control, while 10 μM GA_3 showed the lowest rate, which was 1.51-fold lower. Treatment with 0.1 μM GA_3 decreased the Tr but increased the biosynthesis of chlorophyll by 21.30% and the uptake of nitrogen by 14.80% (Fig 3c,d).

Influence of hormones on the activity of ACP

Hormones differentially modulated ACP activity: Early IAA or GA_3 suppressed shoot ACP but enhanced root secretion. After 12 d, 10 μM IAA elevated shoot ACP to 91.74 (P<0.05). The roots treated with 1 μM ACC had 10.51 μmol/min/g ACP, which was 3.06-fold that of the control. 10 μM GA_3 increased shoot ACP, whereas 5-10 μM GA_3 reduced root ACP (Fig 4).

Correlation analysis of the different characters and a membership function analysis

Under P deficiency, a Pearson correlation analysis showed that plant height (-0.79) and root-to-shoot ratio were inversely related; lateral-root number correlated positively

Table 1: Gene primers for the real-time quantitative reverse-transcription PCR.

Number	Gene name	Primer name	Gene sequence (5'-3')
1	*Actin*	Actin-F	CACGAGACCACCTACAACTCTATC
		Actin-R	CATACGGTCAGCAATACCTGG
2	*PHT1-4*	PHT1-4-F	TTCTTTGGCTGGCTTGGTGA
		PHT1-4-R	TGTCCAAAAGAAAGGCCGGA
3	*PAP23*	PAP23-F	GTGAAGGCCGTTTTCGTTGG
		PAP23-R	CGAGACCATCCAGCCTTTCC
4	*SQD2*	SQD2-F	AGCACGAATCGCTATTGTTGG
		SQD2-R	GAGATAATTCTTCCCCGCTTA
5	*AUX*	AUX-F	GACCAAGACACTGTCCAACCTCC
		AUX-F	GCCATGCTCACTTTCACATACAT
6	*ACO*	ACO-F	ACTGGGGATTCTTTGAGCTGG
		ACO-R	AACAGCCTCTAGCCCTTTGG
7	*DELLA*	DELLA-F	TATTACTCGACCGTTTTTGACTCG
		DELLA-R	GAGCCGGTCCCTCGCA

Note: *PHT1-4*,*organic phosphate transporter1-4*; *SQD2*, *sulfoquinovosyl diacylglycerol*; *PAP23*,*purple acid phosphatase 23*; *AUX*,*auxin*; *ACO*,*1-aminocyclopropane-1-carboxylate oxidase*; *DELLA*, *DELLA protein*, the sequence is the 5'-3' end of the gene.

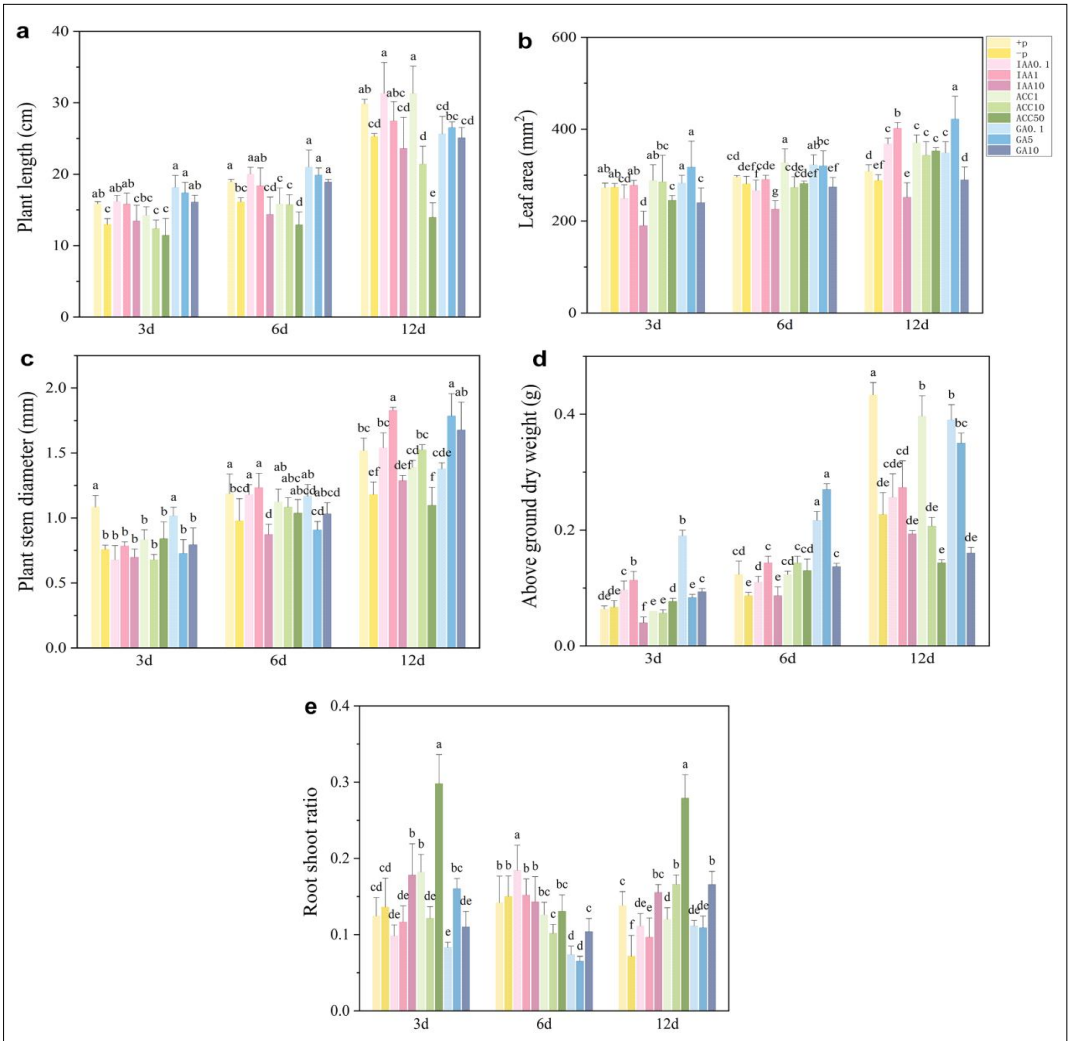
with ACP (0.62), root length (0.93) and surface area (0.76). Chlorophyll and nitrogen contents were tightly linked (0.97) and root surface area associated with Pn (0.64), which suggested the development of a root system that affects the photosynthetic efficiency and overall growth (Fig 5).

Membership-function analysis of nine morphological traits across 11 treatments (≥85% cumulative variance) extracted four principal components (≥89.45% contribution) to rank the optimal hormone concentrations for low-P-stressed alfalfa seedlings.1 µM ACC (*D* = 0.7303), 10 µM ACC (0.6258) and 5 µM GA_3 (0.6004) were the top three hormone concentrations for promoting shoot growth under P deficiency. Six treatments (all ethylene, 0.1 and 5 µM GA_3, 1 µM IAA) exceeded normal-P controls. Low-P ethylene, especially 1 µM ACC, surpassed normal growth. The concentrations of auxin promoted growth, whereas 10 µM GA_3 inhibited it, yielding poorer performance than P-stressed plants (Table 2).

Effects of hormones on the *PSI* genes and the levels of expression of the key hormone signaling genes

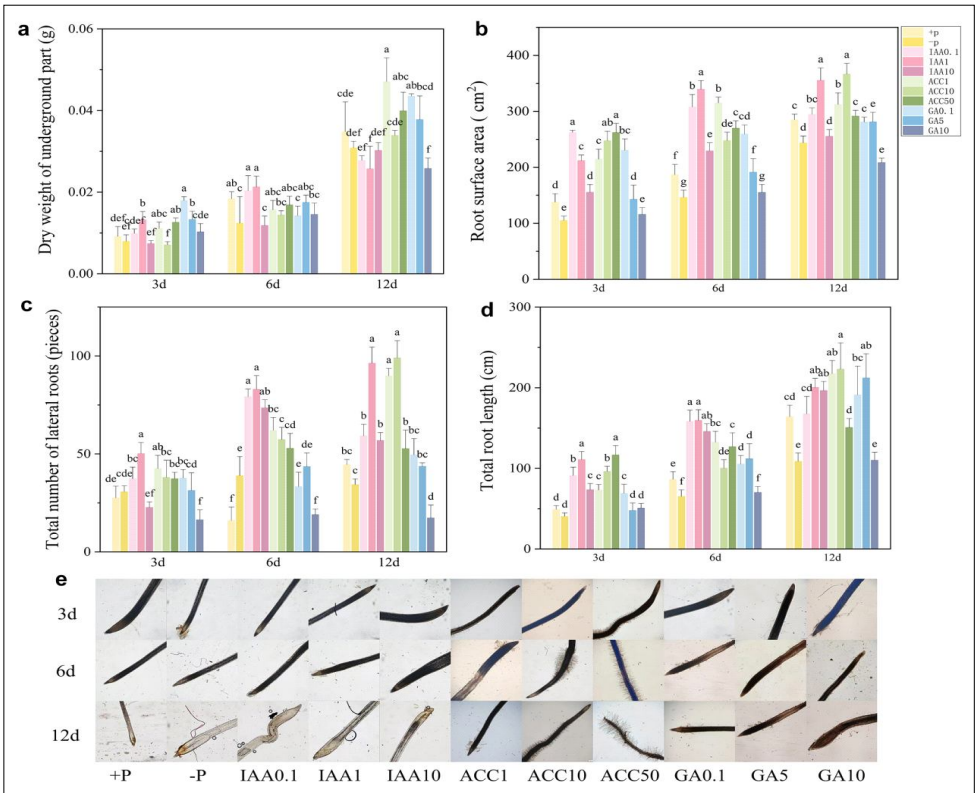
Roots expressed key *PSI* genes (*PHT1-4*, *PAP23*, *SQD2*). IAA (10 µM) progressively up-regulated *PHT1-4* (*P*<0.05); 1 µM ACC down-regulated *PHT1-4*, whereas 5 µM GA_3 up-regulated it. *PAP23* was induced by LP stress compared to the conditions of normal phosphate. Seedlings treated with 10 µM IAA or ACC for 12 h significantly up-regulated *PAP23*. The highest *PAP23* level was reached at 6 d with 5 µM GA_3. *SQD2* expression increased with auxin and ethylene concentrations, was lowest under 1 µM ACC and was significantly higher with 5 µM GA_3 than all other treatments (Fig 6).

1 µM IAA optimally induced *AUX* expression. Peak AUX expression at 3 d under 0.1 or 1 µM IAA.*ACO* was also



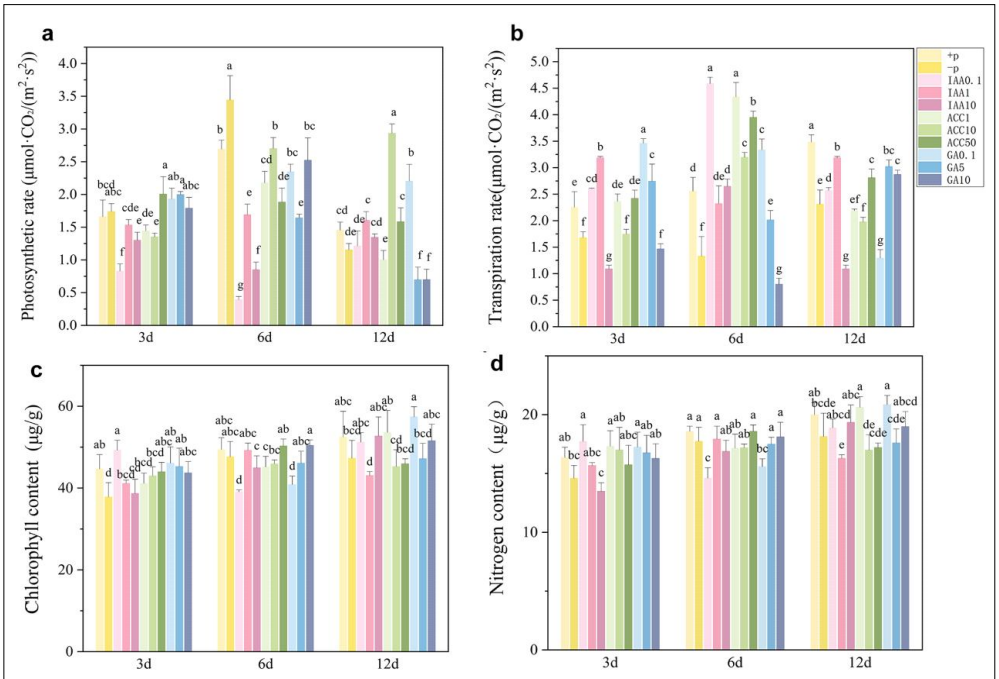
(a) Plant length. (b) Leaf area. (c) Plant stem diameter. (d) Aboveground dry weight. (e) Root-to-shoot ratio. The data were the mean ± SD and different lowercase letters denote statistically significant differences (*P*<0.05). +P, normal phosphorus; -P, low phosphorus; IAA1, 1 ìM of IAA and the same applies to the following. IAA: Indole-3-acetic acid.

Fig 1: Influence of plant hormones on the agronomic traits of alfalfa.



(a) Dry weight of underground part. (b) Root surface area. (c) Total number of lateral roots. (d) Total root length. (e) Microscopic view of the alfalfa roots.The data were expressed as the mean ± SD and different lowercase letters denote statistically significant differences (P<0.05).

Fig 2: Influence of exogenous hormones on the morphological characteristics of alfalfa roots.



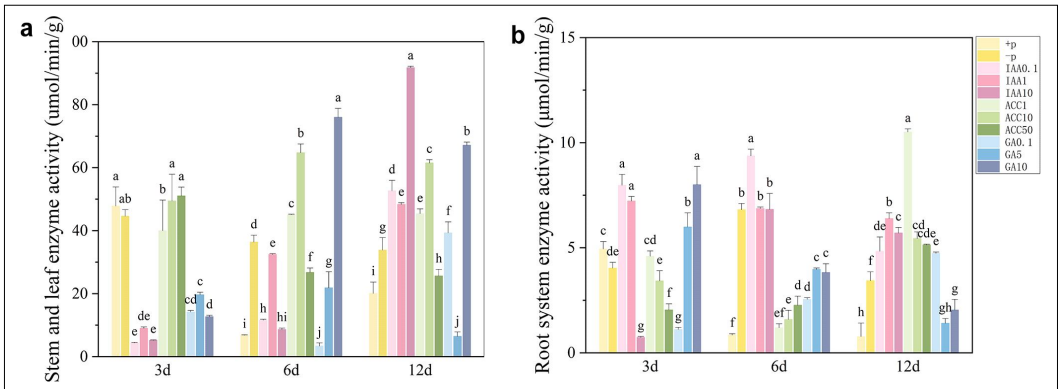
(a) Photosynthetic rate. (b) Transpiration rate. (c) Chlorophylll content. (d) Nitrogen content. The data were expressed as the mean ± SD and different lowercase letters denote statistically significant differences (P<0.05).

Fig 3: Influence of exogenous hormones on the photosynthetic traits of alfalfa.

induced by ACC treatments from 12 h to 12 d. 1 μM ACC elicited maximal ACO expression at 3 d. *DELLA* was expressed at the highest levels in seedlings that appeared to have been exposed to 0.1 μM GA_3. However, 5 μM GA_3 induced the peak expression after 3 days (Table 3).

The supply of phosphate directly affects the growth and development of plants through the biosynthesis of nucleic acids, proteins, energy-rich phosphate and the enhancement of photosynthesis and others (Iqbal *et al*., 2023; Salim *et al*. 2023). Phosphate starvation stress led to the increase in the activity of ACP, the elongation of roots and the production of organic acids and phosphate transporters (Xu *et al*., 2022; Dokwal *et al*., 2022). Increased phosphate uptake and transport contribute to the maintenance of stable phosphorus levels within the plant, thereby facilitating an improved adaptation to phosphate deficiency (Soumya *et al*., 2022; Lian *et al*., 2023). In this study, the height, stem thickness and leaf area of alfalfa were significantly reduced in conditions of phosphorus deficiency compared to plants grown under normal levels. This is probably because the alfalfa plants prioritized the allocation of phosphorus by restricting the growth of their stems and leaves to maintain the normal development of the plants. Nevertheless, the application of phytohormones enhanced the allocation of nutrients among the stems, leaves and root systems (Du *et al*., 2024; Yan *et al*., 2024; Nambara, 2021).

Exogenous phytohormones modulate biomass partitioning and root–shoot allocation under LP stress (Groenewald *et al*. 2019; Shi *et al*. 2017; Lei *et al*., 2022). Whereas 5 μM GA_3 markedly increased shoot height and stem diameter-effects counteracted by 50 μM ACC-low-



(a) Stem and leaf enzyme activity. (b) Root system enzyme activity.The data were expressed as the mean ± SD and different lowercase letters denote statistically significant differences (P<0.05).

Fig 4: Influence of exogenous hormones on the physiological parameters of alfalfa.

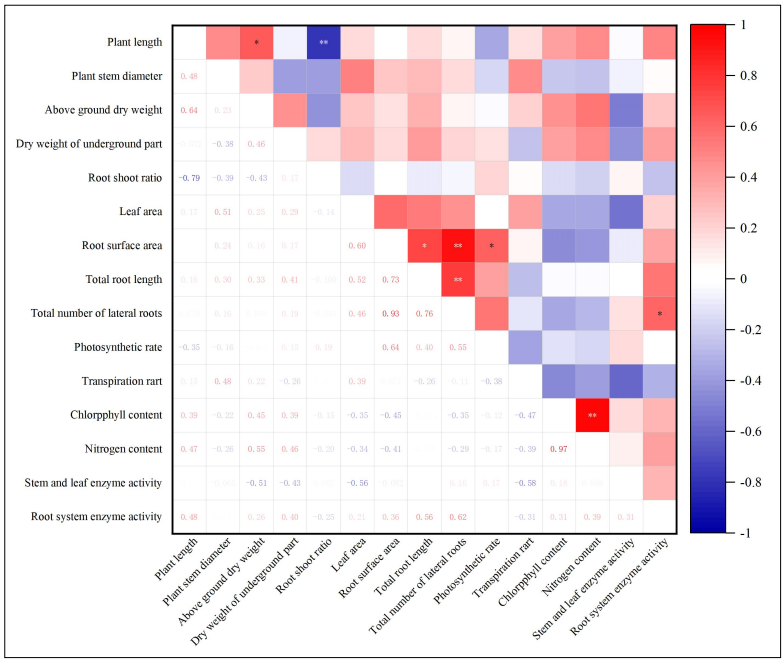


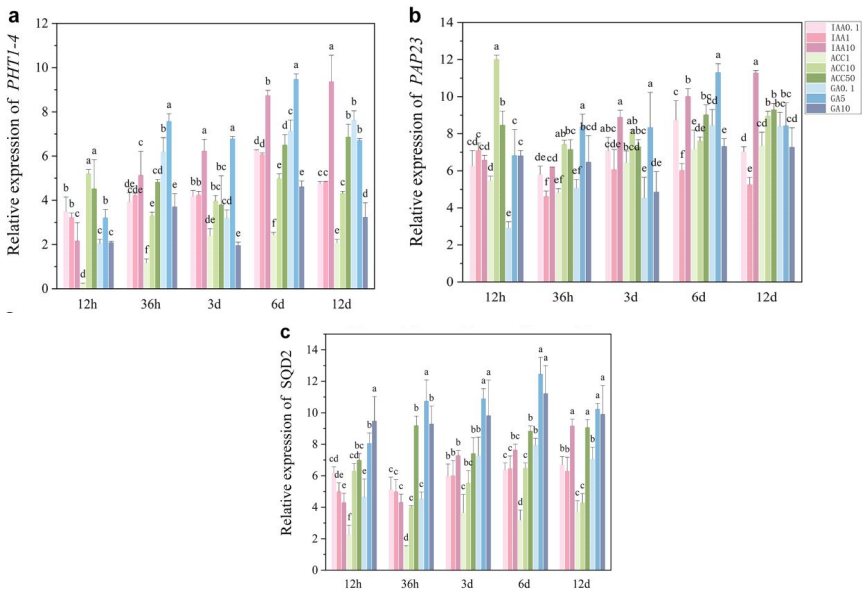
Fig 5: Correlation analysis of the morphological and physiological parameters in alfalfa.

phosphate stress alone elevated the alfalfa root-to-shoot ratio by 9.60%, highlighting adaptive resource reallocation. Phytohormones further remodel root architecture for improved P acquisition (Nussaume *et al*. 2024). GA_3 inhibited shoot and lateral-root growth yet elongated primary roots, whereas 1 μM IAA and 10 μM ACC most effectively stimulated lateral-root proliferation and expanded root surface area, respectively. In LP-stressed plants, 10 μM ACC maximized transpiration and photosynthesis; 1 μM ACC and 0.1 μM GA_3 restored chlorophyll and nitrogen contents; and 1 μM ACC elevated acid phosphatase activity and Pi uptake. Optimal shoot biomass accrued with 5 μM GA_3, but the most robust root system-greater surface area, lateral-root number and total length-was achieved with 1 μM IAA and 10 μM ACC.

Phytohormones modulate gene expression and metabolic pathways, thereby shaping plant growth, development and responses to LP. The *PHT1* gene, part of the phosphorus transport protein family, is crucial for the uptake, function and utilization of phosphorus. These effects promote the translocation of phosphorous to specific tissues. The *PAPs* are the key to the utilization of organic phosphorus in the soil and its redistribution within the plants. qRT-PCR quantification of *PHT1-4*, *PAP23*, *SQD2*, *AUX*, *ACO* and *DELLA* revealed that hormone treatments elevated Pi-transporter abundance and up-regulated *PAP23* and *SQD2*

Table 2: Affiliation function values of the different treatments for the morphological traits in alfalfa.

Process	Plant height	Plant stem diameter	Above ground dry weight	Dry weight of under-ground part	Root shoot ratio	Leaf area	Root surface area	Total root length	Total number of lateral roots	*D* value	order
+P	29.83	1.52	0.5333	0.0348	0.14	307.93	284.83	163.99	45	0.4610	7
-P	25.27	1.18	0.2267	0.0308	0.07	288.20	243.60	108.48	34	0.1907	10
IAA0.1	31.34	1.54	0.2567	0.0278	0.11	367.77	294.48	167.61	55	0.3867	8
IAA1	27.44	1.83	0.2733	0.0257	0.10	401.87	355.31	200.43	96	0.5430	6
IAA10	23.60	1.29	0.1933	0.0302	0.16	251.60	255.30	196.53	53	0.3113	9
ACC1	31.30	1.39	0.3967	0.0471	0.12	370.00	312.00	217.32	90	0.7303	1
ACC10	21.40	1.52	0.2067	0.0339	0.17	342.93	366.51	222.93	99	0.6258	2
ACC50	13.92	1.10	0.1433	0.0399	0.28	352.60	291.47	150.65	53	0.5432	5
GA0.1	25.62	1.38	0.3900	0.0435	0.11	348.10	280.83	191.06	50	0.5790	4
GA5	26.50	1.78	0.3500	0.0378	0.11	421.63	281.14	211.99	44	0.6004	3
GA10	25.08	1.68	0.1600	0.0258	0.17	289.27	208.32	110.06	17	0.1141	11



PHT1-4 (a), *PAP23* (b) and *SQD2* (c). The data are expressed as the mean ± SD and different lowercase letters denote statistically significant differences (P<0.05).

Fig 6: The relative levels of expression of genes.

Table 3: Expression of different exogenous hormones on the genes related to the roots of alfalfa.

Related gene	hormones	12 h	36 h	3 d	6 d	12 d
AUX	IAA0.1	5.9±0.28c	6.38±0.62c	8.13±1.41b	5.72±0.34c	6.48±0.36b
	IAA1	9.7±0.66a	9.7±0.13a	11.07±0.28a	10.94±0.34a	9.33±0.17a
	IAA10	7.0±0.28b	8.27±0.63b	6.95±0.24b	7.29±0.71b	8.59±0.89a
ACO	ACC1	5.3±0.27a	5.45±0.12a	6.5±0.04a	5.72±0.05a	5.64±0.09a
	ACC10	5.8±0.33a	4.63±0.23b	3.07±0.46c	4.17±0.58b	4.14±0.26c
	ACC50	3.6±0.51b	3.06±0.3c	3.96±0.05b	3.87±0.4b	4.82±0.29b
DELLA	GA0.1	4.94±0.5a	6.14±0.93a	3.23±0.45b	4.67±1.17b	4.05±0.29b
	GA5	3.58±1.5ab	5.78±1.12a	4.9±0.33a	6.47±0.67a	5.83±0.57a
	GA10	2.94±0.39b	2.87±0.15b	1.34±0.18c	2.5±0.06c	1.71±0.26c

Note: Different lowercase letters (a, b, c) within the same column for the same gene denote statistically significant differences.

in alfalfa. Various hormone treatments modulated the expression of these genes, which affected the growth and adaptation to LP stress. The expression of *AUX* peaked with 1 μM IAA, while *PHT1-4* and *PAP23* peaked with 10 μM IAA. Treatment with 1 μM ACC upregulated *ACO* and 5 μM GA_3 upregulated *SQD2* and *DELLA*. *SQD2* induction under LP reallocates membrane lipids and improves P recycling. Transcription factors within hormone signaling cascades integrate these responses and optimized hormone doses enhance Pi acquisition, alleviating growth inhibition under P deficiency.

CONCLUSION

Under phosphate deficiency, IAA enhances lateral-root initiation and elongation while suppressing primary-root extension. ACC promotes both lateral-root emergence and root-hair elongation. GA_3 stimulates primary-root elongation yet inhibits lateral-root formation. Hormonal priming with ACC, GA_3 and IAA alleviated P stress in alfalfa. IAA up-regulated *PHT1-4, PAP23* and *AUX*; ethylene induced *ACO*; and GA_3 elevated *SQD2* and *DELLA*. Lateral-root abundance correlated positively with root enzyme activity. Membership-function analysis identified 1 μM ACC as the most effective treatment, significantly enhancing seedling height, stem diameter, lateral-root number, chlorophyll content, nitrogen uptake and ACP activity, thereby boosting LP tolerance.

ACKNOWLEDGEMENT

We acknowledge the platform of Key Laboratory of Grassland Germplasm Innovation and Sustainable Utilization of Grassland Resources in Inner Mongolia, Key Laboratory of Forage Cultivation, Processing and High Efficient Utilization of Ministry of Agriculture, China.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Funding

The work was supported by National Natural Science Foundation of China (32301484), Inner Mongolia Autonomous Region Natural Science Foundation General Program (2025MS03136), Grassland Talents Program of Inner Mongolia Autonomous Region, Scientific Research Foundation for Advanced Talents by Inner Mongolia Agricultural University (NDYB2022-51), The First-Class Discipline Scientific Research Program of Inner Mongolia (IMAUCXQJ2023015), Capacity Building Project of the National Forestry and Grassland Engineering Technology Research Center for the Breeding of Drought- and Cold-Tolerant Grass Varieties in Northern China (BR221029, BR251016), 2023 National Center of Pratacultural Technology Innovation (under preparation) Major Innovation Platform Construction Project (CCPTZX2023B0701), National Key R and D Program-Intergovernmental International Science and Technology Innovation Cooperation: China-Mongolia Joint Research Project (2024YFE0113100) and Scientific Research Funding for Universities Directly under the Inner Mongolia Autonomous Region (BR22-12-07).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Anfang, M., Shani, E. (2021) Transport mechanisms of plant hormones. Curr. Opin. *Plant Biol.* **63:** 102055.

Balboa, G.R., Ferguson, R., Punte,l L. (2024) Irrigated corn yield and soil phosphorus response to long term phosphorus fertilization. *Agron. J.* **116:** 2588-2598

Brini, F., Mseddi, K., *et al.* (2022) Hormone-mediated plant responses to light quality and quantity. *Environ. Exp. Bot.* **202:** 105026.

Chen, F., Zhang, J., Ha, X. (2023) Genome-wide identification and expression analysis of the Auxin-Response factor (ARF) gene family in *Medicago sativa* under abiotic stress. *BMC Genomics.* **24:** 498.

Chen, S., Han, X., Fang, J. (2017) Sedum alfredii SaNramp6 Metal Transporter Contributes to Cadmium Accumulation in Transgenic Arabidopsis thaliana. *Sci Rep.* **7(1):** 13318.

Chiou, T.J., Lin, S.I. (2011) Signaling network in sensing phosphate availability in plants. *Annu. Rev. Plant Biol*. **62:** 185-206.

Comte, M., Coron, J., Guerrero, S. (2013) Analysis of a model of phosphorus uptake by plant roots. *J. Evol. Equ.* **13(3):** 595-615.

Di, D., Sun, L., Zhang, X. (2018) Involvement of auxin in the regulation of ammonium tolerance in rice (*Oryza sativa* L.). *Plant Soil.* **432:** 373-387.

Dokwal, D., Cocuron, J., Alonso, A.P. (2022) Metabolite shift in Medicago truncatula occurs in phosphorus deprivation. *J. Exp. Bot*. **73:** 2093-2111.

Du, T., Zhao, S., Zheng, X. (2024) External phosphorus enhances the efficient acquisition of phosphorus by alfalfa (*Medicago sativa* L.) through its interaction with root morphological traits and rhizosphere carboxylates. *Rhizosphere.* **29:** 100861.

Fonouni-Farde, C., Miassod, A., Laffont, C. (2019) Gibberellins negatively regulate the development of *Medicago truncatula* root system. *Sci. Rep*. **9:** 2335.

Gong, J., Song, L., Zhang, Z. (2024) Correlations between root phosphorus acquisition and foliar phosphorus allocation reveal how grazing promotes plant phosphorus utilization. *Plant Physiol. Biochem*. **208:** 108467.

Groenewald, L., Morcillo, R.J.L, Midgley, G.F. (2019) Partitioning of above and below ground costs during phosphate stress in Medicago truncatula. *J. Plant Nutr*. **42:** 759-771.

Gusain, S., Kumari, K., Joshi, R. (2024) Physiological, hormonal and molecular dynamics of root system architectural response to drought stress signaling in crops. *Rhizosphere.* **31:** 100922.

Han, Y., White, P.J., Cheng, L. (2022) Mechanisms for improving phosphorus utilization efficiency in plants. *Ann. Bot.* **129:** 247-258.

Hynšt, J., Urbánková, E., Obdržálková, E. (2024) Effect of increasing rate of P fertilization on available P content in soil and plant uptake: Implications for P management. *J. Plant Nutr*. **47:** 1834-1849.

Iqbal, A., Huiping, G., Qiang, D. (2023) Differential responses of contrasting low phosphorus tolerant cotton genotypes under low phosphorus and drought stress. *BMC Plant Biol.* **23:**168.

Jain, A., Poling, M.D., Karthikeyan, A.S. (2007). Differential effects of sucrose and auxin on localized phosphate deficiency-induced modulation of different traits of root system architecture in arabidopsis. *Plant Physiol.* **144:** 232-247.

Jiang, C., Gao, X., Liao, L. (2007) Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in Arabidopsis. *Plant Physiol*. **145:** 1460-1470.

Journals, A. (2024). Effects of excessive application of P and Zn fertilizer on growth and yield of maize under hydroponic conditions. *Indian Journal of Agricultural Research.* **58(3):** 431-437. doi: 10.18805/IJARe.AF-833.

Kumar, A., Shahbaz, M., Koirala, M. (2019) Root trait plasticity and plant nutrient acquisition in phosphorus limited soil. *J. Plant Nutr. Soil Sci.* **182:** 945-952.

Lei, K.J., Zhou, H., Gu, D.L. (2022) The involvement of abscisic acid-insensitive mutants in low phosphate stress responses during rhizosphere acidification, anthocyanin accumulation and Pi homeostasis in Arabidopsis. *Plant Sci*. **322:** 111358.

Lian, W., Yu, Q., Zhang, P. (2023) NtPIN3 positively regulates low phosphorus tolerance by changing root elongation, Pi concentration and antioxidant capacity in tobacco. *Environ. Exp. Bot*. **208:** 105257.

Ma, Z., Yang, K., Wang, J. (2023) Exogenous melatonin enhances the low phosphorus tolerance of barley roots of different genotypes. *Cells*. **12:** 1397.

Mimura, T., Reid, R. (2024) Phosphate environment and phosphate uptake studies: Past and future. *J. Plant Res*. **137:** 307-314.

Nacry, P., Canivenc, G., Muller, B. (2005) A role for auxin redistribution in the responses of the root system architecture to phosphate starvation in Arabidopsis. *Plant Physiol*. **138:** 2061-2074.

Nahar, K., Bovill, W., Mcdonald, G. (2022) Assessing the contribution of seedling root traits to phosphorus responsiveness in wheat. *J. Plant Nutr.* **45:** 2170-2188.

Nambara, E., Wees, S.C.M.V. (2021) Plant hormone functions and interactions in biological systems. *The Plant Journal.* **105:** 287-289.

Nasr Esfahani, M., Sonnewald, U. (2024). Unlocking dynamic root phenotypes for simultaneous enhancement of water and phosphorus uptake. *Plant Physiol. Biochem.* **207:** 108386.

Nussaume, L., Kanno, S. (2024). Reviewing impacts of biotic and abiotic stresses on the regulati-on of phosphate homeostasis in plants. *J. Plant Res*. **137:** 297-306.

Puga, M.I., Poza-Carrión, C., Martinez-Hevia, I. (2024) Recent advanc-es in research on phosphate starvation signaling in plants. *J. Plant Res*. **137:** 315-330.

Ren, M., Li, Y., Zhu, J., Zhao, K. (2023) Phenotypes and molecular mechanisms underlying the root response to phosphate deprivation in plants. *International Journal of Molecular Sciences*. **24(6):** 5107. doi: 10.3390/ijms24065107.

Prakash, G., Yadav, S., Prachi, Rai, N. (2025). Phosphorus deficiency induced physiological and antioxidant response in mungbean. *Legume Research*. doi: 10.18805/LR-4586.

Salim, M., Chen, Y., Solaiman, Z.M., Siddique, K.H.M. (2023). Phosphorus application enhances root traits, root exudation, phosphorus use efficiency and seed yield of soybean genotypes. *Plants.* **12:** 1110.

Shi, T.Q., Peng, H., Zeng, S.Y. (2017). Microbial production of plant hormones: Opportunities and challenges. *Bioengineered.* **8:** 124-128.

Soumya, P.R., Vengavasi, K., Pandey, R. (2022). Adaptive strategies of plants to conserve internal phosphorus under P deficient condition to improve P utilization efficiency. *Physiology and Molecular Biology of Plants : An International Journal of Functional Plant Biology*. **28 11-12:** 1981-1993.

Sulieman, S., Ha, C.V., Schulze, J., Tran, L.P. (2013) Growth and nodulation of symbiotic Medic-ago truncatula at different levels of phosphorus availability. *J. Exp. Bot*. **64(10).** doi: 10.1093/jxb/ert122.

Wang, Y., Sun, Z., Wang, Q. (2023). Winter survival, yield and yield components of alfalfa as affected by phosphorus supply in two Alkaline soils. *Agronomy*. **13:** 1565.

Wong, C., Alabadí, D., Blázquez, M.A. (2023). Spatial regulation of plant hormone action. *J. Exp. Bot.* **74:** 6089-6103.

Xia, J., Nan, L., Wang, K., Yao, Y. (2024). Comprehensive dissection of metabolites in response to low phosphorus stress in different root-type alfalfa at seedling stage. *Agronomy.* **14(8):** 1697.

Xu, H., Zhang, H., Fan, Y. (2022). The purple acid phosphatase GmPAP17 predominantly enhances phosphorus use efficiency in soybean. *Plant Sci.* **320:** 111283.

Xu, Y., Li, K., Zhu, K. (2020). Effect of exogenous plant hormones on agronomic and physiological performance of a leaf early-senescent rice mutant osled. *Plant Growth Regul.* **92:** 517-533.

Yan, W., Lu, P., Liu, Y. (2024). Comprehensive evaluation of phosphate deficiency tolerance in common vetch germplasms and the adaption mechanism to phosphate deficiency. *J. Plant Physiol.* **302:** 154317.

Yang, K., Li, S., Sun, Y. (2024) Effects of combined nitrogen and phosphorus application on soil phosphorus fractions in alfalfa (*Medicago sativa* L.) production in China. Front. *Plant Sci.* **15.**

Zhang, H., Wang, N., Zheng, S. (2021) Effects of exogenous ethylene and cobalt chloride on root growth of Chinese fir seedlings under Phosphorus-Deficient Conditions, Forests.

Zhao, X., Zhao, L., Huang, L. (2021) Response of growth characteristics and endogenous hormones of Sophora davidii to low-phosphorus stress. *Acta Physiol. Plant.* **43:** 118.

Zhou, Q., Jia, C., Ma, W. (2019). MYB transcription factors in alfalfa (Medicago sativa): Genome-wide identification and expression analysis under abiotic stresses. *PeerJ.* **7:** e7714.

Zhu, J.N., Huang, H., Du, Y. (2022).The transgenerational effect of arbuscular mycorrhizal fungi on root phosphatase activity of host plant *Medicago truncatula*. *Chinese Journal of Ecology*. **41(5):** 912-918.