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Effects of Phosphate Solubilizing Bacteria and Arbuscular Mycorrhizal Fungi on the Production of Alfalfa under Phosphorus Fertigation

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Abstract

Alfalfa (*Medicago sativa* L.) is an important forage legume in semiarid areas of China. Comprehensive studies on the growth, nutritional quality and underground biomass of alfalfa under water-phosphorus coupling with the simultaneous inoculation of phosphate-solubilizing bacteria (PSB) and arbuscular mycorrhizal fungi (AMF) are scarce. An orthogonal experimental design [L16 (4^3)] was used and the soil water holding capacity, phosphorus (P) application rate and bacterial inoculation treatments each had 4 levels. The four water holding capacities were 35% (W_1), 50% (W_2), 65% (W_3), and 80% (W_4). The four P rates were 0 mg·kg^{-1} (P_0), 50 mg kg^{-1} (P_1), 100 mg kg^{-1} (P_2) and 150 mg kg^{-1} (P_3). The four inoculation treatments were no inoculation (J_0), *Bacillus megaterium* (J_1), *Funneliformis mosseae* (J_2) and double inoculation (*B. megaterium* + *F. mosseae*) (J_3). The results showed that the dry matter yield, plant height and stem diameter in all treatments were significantly higher than those in the CK treatment ($P \leq 0.05$), and were the highest under the $W_3P_2J_0$ treatment. At the same water holding capacity, the taproot length and underground biomass in the J_0 treatment were significantly greater than those in the J_1, J_2 and J_3 treatments ($P \leq 0.05$). The soil pH values in the J_0 treatment were significantly lower than those in th J_1, J_2 and J_3 treatments ($P \leq 0.05$). The aboveground biomass, plant height, crude protein, neutral detergent fibre, acid detergent fibre, taproot length and underground biomass were influenced by factors in the order of water > P > bacteria, and these factors affected the stem diameter and P concentration of the alfalfa from most to least as P > water > bacteria. The dry matter yield of alfalfa was positively correlated with the plant height, taproot length and underground biomass ($P \leq 0.01$) and was negatively correlated with the neutral detergent fibre and acid detergent fibre contents ($P \leq 0.05$). The four treatments that had the greatest influence on the production performance of alfalfa were $W_3P_1J_3 > W_3P_2J_0 > W_3P_3J_1 > W_3P_0J_2$. We concluded that a soil water capacity of 65%, a P application rate of 44.6 mg kg^{-1}, and inoculation with AMF and PSB were the most effective conditions for promoting alfalfa growth.

Keyword: Alfalfa; Water-phosphorus coupling; Arbuscular mycorrhizal fungi; Production performance; Underground biomass

Introduction

Alfalfa (*Medicago sativa* L.) is perennial leguminous forage with high yields, a strong regenerative capacity, multiple harvests in one growing season, and high crude protein concentration. Alfalfa can adapt to different regional environments. It has played an important role in the adjustment of the animal husbandry structure in China (Brink *et al*. 2015). Alfalfa roots can reach more than ten meters long and absorb water from the deep soil (Sim *et al*. 2017). Alfalfa planting is of great significance in arid and semiarid areas (Gu *et al*. 2018). Xinjiang has a temperate continental arid climate with low rainfall, uneven seasonal rainfall distribution, and shortages of surface water and groundwater resources (Zhang *et al*. 2020). Alfalfa consumes large amounts of water, and artificial irrigation is necessary for alfalfa cultivation in arid areas. Therefore, water is one of the main factors limiting the development of alfalfa in Xinjiang. Some studies demonstrated that sufficient irrigation could increase the photosynthetic activity of alfalfa, resulting in increased alfalfa dry matter yield would increase. However, the water absorption capacity in the roots of alfalfa gradually decreased under drought stress, and the transpiration rate and photosynthetic rate decreased, which led to a decline in crop yield (Brookshire and Weaver 2015). Therefore, identifying the appropriate irrigation amount is the key to improving alfalfa production performance.

Phosphorus (P) is an essential element for plant growth and development, because it is involved in a wide range of physiological, biosynthetic, and metabolic processes (Lissbrant *et al.* 2009). It has been reported that the dry matter yield, nutrient quality and root growth of alfalfa are significantly affected by the soil P concentration (Mallarino and Rueber 2013). One study showed that water-P coupling can affect the level of P in the soil environment

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because of the unique properties of P in soil, such as its low solubility, low mobility, and high fixation by the soil matrix, and the recovery of applied P by crops in one growing season is often low (Pizzeghello *et al.* 2014). Most of the P remains in the soil in the form of poorly soluble P, which increases the concentration of P in the soil, limits the growth and development of alfalfa and causes P pollution in the soil (Thuynsma *et al*. 2014).

Low phosphorus use efficiency (PUE) seriously hinders further improvements in crop yield and nutritional quality. Phosphorus-solubilizing bacteria (PSB) can increase P availability to plants by releasing organic acids and phosphatases that enhance the solubility of various inorganic P forms in soil. Therefore, PSB may provide a good way to solve the problem of P limitation. Some studies have demonstrated that PSB play a crucial role in soil P solubilization and increase the bioavailability of soil P for plants (Shi *et al*. 2017). PSB can transform insoluble P to available phosphorus (AP) in the soil for plant absorption and utilization, and promote plant growth by increasing the plant P concentration (Heijden *et al*. 2008). P nutrient uptake by plants mainly occurs through the roots in contact with soil. Arbuscular mycorrhizal fungi (AMF) can form symbiotic relationships with more than 80% of terrestrial plants (Meena *et al*. 2018) and promote mineral nutrient uptake by host plants (especially P) and soil fertility. Compared with single AMF inoculation, simultaneous inoculation with two rhizotrophic bacteria can significantly improve the yield and P concentration in alfalfa, enhance the ability of plant roots to resist drought (Rodriguez-Caballero *et al*. 2017) and increase the effectiveness of the microorganisms in saving P and increasing crop production (Zhang *et al*. 2014).

At present, research on water and P mainly focuses on the performance of rice and other crops (Song *et al*. 2018), while there are relatively few studies on the effects on the production performance of alfalfa. In particular, there are few reports regarding the effects of simultaneous inoculation with PSB and AMF under water-P coupling conditions on the production performance and roots of alfalfa. Therefore, this study aimed to investigate the effects of water-P coupling on the simultaneous inoculation of PSB and AMF on the growth, nutrient quality and underground biomass of alfalfa to provide a theoretical basis for the development of a rational water and fertilizer management system as well as compound microbial fertilizers for alfalfa cultivation in the Xinjiang oasis region of China.

Materials and Methods

Experimental details and treatments

Experimental site: The pot experiment was conducted in 2018 at the experimental station of the Agricultural College of Shihezi University (44°18′ N, 86°03′ E), Xinjiang, China. The experimental site was located in a temperate continental climate zone that is dry and rainless. The diurnal temperature varied greatly; the mean annual temperature was 11.2–13.9ºC and the annual precipitation was 203.1–394.9 mm. The annual pan evaporation was approximately 1000–1500 mm. The test soil was collected from the experimental station of the Agricultural College of Shihezi University, Shihezi, China. The soil (0–20 cm layer) at the experimental site was a grey desert soil. The collected soil was air-dried and then passed through a 2 cm sieve to remove roots, stones and other fine plants material in the soil and was brought back to the laboratory for the determination of physical and chemical properties. The specific physical and chemical properties of the soil are shown in Table 1.

Treatments: An orthogonal experimental design ($L16(4^3)$) was adopted in this study. There were four levels of moisture concentration, P application and bacterial inoculation in the potted plants (without considering the interactions of various factors). The experimental treatments were the factorial combinations of the different treatment factors. The four-soil water holding capacities were 35% (W_1), 50% (W_2), 65% (W_3), and 80% (W_4). The four P rates were 0 mg kg^{-1} (P_0), 50 mg kg^{-1} (P_1), 100 mg kg^{-1} (P_2) and 150 mg kg^{-1} (P_3). The four inoculation treatments were no inoculation (J_0), *B. megaterium* inoculation (J_1), *F. mosseae* inoculation (J_2), and double inoculation (*B. megaterium* + *F. mosseae*) (J_3). Each treatment was repeated 6 times and the treatments were randomly arranged, as shown in Table 2. To ensure that the test was only affected by the phosphate fertilizer, based on the monoammonium phosphate ($NH_4H_2PO_4$) containing nitrogen fertilizer, the effect of the nitrogen fertilizer on the production of alfalfa was offset by adding urea (CN_2H_4O) to maintain the consistency of the test, as shown in Table 3.

In this experiment, the *B. megaterium* strain was purchased from the Agricultural Culture Collection of China (ACCC). *B. megaterium* can grow and form soluble P circles in NBRIP liquid medium with $Ca_3(PO_4)_2$ as the P source. *F. mosseae* was purchased from Qingdao Agricultural Mycorrhizal Research Institute of China. The inoculum of this fungus was a mixture of spores, hyphae, sand and root segments of its host plants. The density of spores was 25–35 g^{-1}. The alfalfa variety tested was WL354HQ.

The composition of the beef extract peptone liquid medium was: beef extract 5 g L^{-1}, peptone 10 g L^{-1}, NaCl 5 g L^{-1}, agar 30 g L^{-1}, pH 7.0. The composition of Hoagland's nutrient solution was: $Ca(NO_3)_2$ 945 mg L^{-1}, KNO_3 607 mg L^{-1}, $MgSO_4$ 493 mg L^{-1}, iron salt solution 2.5 mg L^{-1}, trace element 5 mg L^{-1}, pH 6.0.

The strains of *B. megaterium* were rejuvenated and inoculated into beef extract peptone liquid medium for propagation. A plate with a colony count of 30–300 was used as the effective counting plate, and the colony count in the bacterial solution was approximately 10^9 cfu mL^{-1} for backup use. The soil was sterilized at high temperature and humidity and was heated at 121ºC and stored.

Table 1: Basic physical and chemical properties of the test soils

Organic matter g kg^{-1}	Alkali-hydrolyzed N (mg kg^{-1})	Total N (g kg^{-1})	Available P (mg kg^{-1})	Total P (g kg^{-1})	Available K (mg kg^{-1})	Field Capacity (%)	Soil bulk Density (g cm^{-3})	pH value
24.9	68.3	1.53	15.7	0.22	132.6	24.2	1.58	7.83

Table 2: Experimental design and implementation plan

Number	Treatments	Soil water holding capacity	Phosphorus application rate	Bacteria
1	$W_1P_0J_0$	W_1 (35%, Severe water shortage)	P_0 (No-phosphorus)	J_0 (No-bacteria)
2	$W_1P_1J_1$	W_1 (35%, Severe water shortage)	P_1 (50 mg kg^{-1})	J_1 (*B. megaterium*)
3	$W_1P_2J_2$	W_1 (35%, Severe water shortage)	P_2 (100 mg kg^{-1})	J_2 (*F. mosseae*)
4	$W_1P_3J_3$	W_1 (35%, Severe water shortage)	P_3 (150 mg kg^{-1})	J_3 (*B. megaterium* + *F. mosseae*)
5	$W_2P_0J_1$	W_2 (50%, Mild water shortage)	P_0 (No-phosphorus)	J_1 (*B. megaterium*)
6	$W_2P_1J_0$	W_2 (50%, Mild water shortage)	P_1 (50 mg kg^{-1})	J_0 (No-bacteria)
7	$W_2P_2J_2$	W_2 (50%, Mild water shortage)	P_2 (100 mg kg^{-1})	J_2 (*F. mosseae*)
8	$W_2P_3J_3$	W_2 (50%, Mild water shortage)	P_3 (150 mg kg^{-1})	J_3 (*B. megaterium* + *F. mosseae*)
9	$W_3P_0J_2$	W_3 (65%, Moderate irrigation)	P_0 (No-phosphorus)	J_2 (*F. mosseae*)
10	$W_3P_1J_3$	W_3 (65%, Moderate irrigation)	P_1 (50 mg kg^{-1})	J_3 (*B. megaterium* + *F. mosseae*)
11	$W_3P_2J_0$	W_3 (65%, Moderate irrigation)	P_2 (100 mg kg^{-1})	J_0 (No-bacteria)
12	$W_3P_3J_1$	W_3 (65%, Moderate irrigation)	P_3 (150 mg kg^{-1})	J_1 (*B. megaterium*)
13	$W_4P_0J_3$	W_4 (80%, Over-irrigation)	P_0 (No-phosphorus)	J_3 (*B. megaterium* + *F. mosseae*)
14	$W_4P_1J_2$	W_4 (80%, Over-irrigation)	P_1 (50 mg kg^{-1})	J_2 (*F. mosseae*)
15	$W_4P_2J_1$	W_4 (80%, Over-irrigation)	P_2 (100 mg kg^{-1})	J_1 (*B. megaterium*)
16	$W_4P_3J_0$	W_4 (80%, Over-irrigation)	P_3 (150 mg kg^{-1})	J_0 (No-bacteria)

Table 3: Amount of fertilizer application (mg kg^{-1})

Treatments	$NH_4H_2PO_4$	$NH_4H_2PO_4$ (Containing P 52%)	$NH_4H_2PO_4$ (Containing N 12.2%)	CN_2H_4O	CN_2H_4O (Containing N 46%)	Total N %
P_0	0	0	0	76.5	35.1	35.1
P_1	96	50	11.7	51	23.4	35.1
P_2	192	100	23.4	25.5	11.7	35.1
P_3	288	150	35.1	0	0	35.1

Note: P_0, P_1, P_2, and P_3 represent 0 mg kg^{-1}, 50 mg kg^{-1}, 100 mg $kg^{-1}$and 150 mg P kg^{-1}, respectively

Large seeds were selected, sterilized with 75% alcohol for 30 s, sterilized with 5% hypochlorite for 12 min, rinsed with sterile water many times and sown in the seedling tray. The seedling tray had a size of 72 holes $plate^{-1}$ and each hole had a diameter of 4 cm. One seed was planted per well. The seeding depth in the seedling pots was 1–2 cm. After the seeds were sown, the bacteria mentioned above were added *B. megaterium* was added in 10 mL volumes to the seedling tray, and *F. mosseae* was spread all around the seeds. On March 24, 2018, the seedling tray was placed in a constant temperature incubator to accelerate germination at 25°C. The culture conditions were as follows: 12 h of light (at 25°C), 12 h of darkness (at 20°C), 300 micromol m^{-2} S^{-1} light intensity, and 55% air humidity. Meanwhile, a black plastic basin of 24 cm × 16 cm × 19 cm (basin diameter × bottom diameter × height) was soaked in alcohol for 20 min and stored. On April 6, 10 seedlings with uniform growth were selected and placed in pot boxes. Five kilograms of sterile soil was added to each pot during transplantation, and the bacteria were added again (as above). Hoagland's solution without phosphoric acid was added every 10 days (100 mL per pot) for each treatment. The specific addition dates were March 24, March 30, April 9, April 19 and April 29, 2018. The application was stopped after adding phosphate fertilizer. The P fertilizer used in this study was monoammonium phosphate (P 52%), which has good water solubility. The P fertilizer was applied together with the irrigation water beginning at the branching period and after each cut. The specific fertilization dates were May 11 and July 4, 2018, and the distance between the pots was 20 cm. The water holding capacity of the pot soil was controlled by the weighing method at 35–80% at 10:00 every morning. Each treatment was repeated 6 times for a total of 96 pots. Each pot was surrounded by supports with a white plastic tarp on them. If it was rainy, the tarp was spread out to prevent the rain from influencing the pot experiment.

Alfalfa biomass

Taking each pot as a unit, 3 pots with the same growth of the 6 pots in each treatment were selected. The first crop was cut on June 30, 2018, and the second crop was cut on August 19, 2018. The alfalfa plants were cut (5 cm) with scissors and weighed, and the yield of fresh alfalfa forage was recorded. The roots of the alfalfa were rinsed and weighed, and the fresh weight was recorded. This was repeated 3 times. The absolute length of each taproot was measured. The aboveground and underground biomass samples were taken back to the laboratory. The sample were first oven-dried at 105°C for 30 min and then at 65°C to a constant mass. The aboveground biomass (g pot^{-1}) and

underground biomass (g pot^{-1}) of alfalfa were calculated according to the following formula.

Aboveground biomass of alfalfa = Fresh yield of alfalfa × (1-moisture concentration) (1).
Underground biomass of alfalfa = Fresh root of alfalfa × (1-moisture concentration) (2).

Plant height

At the same time as the biomass measurement, 10 alfalfa plants with uniform growth were randomly selected in three pots. The vertical height of the alfalfa plants to the surface was measured by a steel tape, and the average height (cm) was calculated.

Stem diameter determination

At the same time as the plant height measurements, the stems of 10 alfalfa plants along the height of the plant were measured, the stem diameter at 5 cm from the ground was measured with a Vernier calliper, and the average values (mm) were determined.

Nutrient quality

Crude protein was determined by the semimicro Kjeldahl method. The neutral detergent fibre and acid detergent fibre were determined according to the procedures of Soest *et al.* (1991).

Phosphorus concentration

The fresh alfalfa grass and root samples were dried and crushed. The samples were placed in a 600°C Maofu furnace to burn to a white ash, and the ash was dissolved by hydrochloric acid. After filtration, the P concentration was determined using the molybdenum-antimony anti-spectrophotometric method (Fan *et al*. 2016). The soil in the pots was removed from the second cut, sieved through a 2 mm sieve and placed in a self-sealing plastic bag for the determination of total phosphorus (TP) and AP in the soil. TP was determined by the sulfuric acid-perchloric acid decoction molybdenum antimony colorimetric method and AP was determined by the $NaHCO_3$ extraction molybdenum antimony colorimetric method (Mehlich 1984).

Taproot length

After the soil was removed from the pots, 10 alfalfa roots from plants whose plant height and stem diameter had been measured were washed with water. The length of the main root was measured by straightening the main root with a steel tape measure and the average taproot length (cm) was determined.

Statistical analysis

Microsoft Excel 2010 was used for data processing, and all the plant data collected were statistically analysed in S.P.S.S. 20.0 using analysis of variance. The obtained results were tested withFisher's least significant difference (Duncan's) test with significance determined at the 5% level. The principal component analysis method was used to identify the best treatment. The principal component analysis method formula is as follows:

$$F_i = \sum_{j=1}^{n} A_{ij}Z_{ij},\ i=1,\ 2,\ 3\ldots n. \quad (3)$$

Where A is the eigenvector value and Z is the standardized value of the alfalfa index for each treatment.

The principal component synthesis model formula is as follows (Tang and Feng 2002):

$$F = \sum_{i=1}^{n} F_i\lambda_i,\ i=1,\ 2,\ 3\ldots n. \quad (4)$$

Where λ_i represents the proportion of the variance contribution rate of the i-th principal component to the total extracted variance contribution rate.

Pearson's correlation analysis was used to analyse the correlation of each growth index of alfalfa to the treatments.

Results

Aboveground biomass, plant height and stem diameter

The aboveground biomass, plant height and stem diameter of alfalfa were significantly higher in all treatments than in the CK treatment ($P \leq 0.05$) and reached a maximum under the $W_3P_2J_0$ treatment (Table 4). The dry matter yield of alfalfa in the W_2, W_3 and W_4 treatments was significantly higher than that in the W_0 treatment ($P \leq 0.05$). The aboveground biomass, plant height and stem diameter of alfalfa first increased and then decreased with increasing P application under the same water holding capacity and reached a maximum under the P_2 treatments in the first cut. The aboveground biomass, plant height and stem diameter of alfalfa were significantly different between the P_2 and P_3 and the P_0 and P_1 treatments under the W_2 and W_4 conditions ($P \leq 0.05$), but there was no significant difference between the P_2 and P_3 and the P_0 and P_1 treatments ($P \geq 0.05$). The aboveground biomass, plant height and stem diameter of the P_2 and P_3 treatments were significantly greater than those of the P_0 treatments under W_3 conditions ($P \leq 0.05$). The aboveground biomass, plant height and stem diameter of alfalfa in the first cut were higher than those in the second cut.

Nutritional quality

The nutritional quality of alfalfa was determined by inoculating PSB and AMF under water-P coupling

Table 4: Production performance of alfalfa under different treatments

Treatments	Dry matter yield (g pot^{-1})		Plant height (cm)		Stem diameter (mm)	
	First cut	Second cut	First cut	Second cut	First cut	Second cut
$W_1P_0J_0$	9.62 ± 0.45k	7.33 ± 0.31h	31.75 ± 0.23i	28.26 ± 0.59g	2.53 ± 0.07h	2.37 ± 0.03g
$W_1P_1J_1$	10.95 ± 0.58j	8.41 ± 0.38g	33.07 ± 0.51h	30.42 ± 0.58f	2.88 ± 0.04def	2.57 ± 0.06ef
$W_1P_2J_2$	11.93 ± 0.30j	9.23 ± 0.30g	35.75 ± 0.50efg	31.03 ± 0.47ef	3.28 ± 0.06b	2.79 ± 0.02c
$W_1P_3J_3$	11.69 ± 0.51j	8.87 ± 0.27g	33.45 ± 0.41h	30.91 ± 0.68ef	3.01 ± 0.02cde	2.72 ± 0.01cd
$W_2P_0J_1$	18.05 ± 0.45hi	14.92 ± 0.58e	34.66 ± 0.37g	32.11 ± 0.45de	2.69 ± 0.09gh	2.42 ± 0.04g
$W_2P_1J_0$	18.71 ± 0.52gh	15.34 ± 0.47e	36.3 ± 0.55def	32.85 ± 1.05d	2.75 ± 0.04fg	2.53 ± 0.03ef
$W_2P_2J_2$	20.09 ± 0.41ef	17.13 ± 0.55bc	40.08 ± 0.48c	36.74 ± 0.92b	3.31 ± 0.06ab	2.95 ± 0.03b
$W_2P_3J_3$	19.24 ± 0.49fg	16.65 ± 0.34cd	36.75 ± 0.35de	34.91 ± 0.20c	3.15 ± 0.05bc	2.74 ± 0.04cd
$W_3P_0J_2$	22.90 ± 0.72cd	16.24 ± 0.44d	42.21 ± 0.54b	36.72 ± 0.62b	2.92 ± 0.03def	2.56 ± 0.04ef
$W_3P_1J_3$	23.50 ± 0.33bc	17.81 ± 0.31b	43.25 ± 0.48b	39.11 ± 0.65a	2.97 ± 0.05de	2.63 ± 0.03de
$W_3P_2J_0$	25.13 ± 0.38a	19.34 ± 0.24a	45.39 ± 0.64a	40.35 ± 0.52a	3.47 ± 0.07a	3.09 ± 0.06a
$W_3P_3J_1$	24.35 ± 0.51ab	18.79 ± 0.45a	44.37 ± 0.72a	39.75 ± 1.13a	3.26 ± 0.04b	2.93 ± 0.07b
$W_4P_0J_3$	17.13 ± 0.44i	13.11 ± 0.42f	35.38 ± 0.47fg	32.33 ± 0.61de	2.87 ± 0.03ef	2.58 ± 0.08ef
$W_4P_1J_2$	18.61 ± 0.40gh	13.69 ± 0.30f	35.65 ± 0.61efg	30.29 ± 0.31f	2.62 ± 0.09gh	2.47 ± 0.04fg
$W_4P_2J_1$	22.34 ± 0.48d	15.36 ± 0.27e	42.91 ± 0.54b	36.25 ± 0.38bc	3.30 ± 0.05ab	2.77 ± 0.04c
$W_4P_3J_0$	20.93 ± 0.42e	14.73 ± 0.38e	37.41 ± 0.75d	31.25 ± 0.31ef	3.06 ± 0.02cd	2.71 ± 0.09cd

Note: Different small letters within the same column indicate significant differences at the 0.05 level

Table 5: Nutrition quality of alfalfa under different treatments

Treatments	Crude protein (%)		Neutral detergent fiber (%)		Acid detergent fiber (%)		P concentration in alfalfa (%)	
	First cut	Second cut	First cut	Second cut	First cut	Second cut	First cut	Second cut
$W_1P_0J_0$	17.29 ± 0.06ij	18.02 ± 0.11f	43.99 ± 0.17a	43.78 ± 0.75a	33.12 ± 0.31ab	32.98 ± 0.62a	0.2130 ± 0.0030h	0.2066 ± 0.0076h
$W_1P_1J_1$	17.56 ± 0.07gh	18.15 ± 0.08def	41.57 ± 0.27bc	40.4 ± 0.69cd	31.54 ± 0.52bcd	31.94 ± 0.55b	0.2467 ± 0.0061de	0.2292 ± 0.0058ef
$W_1P_2J_2$	18.18 ± 0.11cd	18.54 ± 0.17c	40.19 ± 0.52de	41.28 ± 0.54bc	30.47 ± 0.78de	30.88 ± 0.40c	0.2597 ± 0.0016bc	0.2584 ± 0.0025ab
$W_1P_3J_3$	17.87 ± 0.10ef	18.35 ± 0.13cd	39.42 ± 0.27def	40.99 ± 0.44bc	32.22 ± 0.65bc	30.81 ± 0.35c	0.2550 ± 0.0074cd	0.2330 ± 0.0045de
$W_2P_0J_1$	18.05 ± 0.07de	18.38 ± 0.17cd	39.37 ± 0.41ef	39.61 ± 0.38de	32.39 ± 0.48abc	28.87 ± 0.51def	0.2341 ± 0.0052fg	0.2131 ± 0.0033h
$W_2P_1J_0$	18.45 ± 0.13b	18.86 ± 0.11b	38.96 ± 0.41efg	39.49 ± 0.33de	30.52 ± 1.01de	29.03 ± 0.44def	0.2390 ± 0.0031ef	0.2273 ± 0.0058efg
$W_2P_2J_2$	18.87 ± 0.08a	19.19 ± 0.16a	37.98 ± 0.48gh	38.96 ± 0.55ef	30.80 ± 0.88cde	28.26 ± 0.35fg	0.2728 ± 0.0048a	0.2590 ± 0.0068ab
$W_2P_3J_3$	18.34 ± 0.13bc	18.52 ± 0.04c	38.4 ± 0.16fgh	39.43 ± 0.30de	31.09 ± 1.10cde	28.52 ± 0.51efg	0.2648 ± 0.0034abc	0.2480 ± 0.0045bc
$W_3P_0J_2$	17.84 ± 0.11ef	18.23 ± 0.09def	37.57 ± 0.30h	38.78 ± 0.52ef	28.21 ± 0.34fg	27.14 ± 0.52hi	0.2571 ± 0.0045cd	0.2183 ± 0.002fgh
$W_3P_1J_3$	17.96 ± 0.10e	18.33 ± 0.10cde	36.37 ± 0.85i	37.87 ± 0.40fg	26.83 ± 0.82g	26.01 ± 0.30j	0.2681 ± 0.0062ab	0.2329 ± 0.0041de
$W_3P_2J_0$	18.32 ± 0.08bc	18.60 ± 0.18c	35.94 ± 0.14i	37.59 ± 0.55g	27.06 ± 0.88g	26.65 ± 0.48ij	0.2710 ± 0.0045a	0.2673 ± 0.0051a
$W_3P_3J_1$	18.24 ± 0.08bcd	18.39 ± 0.08cd	37.71 ± 1.06gh	38.03 ± 0.68fg	29.67 ± 0.41ef	27.75 ± 0.31gh	0.2692 ± 0.0018ab	0.2548 ± 0.0061b
$W_4P_0J_3$	17.19 ± 0.01j	17.38 ± 0.06h	41.59 ± 0.91bc	41.62 ± 0.37b	30.44 ± 0.48de	29.73 ± 0.37d	0.2279 ± 0.0035g	0.2153 ± 0.0065gh
$W_4P_1J_2$	17.69 ± 0.08fg	17.75 ± 0.11g	40.05 ± 0.34de	41.47 ± 0.48bc	33.90 ± 1.06a	29.63 ± 0.64d	0.2470 ± 0.0045de	0.2302 ± 0.0049def
$W_4P_2J_1$	17.74 ± 0.10fg	18.07 ± 0.06ef	40.67 ± 0.51cd	40.88 ± 0.47bc	30.78 ± 0.47cde	28.98 ± 0.38def	0.2653 ± 0.0034abc	0.2691 ± 0.0076a
$W_4P_3J_0$	17.42 ± 0.08hi	17.54 ± 0.08gh	42.37 ± 0.88b	41.65 ± 0.40b	33.12 ± 0.31ab	29.46 ± 0.54de	0.2573 ± 0.0055cd	0.2420 ± 0.0058cd

Note: Different small letters within the same column indicate significant differences at the 0.05 level

conditions (Table 5). The crude protein and P concentration of alfalfa first increased and then decreased with increasing P application under the same water holding capacity and reached a maximum under the $W_2P_2J_2$ treatment. The crude protein in the W_2 treatments was significantly higher than that in the W_4 treatments ($P \leq 0.05$). The P concentration of alfalfa in the P_2 treatment was significantly higher than those in the P_0, P_1 and P_3 treatments ($P \leq 0.05$). The neutral detergent fibre and acid detergent fibre in the water, P and bacteria treatments were significantly lower than those in the CK treatment ($P \leq 0.05$) and they increased first and then decreased with increasing P application under the W_2, W_3 and W_4 treatments. The crude protein in the first cut was lower than that in the second cut, and the P concentration was higher in the first cut than in highly than the second cut under the different treatments.

Underground biomass and soil phosphorus

The taproot length and underground biomass of alfalfa first increased and then decreased with increasing P application under the same water holding capacity and reached a maximum under the $W_1P_2J_2$, $W_2P_2J_2$ and $W_4P_2J_1$ treatments in the P_2 treatment, except for the $W_2P_1J_3$ treatment in the W_2 treatment (Table 6). The TP and AP increased gradually with increasing P application under the same water holding capacity and reached a maximum under the $W_1P_3J_3$, $W_2P_3J_3$, $W_3P_3J_3$ and $W_4P_3J_0$ treatments in the P_3 treatment. The TP and AP in the P_1, P_2 and P_3 treatments were significantly higher than those in the P_0 treatments ($P \leq 0.05$).

The taproot length and AP of the water, P and bacteria treatments were significantly higher than those of the CK treatment ($P \leq 0.05$), and the highest were $W_3P_1J_3$ and $W_2P_3J_3$, which increased 69.7 and 138.2%, respectively. The taproot length and underground biomass in the W_3 treatments were significantly higher than those in the W_1 treatment ($P \leq 0.05$), and the underground biomass reached a maximum under the P_2 treatment. The taproot length and underground biomass in the J_1, J_2 and J_3 treatments were significantly higher than those in the J_0 treatment ($P \leq 0.05$). The soil pH values in the J_1, J_2 and J_3 treatments were significantly less than those in the J_0 treatment ($P \leq 0.05$).

Table 6: Underground biomass of alfalfa and soil phosphorus concentration under different treatments

Treatments	Taproot length (cm)	Underground Biomass (g pot^{-1})	pH value	Total P (g kg^{-1})	Available P (mg kg^{-1})
$W_1P_0J_0$	21.44 ± 0.48j	6.42 ± 0.31g	7.70 ± 0.01a	0.282 ± 0.016def	16.08 ± 1.09j
$W_1P_1J_1$	24.83 ± 0.81i	7.29 ± 1.22g	7.58 ± 0.06b	0.342 ± 0.010bcd	22.56 ± 1.32fg
$W_1P_2J_2$	26.68 ± 1.19h	8.32 ± 0.82fg	7.44 ± 0.05c	0.339 ± 0.011bcd	32.01 ± 0.78cd
$W_1P_3J_3$	25.87 ± 0.79hi	7.64 ± 0.51g	7.25 ± 0.02de	0.484 ± 0.044a	35.14 ± 0.62ab
$W_2P_0J_1$	29.26 ± 0.66fg	17.16 ± 0.72bc	7.29 ± 0.03de	0.264 ± 0.047ef	18.13 ± 0.35i
$W_2P_1J_0$	31.93 ± 0.47de	16.68 ± 0.95bcd	7.73 ± 0.08a	0.384 ± 0.009bc	21.98 ± 0.45g
$W_2P_2J_2$	34.25 ± 0.99bc	17.21 ± 0.65bc	7.40 ± 0.03c	0.357 ± 0.023bc	33.53 ± 1.07bc
$W_2P_3J_3$	32.87 ± 0.88cd	15.12 ± 0.83cde	7.13 ± 0.03f	0.492 ± 0.033a	36.30 ± 0.33a
$W_3P_0J_2$	35.66 ± 0.68ab	18.60 ± 1.16ab	7.39 ± 0.04c	0.275 ± 0.013ef	20.02 ± 0.93h
$W_3P_1J_3$	36.40 ± 0.54a	20.33 ± 2.05a	7.07 ± 0.03f	0.321 ± 0.024cde	24.28 ± 0.71f
$W_3P_2J_0$	35.80 ± 1.29ab	18.19 ± 1.34ab	7.68 ± 0.06a	0.391 ± 0.031b	29.11 ± 1.36e
$W_3P_3J_1$	33.49 ± 0.50cd	17.64 ± 1.10bc	7.26 ± 0.02de	0.495 ± 0.034a	34.52 ± 1.27ab
$W_4P_0J_3$	29.57 ± 0.45fg	10.28 ± 0.88f	7.09 ± 0.02f	0.245 ± 0.033f	22.19 ± 0.55g
$W_4P_1J_2$	30.88 ± 1.15ef	10.65 ± 0.82f	7.30 ± 0.04d	0.366 ± 0.023bc	22.42 ± 0.51fg
$W_4P_2J_1$	32.80 ± 0.88cd	14.10 ± 1.57de	7.21 ± 0.05e	0.349 ± 0.016bc	31.57 ± 0.37d
$W_4P_3J_0$	28.52 ± 0.55g	13.58 ± 2.04e	7.65 ± 0.08ab	0.518 ± 0.035a	34.49 ± 1.15ab

Note: Different small letters within the same column indicate significant differences at the 0.05 level.

Table 7: Variance analyses of the effects of water, phosphorus and bacteria on the indicators of alfalfa

Factor	W		P		J	
	F-value	Pr > *F*	*F*-value	Pr > *F*	*F*-value	Pr > *F*
Aboveground biomass	1006.335	< 0.001	61.031	< 0.001	3.061	0.113
Plant height	81.331	< 0.001	19.261	0.002	2.378	0.169
Stem diameter	4.431	0.058	27.886	0.001	0.529	0.679
Crude protein	46.543	< 0.001	15.562	0.003	1.284	0.362
Neutral detergent fiber	33.304	< 0.001	4.622	0.053	2.188	0.190
Acid detergent fiber	42.428	< 0.001	5.729	0.034	4.042	0.069
P concentration in alfalfa	19.439	0.002	123.568	< 0.001	4.404	0.058
Taproot length	78.978	< 0.001	7.975	0.016	4.584	0.054
Underground biomass	37.954	< 0.001	0.459	0.721	0.100	0.957
pH value	4.419	0.058	0.561	0.660	36.396	< 0.001
Total P	0.876	0.504	277.791	< 0.001	10.966	0.008
Available P	0.846	0.517	153.617	< 0.001	5.546	0.036

Note: W: soil water holding capacity; P: phosphorus application; J: bacterium inoculated, $P < 0.05$ was significant; $P < 0.01$ was extremely significant.

Variance analysis

The aboveground biomass, plant height, crude protein, neutral detergent fibre, acid detergent fibre, taproot length, and underground biomass were influenced by factors in the order of water > P > bacteria (Table 7). The effects of water, P and bacteria on the stem diameter and P concentration of alfalfa decreased in the order P > water > bacteria; the effects on pH value were bacteria > water > P, and the effects on soil TP and AP were in the order P > bacteria > water.

The soil water holding capacity had a highly significant effect on aboveground biomass, plant height, stem diameter, crude protein, neutral detergent fibre, acid detergent fibre, plant P concentration, taproot length, and underground biomass ($P \leq 0.01$). The P application rate had a highly significant effect on aboveground biomass, plant height, stem diameter, crude protein, plant P concentration, taproot length, underground biomass, TP and AP ($P \leq 0.01$), which had a highly significant effect on acid detergent fibre and taproot length ($P \leq 0.05$). The P application rate had a highly significant effect on soil pH and soil TP ($P \leq 0.01$) and had a significant effect on AP ($P \leq 0.01$).

Pearson's correlation analysis

The plant height, stem diameter, taproot length and underground biomass were significantly positively correlated with the dry matter yield ($P \leq 0.05$) (Table 8). The P concentration in alfalfa was positively correlated with dry matter yield ($P \leq 0.01$) and neutral detergent fibre and acid detergent fibre were significantly negatively correlated with dry matter yield ($P \leq 0.01$). Dry matter yield, plant height, stem diameter and plant P concentration, taproot and underground biomass were positively ($P \leq 0.01$) or significantly negatively correlated with neutral detergent fibre and acid detergent fibre, respectively, while neutral detergent fibre was significantly positively correlated with acid detergent fibre. The stem diameter, P concentration and TP were significantly positively correlated with AP ($P \leq 0.01$), and the other indexes were not significantly correlated with AP ($P \geq 0.01$). The underground biomass was significantly negatively correlated with crude protein ($P \leq 0.01$) and there were no significant correlations between underground biomass and the other indicators ($P \geq 0.05$).

Principal component analysis

Since each treatment performed differently for the different

Table 8: The correlation analysis of each index of alfalfa under different treatments

Index	Above ground biomass	Plant height	Stem diameter	Crude protein	Neutral detergent fiber	Acid detergent fiber	P concentration in alfalfa	Taproot length	Underground biomass	pH value	Total P
Plant height	0.886**										
Stem diameter	0.480	0.685**									
Crude protein	0.323	0.410	0.486								
Neutral detergent fiber	-0.752**	-0.815**	-0.529*	-0.671**							
Acid detergent fiber	-0.810**	-0.904**	-0.517*	-0.411	0.885**						
P concentration in alfalfa	0.541*	0.695**	0.920**	0.523*	-0.569*	-0.503*					
Taproot length	0.935**	0.885**	0.485	0.450	-0.854**	-0.892**	0.572*				
Underground biomass	-0.222	-0.214	-0.068	0.106	0.225	0.245	-0.128	-0.322			
pH value	0.919**	0.817**	0.341	0.498*	-0.826**	-0.842**	0.404	0.901**	-0.161		
Total P	0.194	0.147	0.459	0.199	-0.153	0.038	0.526*	0.074	0.070	0.051	
Available P	0.244	0.326	0.793**	0.336	-0.242	-0.129	0.805**	0.219	-0.207	0.085	0.803*

Note: *Significant correlation was found at the 0.05 level (bilateral), **significant correlation was found at the 0.01 level (bilateral).

Table 9: The principal component analysis of each index of alfalfa under different treatments

Index	Component			Treatments	Synthesis score				Rank
	1	2	3		Y_1	Y_2	Y_3	Y_T	
Aboveground biomass	0.880	0.275	0.076	$W_1P_0J_0$	-3.089	0.119	-0.092	-3.062	16
Plant height	0.938	0.160	0.060	$W_1P_1J_1$	-1.667	-0.134	-0.059	-1.860	15
Stem diameter	0.752	-0.517	0.034	$W_1P_2J_2$	-0.564	-0.387	-0.030	-0.980	13
Crude protein	0.603	-0.176	-0.526	$W_1P_3J_3$	-0.858	-0.469	0.063	-1.265	14
Neutral detergent fiber	0.898	0.217	-0.152	$W_2P_0J_1$	-0.712	0.448	-0.027	-0.290	9
Acid detergent fiber	0.857	0.393	-0.007	$W_2P_1J_0$	-0.032	0.143	-0.218	-0.107	8
P concentration in alfalfa	0.797	-0.525	0.062	$W_2P_2J_2$	1.460	-0.182	-0.081	1.197	5
Taproot length	0.913	0.317	0.074	$W_2P_3J_3$	0.882	-0.270	0.076	0.689	7
Underground biomass	0.842	0.420	-0.121	$W_3P_0J_2$	0.760	0.528	-0.027	1.261	4
pH value	0.229	0.149	0.875	$W_3P_1J_3$	1.667	0.443	0.083	2.193	1
Total P	-0.364	0.777	0.075	$W_3P_2J_0$	2.311	-0.012	-0.135	2.164	2
Available P	0.499	-0.811	0.245	$W_3P_3J_1$	1.783	-0.167	0.048	1.664	3
The eigenvalues of component	6.737	2.438	1.166	$W_4P_0J_3$	-1.255	0.353	0.196	-0.707	10
The cumulative contribution rate (%)	56.144	76.46	86.176	$W_4P_1J_2$	-0.971	0.078	0.059	-0.834	12
				$W_4P_2J_1$	0.699	-0.130	0.132	0.701	5
				$W_4P_3J_0$	-0.412	-0.360	0.009	-0.763	11
				Contribution rate (%)	56.144	20.316	9.716		

indicators, it was not sufficient to evaluate the optimal treatment based on any single indicator. The aboveground biomass, plant height, crude protein, plant P concentration, stem diameter, main root length and underground biomass were positive indicators of plant performance, while neutral detergent fibre, acid detergent fibre, pH value and total P concentration were negative indicators (Table 9). The total accumulation rate was 86.18%. The comprehensive evaluation model was constructed as $Y_T = 0.561Y_1 + 0.203Y_2 + 0.097Y_3$, where Y_T stands for the comprehensive score, and a larger Y comprehensive value indicated a better growth performance in the treatment. The nutritional quality and underground biomass had the greatest impact. The top four treatments were $W_3P_1J_3 > W_3P_2J_0 > W_3P_3J_1 > W_3P_0J_2$.

Discussion

In this study, sufficient irrigation increased the aboveground biomass and plant height of alfalfa. The growth of alfalfa was inhibited when the soil water holding capacity was lower or higher than 65%, and the lower the soil moisture content was, the lower the aboveground biomass and plant height of alfalfa. When the moisture content in the soil is too low, drought stress occurs in the alfalfa plant. Biomass accumulation in plants occurs through photosynthesis, while drought stress inhibits photosynthesis and reduces plant biomass (Fan *et al.* 2016). On the other hand, when the soil moisture is low, alfalfa root growth is inhibited, which reduces the plant's ability to absorb water and nutrients. This prevents the products required by photosynthesis from being synthesized and thereby reduces the alfalfa biomass (Podlaski *et al.* 2017). In this study, the aboveground biomass and plant height of alfalfa at 80% soil water holding capacity were less than those at 65% soil water holding capacity under the same P application rate. The excess moisture in the pots could not spread into the surrounding soil, resulting in the alfalfa roots being immersed in water for a long time. The immersion of alfalfa roots in water obstructs aerobic respiration and enhances anaerobic respiration, which reduces the aboveground biomass of alfalfa (Zhang *et al*. 2020). However, the soil water holding capacity of the 80% treatment was higher than that of the 35% treatment. This is mainly because the potting box is made of plastic; in the sun from July to August, the temperature in the potting boxes is higher than the normal daily temperature. High temperatures increase water evaporation, which leads to a drier soil environment and forces the alfalfa roots to self-recover. It can be

concluded that too high or too low of water content has a negative impact on the aboveground biomass of alfalfa and that a moderate moisture range should be used in the production of alfalfa.

In this study, P application significantly affected the aboveground biomass, plant height, stem diameter and plant P concentration of alfalfa, but it was not true that "the more P, the better". P application significantly increases the amount of chlorophyll in alfalfa leaves, increases the photosynthetic rate of alfalfa, promotes the growth of alfalfa plants, and increases the dry matter yield of alfalfa (Williams *et al*. 2018). However, excessive P application results in a decrease in dry matter quality (Fan *et al*. 2016). There is a certain threshold for the absorption of P by alfalfa plants. When P is below a certain threshold, additional P promotes alfalfa growth and development. When P level exceeds the maximum absorption of P by alfalfa, the dry matter yield of alfalfa plants decreases (Bai *et al*. 2013). Excess P has a negative impact on plant growth and development and can lead to early growth and premature senescence of alfalfa (Fan *et al*. 2016). Therefore, the reasonable application of P fertilizer can improve the alfalfa growth.

Bacterial inoculation effectively promoted alfalfa growth. Compared with the no-bacteria treatment, inoculation with *B. megaterium* improved the soil AP and the alfalfa biomass. Because the AP in soil increased, the alfalfa roots could immediately absorb and utilize it for the growth and development of roots and then transport the nutrients to the aboveground parts; this promoted an increase in the aboveground and underground biomass of plants (Luduena *et al*. 2018). Inoculation with *F. mosseae* formed a symbiont with the roots of alfalfa. Mycorrhizal hyphae can absorb water directly and increase the surface area of the roots. As a result, the use and absorbance efficiency of nutrients and water increases (Parniske 2008), which in turn increases the aboveground and underground biomass of alfalfa. The taproot length and underground biomass under the 50% water treatment were significantly higher than those under the 80% water treatment. This result indicated that inoculation can alleviate the restrictions on alfalfa root length and root biomass under mild drought conditions. Research has shown that inoculation with AMF can improve the growth environment of alfalfa by delaying the ageing of root nodules under drought stress (Kyriazopoulos *et al*. 2014). Therefore, the effect of drought stress on alfalfa roots can be alleviated under mild drought stress. The taproot length and underground biomass of alfalfa in the 50% water treatment were significantly greater than those in the 35% water treatment, and AMF and PSB could not completely offset the inhibition of drought on plants under severe water stress conditions (Rahimzadeh and Pirzad 2017).

P moves through plants in various forms, but its mobility in soil is poor. Alfalfa has the highest P use efficiency, especially in the absence of P or under suitable P conditions in soil. P transfer occurs earlier and more often under low-P stress. A series of changes will also occur in the transfer and distribution of P in alfalfa plants under P stress (Rodríguez *et al*. 2000). This is the reason why the P concentration of alfalfa still increased under water shortage conditions, and why the P concentration in the plants was related to the crude protein concentration. Therefore, the crude protein increases with the increase in the P concentration, and the nutritional quality of alfalfa is improved. The normal growth and development of alfalfa plants were hindered, the water concentration in the alfalfa plants decreased and the lignification degree increased under the severe water shortage conditions (35% water) (Zhang *et al*. 2016); as a result, the neutral detergent fibre and acid detergent fibre increased significantly. Suitable irrigation rate (65% water) provided an adequate water supply for the alfalfa plants, their growth and development were normal, and their neutral detergent fibre and acid detergent fibre decreased. With a further increase in the soil water holding capacity (80% water), the aboveground biomass of the alfalfa plants also increased. The fibre concentration was the highest in the stem, and the stem diameter increased significantly, which led to an increase in the neutral detergent fibre concentration and a decrease in the nutritional quality of the alfalfa.

The effects of AMF and PSB inoculation on the growth, nutritional quality and underground biomass of alfalfa were different under the different water-P coupling conditions. The evaluation of the optimal water, P and bacteria model through only one indicator does not fully explain the advantages and disadvantages of the different treatments. Principal component analysis can be used to evaluate the optimization of multiple indicators by synthesizing multiple indicators (Song *et al*. 2018). The four treatments that had the greatest influence on the production performance of alfalfa were $W_3P_1J_3 > W_3P_2J_0 > W_3P_3J_1 > W_3P_0J_2$. This indicated that the alfalfa performance was the highest when the soil water holding capacity was 65%, the P application rate was 50 $mg \cdot kg^{-1}$, and AMF and PSB were inoculated simultaneously. This treatment effectively improved the aboveground biomass of alfalfa, dissolved more soil TP, promoted the absorption of AP by alfalfa plants, and improved the nutritional quality of alfalfa compared with the other treatments (Meena *et al*. 2018). PSB and AMF play more important functional roles under low P conditions, when PSB can dissolves more P. AMF uses the dissolved P from the PSB to infect roots and form mycorrhizae to improve the root absorption ability, thereby increasing the P concentration and biomass of alfalfa (Smith *et al*. 2004). Under high P conditions, the cells reach a supersaturated state because the P content in the soil is too high; the PSB themselves contain a large amount of P, which inhibits the functions of PSB and AMF (Rahimzadeh and Pirzad 2017). In addition, PSB and AMF improve drought resistance in plant roots under mild water stress, but AMF and PSB cannot completely offset the inhibitory effect of drought on plants under severe stress conditions

(Shi *et al*. 2017). Therefore, only when suitable water and P coupling conditions were selected could the effects of AMF and PSB inoculation improve alfalfa production performance and nutritional quality as well as soil AP.

Conclusion

Compared with those under high P conditions, the effects of inoculation were more beneficial under low P conditions. Severe water stress (35% soil water holding capacity) seriously inhibited the growth of alfalfa. Simultaneous inoculation with *B. megaterium* (PSB) and *F. mosseae* (AMF) effectively alleviated the damage to alfalfa from mild water stress (50% soil water holding capacity), and the double inoculation effect was better than the single inoculation effect. When the soil water holding capacity was 65%, the P application rate was 50 mg kg^{-1}, and AMF and PSB were inoculated simultaneously, it effectively improved the aboveground biomass of alfalfa, dissolved more soil TP, promoted the absorption of AP by alfalfa plants, and improved the nutritional quality of alfalfa compared to the other treatments.

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