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Effects of the applications of phosphorus and potassium fertilizers at different growth stages on the root growth and bioactive compounds of *Salvia miltiorrhiza* Bunge

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Abstract

Dried roots of *Salvia miltiorrhiza* Bunge have been used in Chinese medicine for over 2000 years for treating cardiovascular disease and improving immunity. The products are favored in Asia, Europe, North America, Africa, and Russia. Consequently, increasing the yield and quality of *S. miltiorrhiza* has become a major concern worldwide. Here, we conducted a 2-year greenhouse experiment to investigate the effects of applying phosphorus (P) and potassium (K) fertilizers at different growth stages on root growth and production of bioactive compounds in *S. miltiorrhiza*. The experiment comprised 8 treatments, including 4 each of P and K fertilizers. The P2 treatment greatly enhanced root growth (root length [RL], 43.6 cm; root diameter [RD], 14.4 mm; number of roots [RN], 15.5; shoot dry weight [SDW], 13.82 g per plant; root dry weight [RDW], 15.77 g per plant; and total plant dry weight [TPDW], 29.59 g per plant) and accumulation of bioactive compounds (danshensu [DSS], 0.055%; salvianolic acid B [SAB], 4.50%; cryptotanshione [CTS], 0.056%; tanshinone II A [TSIIA], 0.127%; and total tanshinone [TTS], 0.226%) compared to the other P treatments. The K4 treatment showed improved root growth (RL, 45.9 cm; RD, 11.2 mm; RN, 17.8; SDW, 14.96 g per plant; RDW, 16.80 g per plant; and TPDW, 31.76 g per plant) and bioactive compound accumulation (DSS, 0.52% and SAB, 4.13%) compared to the other K treatments. Conversely, a negative effect was observed in the accumulation of CTS (0.039%), TSIIA (0.055%), and TTS (0.139%). Significantly increased concentrations of CTS (0.052%), TSIIA (0.114%), and TTS (0.213%) were observed in the K1 treatment compared to the other K treatments. The total DSS, SAB, CTS, TSIIA, and TTS yields were, respectively, the highest in the P2 treatment at 8.61, 709.3, 8.87, 19.98, 35.68 mg per plant, and the K4 treatment at 8.41, 693.2, 6.49, 9.18, and 23.42 mg per plant. Further, they were, respectively, the lowest in the P4 treatment at 2.78, 169.7, 1.96, 3.59, and 7.48 mg per plant, and the K3 treatment at 2.95, 194.5, 2.61, 5.08, and 10.17 mg per plant.

Keywords: bioactive compounds; growth stage; phosphorus and potassium fertilizer; root growth; *Salvia miltiorrhiza*.
Abbreviations: RL-root length; RD-root diameter; RN-number of roots; SDW-shoot dry weight; RDW-root dry weight; TPDW-total plant dry weight; HPLC-high performance liquid chromatography; DSS-danshensu; SAB-salvianolic acid B; CTS-cryptotanshione; TSIIA-tanshinone II A; TTS-total tanshinone; ANOVA-analysis of variance; LSD test-least significant difference test; SPSS-Statistical Package for the Social Sciences.

Introduction

Danshen, the root and rhizome of *Salvia miltiorrhiza* Bunge, is one of the earliest, most important, and most commonly used herbal compound in traditional Chinese medicine (TCM). Danshen is used for treating coronary heart disease, including coronary artery spasm, myocardial infarction, and angina pectoris (Lam et al., 2006), in addition to its use in treating hepatitis, chronic renal failure, bone loss (Lin et al., 2006; Wu et al., 2007), hypertension, dysmenorrhea, hepatocirrhosis (Cheng, 2006), neuroasthenic insomnia, and liver fibrosis (Sze et al., 2005). Further, it is used in combination with other herbal ingredients (radix notoginseng (Sanqi) and borneol [bingpian]) for preventing and treating cardiovascular diseases (Liu et al., 1997). Over 50 chemical constituents have been isolated and identified from *S. miltiorrhiza* (Min, 2000), including hydrophilic phenolic acids, such as salvianolic acid B (SAB), danshensu (DDS), rosmarinic acid, lithospermic acid, and caffeic acid, and lipophilic tanshinones, such as tanshinone I, tanshinone IIA (TSIIA), tanshinone II B, and cryptotanshinone (CTS). The phenolic acids in *S. miltiorrhiza* have the following bioactivities: antioxidant, anti-viral, anti-tumor, coagulation, cell protection, anti-ischemia-reperfusion, anti-hypertension, and anti-fibrosis (Kim et al., 2004). The tanshinones exhibit biological activities, including antioxidant, anti-platelet aggregation, coronary flow enhancement, mutagenic activity modulation, myocardium protection against ischemia, antibacterial, anti-tumor promotion, and significant cytotoxicity against human cancer cells (Kang et al., 2004;

Table 1. Effects of the applications P and K fertilizers at different growing stages on the growth of *Salvia miltiorrhiza*.

Test time (year)	Treatments	Root length (cm)	Root diameter (mm)	Number of roots (*n*)	Dry matter (g per plant DW)		
					Shoot	Root	Total plant
2009	P1	[a]41.3 ± 2.20a	12.2 ± 1.40a	10.3 ± 1.30a	14.03 ± 3.30a	13.60 ± 0.21b	27.63 ± 4.47b
	P2	46.3 ± 1.00a	16.3 ± 1.50a	19.3 ± 3.40b	15.73 ± 1.80b	17.13 ± 1.50c	31.68 ± 1.01c
	P3	49.8 ± 3.90a	13.8 ± 0.80a	16.7 ± 1.60b	14.55 ± 0.14b	15.12 ± 1.19bc	29.85 ± 0.86c
	P4	45.6 ± 0.80a	13.8 ± 0.80a	11.4 ± 0.80ab	8.72 ± 1.15 a	6.12 ± 0.29a	14.84 ± 1.44a
	K1	41.3 ± 2.20a	12.2 ± 1.40ab	10.3 ± 1.30a	14.03 ± 3.30a	13.60 ± 0.21ab	27.82 ± 4.47ab
	K2	44.0 ± 0.90b	12.6 ± 0.50b	14.8 ± 0.10bc	10.17 ± 0.14 a	10.71 ± 0.51ab	21.87 ± 0.39ab
	K3	35.8 ± 0.90a	9.1 ± 0.30a	15.7 ± 3.60ab	10.36 ± 0.45a	7.30 ± 0.41a	17.66 ± 0.43a
	K4	48.5 ± 1.60b	13.0 ± 0.90b	22.0 ± 2.80c	16.73 ± 1.59b	19.49 ± 5.19b	36.22 ± 6.80b
2010	P1	32.7 ± 2.43a	6.5 ± 1.60a	6.1 ± 1.24a	7.95 ± 0.38a	7.43 ± 0.41b	16.28 ± 0.79b
	P2	40.9 ± 1.59a	12.5 ± 1.46a	11.8 ± 2.60b	14.08 ± 1.34b	15.42 ± 1.59c	27.50 ± 0.93c
	P3	44.4 ± 3.43a	10.5 ± 0.45a	10.9 ± 1.00b	11.64 ± 0.23b	11.36 ± 0.85bc	23.00 ± 0.79c
	P4	38.1 ± 0.83a	9.0 ± 0.40a	7.9 ± 0.63ab	6.95 ± 1.29a	3.47 ± 0.26a	10.41 ± 0.54a
	K1	32.7 ± 0.43a	6.5 ± 1.00ab	6.1 ± 0.24a	8.95 ± 0.38a	7.43 ± 0.41ab	16.28 ± 0.79ab
	K2	37.6 ± 0.74b	8.4 ± 0.36b	8.9 ± 0.29bc	9.09 ± 0.47a	7.95 ± 0.46ab	17.04 ± 0.92ab
	K3	30.1 ± 0.57a	6.1 ± 0.26a	10.9 ± 1.24ab	9.36 ± 0.12a	4.72 ± 0.35a	14.08 ± 0.47a
	K4	43.3 ± 3.65b	9.3 ± 0.60b	13.5 ± 0.41c	13.19 ± 0.67c	14.11 ± 2.61b	27.30 ± 4.69b
Mean	P1	37.0	9.3	8.2	10.99	10.52	21.51
	P2	43.6	14.4	15.5	13. 82	15.77	29.59
	P3	42.1	12.2	13.8	13.68	13.74	27.42
	P4	41.9	11.4	8.6	7.83	4.79	12.63
	K1	37.0	9.3	8.2	11.49	10.52	22.01
	K2	40.8	10.5	11.8	9.63	930	18.93
	K3	32.9	7.6	13.3	9.86	6.01	15.87
	K4	45.9	11.2	17.8	14.96	16.80	31.76

DW = dry weight; [a]mean ± standard error (n = 8); different letters within each fertilizer treatment (P or K) indicate differences at a 5% significance level among the different treatments. Mean values are the averages of the results of 2 years (2009 and 2010).

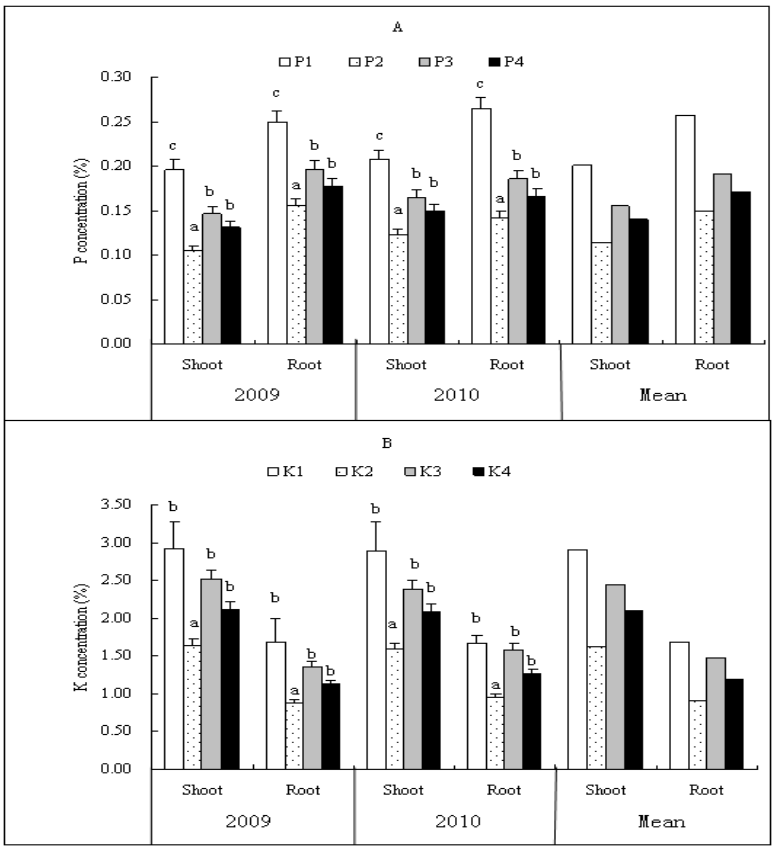

Fig 1. Effects of the applications of P (A) and K (B) fertilizers during the different growth stages on the P and K concentrations (%) in *Salvia miltiorrhiza* tissues. Different letters within each fertilizer treatment (P or K) indicate the differences at a 5% significance level among the different treatments. Mean values are the averages of the results of 2 years (2009 and 2010). Further, the results of 2009 and 2010 years were the mean values of 8 replicates).

Chen et al., 2000). Because of its pharmacological importance, the demand for *S. miltiorrhiza* has increased steadily in recent years. Among the several bioactive components, SAB and TSIIA are used to determining *S. miltiorrhiza* quality (TPC, 2010). DDS and CTS also have important medicinal applications. Approximately 20,000,000 kg of Danshen are required for prescriptions or are exported from China each year (Li et al., 2008). The compound Danshen dropping pill (CSDP) is a registered trademark drug in 34 countries. CSDP is available in mainstream pharmaceutical markets in the Netherlands, South Africa, Russia, South Korea, and 16 other countries as a medicine for treating cardiovascular disease and improving immunity. CSDP has successfully passed the FDAII clinical trials, and the FDAIII trials are underway. Thus, this TCM has become a major international success. The demand for *S. miltiorrhiza* root is increasing, and the plant has been introduced and cultivated in South Korea, Canada, Germany, and other countries (Heuberger et al., 2010). Therefore, production management protocols for the cultivation of high-yield and high-quality Danshen are essential to meet the strong market demand. Fertilizer application has been used to increase the production of numerous medicinal plants, including mint, palmarosa, basil, fennel, and *Bupleurum* spp. (Apiaceae) (Ram et al., 2006; Rajeswara, 2001; Sifola and Barbieri, 2006; Kapoor et al., 2004; Zhu et al., 2009A and 2009B). Phosphorus (P) and potassium (K) are essential elements for plant growth. Fertilizer application significantly influences the biomass yield, active component concentration, and certain morphological traits of medicinal plants. Numerous studies have indicated that P and K fertilizers affect the levels of several secondary metabolites in plants (Grove and Sumner, 1982; Ismail et al., 1994; Rosa et al., 2001). Therefore, the accumulation of bioactive compounds in medicinal crops can be stimulated by exposing the plants to suitable fertilizers. Phosphate is known to influence the root growth and production of secondary metabolites in a large variety of plant species, including photosynthetic carbon metabolism (Anna et al., 2005), morphological and physiological traits (Hans et al., 2006), as well as secondary metabolites in *Salvia officinalis* L. (Monika et al., 2009) and in common bean (*Phaseolus vulgaris*) (Georgina et al., 2007). P also affects the growth, phosphate concentration, and cluster-root formation in 3 *Lupinus* spp. (Ahmad et al., 2010); metabolism regulatory networks in *Arabidopsis* (Renate et al., 2007); and sucrose levels in the phloem of *Arabidopsis* roots (John et al., 2008). Several studies have shown the effect of K on the biomass and secondary metabolites of plants (Partrick et al., 2004). For example, Ashley et al. (2006) reported the effect of K on transport proteins. Anna et al. (2008) revealed that application of K fertilizer had a great impact on the physiologic, metabolic, and hormonal processes of *Arabidopsis thaliana*. Further, Patrick et al. (2009) studied the relationship between K and the metabolism of proteins and amino acids. Some studies in China have focused on the effects of fertilizers on biomass production, root growth, and some bioactive compounds of *S. miltiorrhiza* (Wang et al., 2003; Li et al., 2007; Kirakosyan et al., 2004). However, little is known on the effect of fertilizer application during different growth stages on the root growth and production of bioactive compounds in *S. miltiorrhiza* roots. Therefore, we conducted a 2-year pot experiment in a flat membrane greenhouse to primarily determine the effects of applying P and K fertilizers at different growth stages on the root growth (measured as root length [RL], root diameter [RD], number of roots [RN], and shoot and root biomass production) and the production of DDS, SAB, CTS, TSIIA, and total tanshinone (TTS) in *S. miltiorrhiza*.

Results

Phosphorus and potassium concentrations in plants

The P fertilizer treatments resulted in a generally higher concentration of P in the roots than in the shoots. The mean P concentrations in the roots and shoots were significantly higher by 1.7-fold in the P1 treatment (0.202% and 0.257%), 1.3-fold in P3 (0.156% and 0.191%), and 1.2-fold in P4 (0.141% and 0.172%) than that in P2. The P concentration in the P3 and P4 treatments were significantly lower than that in P1 (Fig. 1A). The K fertilizer treatments resulted in a generally higher concentration of K in the shoots than in the roots. The root and shoot K concentrations were the highest in the K1 treatment, but showed significant increases in K3 and K4 treatments than that in K2. The root and shoot K concentrations were lower in K4 than in K3. The root and shoot K concentrations showed no significant differences between K1, K3, and K4 because of the high variation among the replicates (Fig. 1B).

Effect of P applications during different growth stages on the growth variables and bioactive compound concentrations

The RL increased in the P3 treatment; however, the difference was not statistically significant among the P fertilizer treatments. The RD increased in P2, but again the difference was not significant between the largest root diameters among the P fertilizer treatments. The RL, RD, and RN were the lowest in P1. The RN and shoot and root dry weights (SDW and RDW) were the highest in P2 and were significantly higher than that in the P1 treatment. There were no significant differences between P2 and P3. The overlay of P fertilizer applied during the root swelling stage significantly ($p < 0.05$) decreased the SDW, RDW, and total plant dry weights (TPDW) of *S. miltiorrhiza* compared to the other P treatments (Table 1). The root-to-shoot ratio (R/S) was significantly lower ($p < 0.05$) in the P4 treatment compared to the other P treatments. This finding can be attributed to the fact that the P4 treatment had the lowest RDW among all P fertilizer treatments and a relatively high SDW. R/S was the highest in the P2 treatment, but was not significantly different between the P1, P2, and P3 treatments. The mean R/S was 46% lower in the P4 treatment than in the P2 treatment (Fig. 2). No significant differences were observed in the DDS concentration between the P fertilizer treatments. The CTS, TSIIA, and TTS were significantly reduced in the P4 treatment compared to the other P fertilizer treatments, with no significant differences among P1, P2, and P3 regarding the CTS and TTS concentrations. The SAB and TSIIA concentrations were significantly higher in the P2 treatment than in the other P fertilizer treatments; however, no significant differences in the SAB concentrations were observed among the P1, P3, and P4 treatments. Further, no significant difference was observed in the TSIIA concentration between the P1 and P3 treatments (Figs. 3A and 3C). Statistical analyses of the root characteristics and bioactive compounds for the P fertilizer treatments showed that the P2 treatment of 0.4 g P per plant as basic fertilizer improved growth (RL = 43.6 cm; RD = 14.4 mm; RN = 15.5; SDW = 13.82 g per plant; RDW = 15.77 g per plant; and

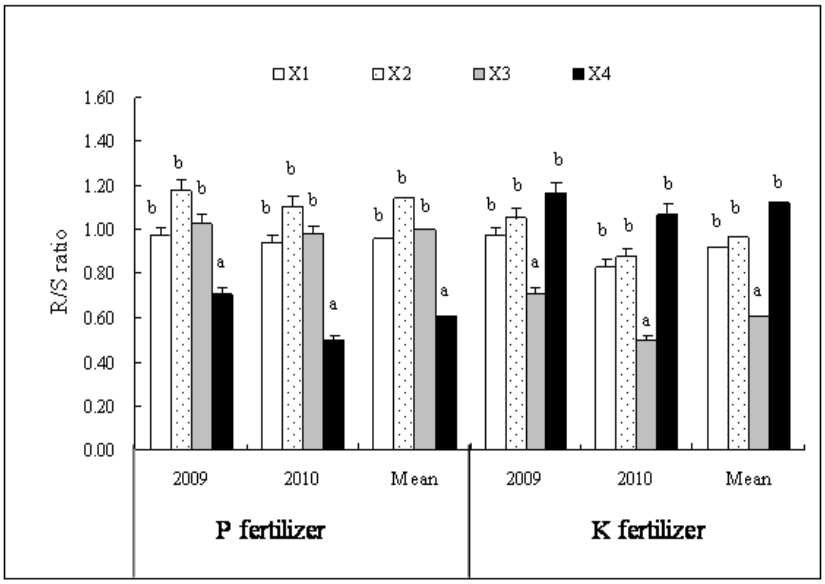

Fig 2. Effects of the application of P and K fertilizers during the different growth stages on the root-to-shoot ratio of *Salvia miltiorrhiza* (as basic fertilizer, X1 = 0.8 g P or 2.5 g K per plant; X2 = 0.4 or 0.8 g P or 1.25 g K per plant; X3 = 0.4 g P or 1.25 g K per plant with 0.4 g P or 1.25 g K per plant overlay applied during the flowering stage; and X4 = 0.4 g P or 1.25 g K per plant with 0.4 g P or 1.25 g K per plant overlay applied during the root swelling stage). Different letters within each fertilizer treatment (P or K) indicate the differences at a 5% significance level among the different treatments. Mean values are averages of the results of 2 years (2009 and 2010). Further, the results of 2009 and 2010 were the mean values of 8 replicates.

TPDW = 29.59 g per plant) and accumulation of bioactive compounds (DSS = 0.055%; SAB = 4.50%; CTS = 0.056%; TSIIA = 0.127%; and TTS = 0.226%) in the roots of *S. miltiorrhiza* compared to the other P fertilizer treatments in this study.

Effect of K applications during different growth stages on growth variables and bioactive compound concentrations

The root characteristics (RL, RD, RN, SDW, RDW, and TPDW) were the highest in the K4 treatment. With the exception of RN, the remaining values were the lowest in the K3 treatment. Further, these parameters differed significantly between the K3 and K4 treatments ($p < 0.05$). No significant differences were observed for SDW, RDW, and TPDW among the K1, K2, and K3 treatments (Table 1). The R/S ratio was significantly lower ($p < 0.05$) in the K3 treatment than in the other K fertilizer treatments, but was the highest in the K4 treatment. The mean R/S ratio was 50% lower in K3 than in K4. This finding can be attributed to the fact that K3 had the lowest RDW among all the K fertilizer treatments and a relatively high SDW. No significant differences were observed in the R/S ratio among the K1, K2, and K4 treatment (Fig. 2). No significant differences in the DDS concentrations were observed among the K fertilizer treatments. The CTS concentration in the K4 treatment was lower than that in the other K treatments and was significantly lower than that in the K1 and K2 treatments. The TSIIA and TTS concentrations were significantly lower in the K4 treatment than in the other K fertilizer treatments. However, the TSIIA concentration was significantly lower in the K3 than in K1 and K2 treatments, whereas the TTS concentration was significantly higher in the K1 than in K2 and K3 treatments. The SAB concentration was significantly lower in the K3 than in the other K treatments with no significant differences among the K1, K2, and K4 treatments (Figs. 3B and 3C). Analysis of the root characteristics and bioactive compounds among the K fertilizer treatments showed that in the K4 treatment of 1.25 g K per plant and 1.25 g K per plant overlay during the root swelling stages showed increases in the root growth (RL = 45.9 cm; RD = 11.2 mm; RN = 17.8; SDW = 14.96 g per plant; RDW = 16.80 g per plant; and TPDW = 31.76 g per plant) and accumulation of bioactive compounds (DSS = 0.52% and SAB = 4.13%). The K4 treatment negatively affected the accumulation of CTS (0.039%), TSIIA (0.055%), and TTS (0.139%) in the roots of *S. miltiorrhiza*. The K1 treatment of 2.5 g K per plant had a greater effect on the accumulation of CTS (0.052%), TSIIA (0.114%), and TTS (0.213%).

Effect of P and K applications at different growing stages on the bioactive compound yield

The total DSS, SAB, CTS, TSIIA, and TTS yields were determined by multiplying the bioactive component concentrations in the roots by the root dry weights. The results showed that the application of P and K at different growing stages had a significant effect on the yields (Fig. 4). The total DSS, SAB, CTS, TSIIA, and TTS yields were the highest in the P2 treatment of 0.4 g P per plant with, respective, yields of 8.61, 709.3, 8.87, 19.98, and 35.68 mg per plant. These compound yields were also high in the K4 treatment of 1.25 g K per plant with 1.25 g K per plant overlay applied during the root swelling stage with, respective, yields of 8.41, 693.2, 6.49, 9.18, and 23.42 mg per plant. The yields were the lowest in the P4 treatment of 0.4 g P per plant with 0.4 g P per plant overlay applied at the root swelling stages with, respective, yields of 2.78, 169.7, 1.96, 3.59, and 7.48 mg per plant and in the K3 treatment of 1.25 g K per plant with 1.25 g K per plant overlay applied at the flowering stages with, respective, yields of 2.95, 194.5, 2.61, 5.08, and 10.17 mg per plant. The DDS and CTS yields were significantly higher in the P2 treatment than in the P1 and P4 treatments. The SAB, TSIIA, and TTS yields were significantly higher in the P2 than in P1, P3, and P4 treatments. We also noted that the total bioactive compound

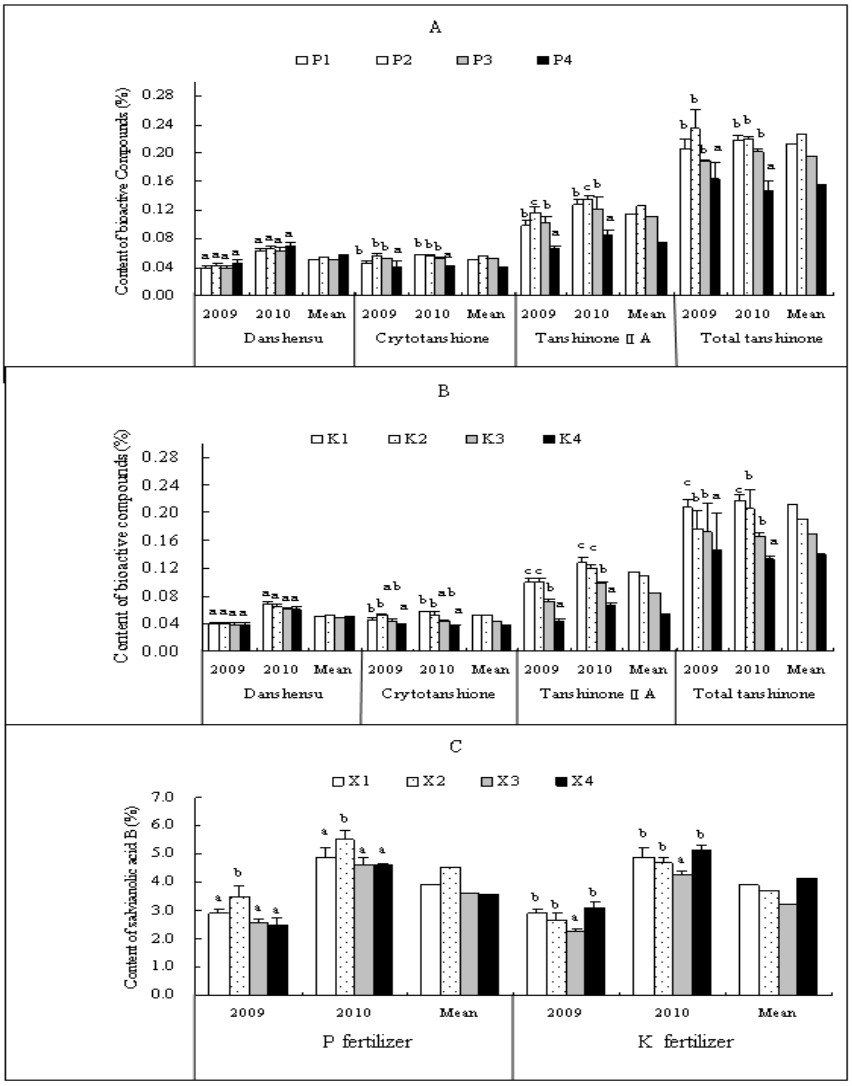

Fig 3. Effects of the applications P and K fertilizers during the different growth stages on bioactive compound concentrations (%) in the roots of *Salvia miltiorrhiza* (as basic fertilizer, X1 = 0.8 g P or 2.5 g K; X2 = 0.4 or 0.8 g P or 1.25 g K per plant; X3 = 0.4 g P or 1.25 g K per plant with 0.4 g P or 1.25 g K per plant overlay applied during the flowering stage; and X4 = 0.4 g P or 1.25 g K per plant with a 0.4 g P or 1.25 g K per plant overlay applied during the root swelling stage). Different letters within each fertilizer treatment (P or K) indicate the differences at a 5% significance level among the different treatments. Mean values are the averages of the results of 2 years (2009 and 2010). Further, the results of 2009 and 2010 were the mean values of 8 replicates.

(DSS, SAB, CTS, TSIIA, and TTS) yields were significantly lower in P4 compared to the other P fertilizer treatments ($p < 0.05$) (Figs. 4A and 4C).Table 1 and Figure 3 showed that both the RDW and bioactive compound concentrations increased in the P2 treatment, resulting in an increase in the total bioactive compound yield, whereas the low root dry weight and bioactive compound concentrations in P4 treatment significantly decreased the total ioactive compound yield. The total DDS and SAB yields significantly increased in the K4 treatment, but remarkably decreased in the K3 treatment compared with the other K fertilizer treatments. The total CTS, TSIIA, and TS yields showed no significant differences among the K1, K2, and K4 treatments (Figs. 4B and 4C). The results of the combined root biomass and bioactive compound concentrations and yield (Table 1; Figs. 3 and 4) can be explained by the high RDW in K4. Consequently, a significant increase in the total bioactive compound yield was observed, especially for CTS, TSIIA, and TTS. However, the K3 treatment showed the lowest yield for total bioactive compounds, which is associated with the significant decrease in RDW.

Discussion

Effects of P application at different growth stages on the growth variables and bioactive compound concentrations and yields

The bioactive compound concentration is one of the most important criteria for determining the quality of *S. miltiorrhiza* (Li et al., 2008). Numerous factors affect the bioactive compound concentrations of *S. miltiorrhiza* root, including growing conditions, harvest time, individual plant characteristics, cultivation methods, and fertilization (Zhu et al., 2009A). Many studies indicate that environmental stressors, such as nutrient deficiency, fertilizer overage, and water stress, affect the level of several secondary metabolites in plants (Kirakosyan et al., 2004). Therefore, accumulation

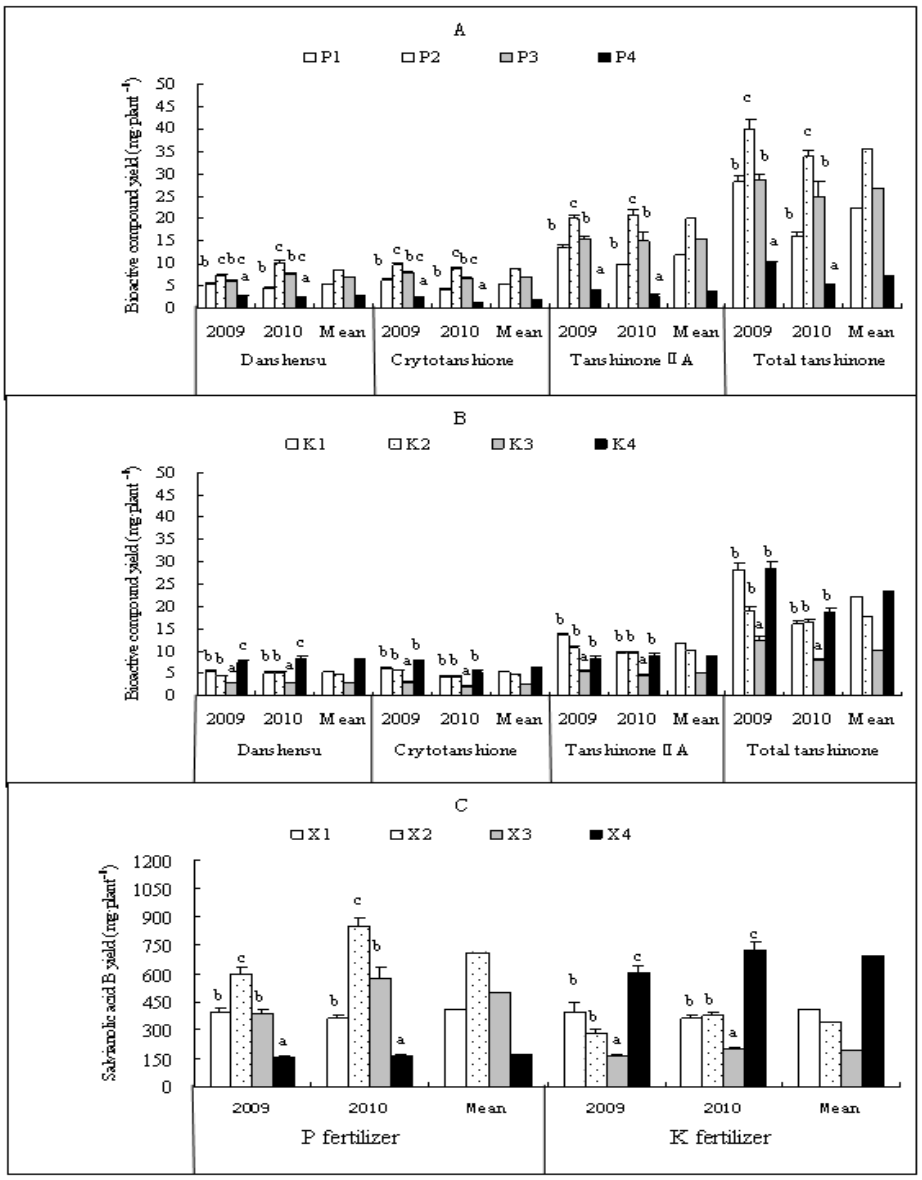

Fig 4. Effect of P and K applications during different growth stages on the total bioactive compound yield (mg per plant dry weight [DW]) in roots of *Salvia miltiorrhiza* (as basic fertilizer, X1 = 0.8 g P or 2.5 g K per plant; X2 = 0.4 or 0.8 g P or 1.25 g K per plant; X3 = 0.4 g P or 1.25 g K per plant and 0.4 g P or 1.25 g K per plant for topdressing at the flowering stage; X4 = 0.4 g P or 1.25 g K per plant and 0.4 g P or 1.25 g K per plant for topdressing at the root swelling stage). Different letters within each fertilizer treatment (i.e., P or K) indicates different values at 5% significance among the different fertilizer treatments. Mean: the averages of the results of 2 years (2009 and 2010); further, the results of 2009 and 2010 were the mean values of 8 replicates.

of bioactive compounds in medicinal crops can be stimulated by exposing the plants to environmental stress. In the present study, we investigated the effects application of P and K fertilizers during different growth stages on growth variables and bioactive compound concentrations and yields.

Our results showed that the application of 0.4 g P per plant (P2) significantly increased the root growth and accumulation of bioactive compounds in the roots of *S. miltiorrhiza* compared to the application of 0.8 g P per plant (P1) and 0.4 g P per plant with 0.4 g P per plant overlay applied at the flowering or root swelling stages (P3 and P4). The P concentrations in the shoot and roots were the lowest in the P2 treatment, whereas the largest concentrations of P were present in P1, followed by P3 and P4 treatments. These results are consistent with those of previous studies in which the root growth and secondary metabolite concentrations increased in P-deficient plants (John et al., 2009; Lei et al., 2011). Excessive fertilizer applications can result in decreased concentrations of medicinal compounds (Zhu et al., 2009B). For example, Georgina et al. (2007) reported a significant increase in the R/S ratio and secondary metabolite concentrations in P-deficient roots compared with P-sufficient roots of the common bean *(P. vulgaris)*. Anna (2005) reported a P-deficient plant with high photosynthetic carbon metabolism. Some studies have shown that P-deficient stress results in the increase of certain secondary metabolite concentrations in *S. officinalis* L. and *Arabidopsis* (Monika et al., 2009; Renate et al., 2007), as well as in the root phloem of *Arabidopsis* (John et al., 2008). The P concentration in plants greatly influences root growth, morphological and physiological traits (Hans et al., 2006), as well as root-cluster formation in 3 *Lupinus* species (Ahmad et al., 2010). The R/S ratio increased significantly in low P soils, and the root morphological and physiological traits differed

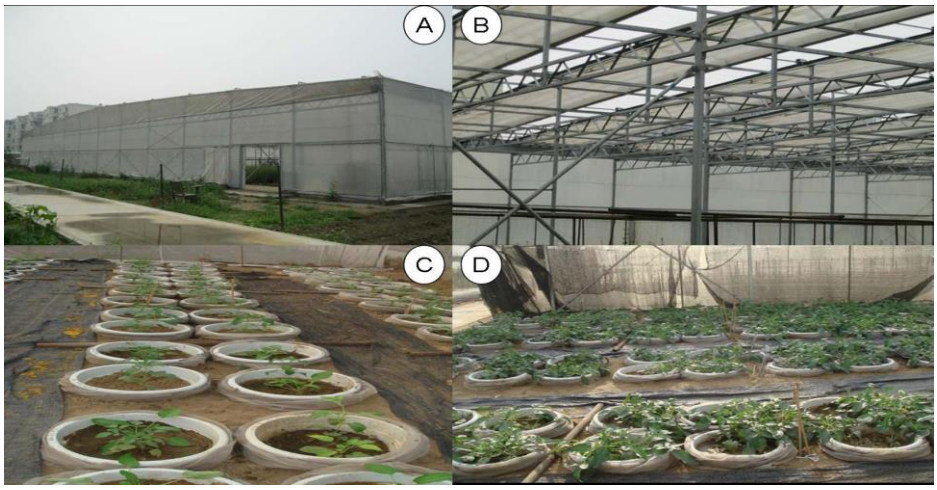

Fig 5. Environment and planting design of greenhouse experiments performed on *Salvia miltiorrhiza* in Beijing (photo credits: L. Lilan). (A & B = flat membrane greenhouse, C & D = design and positioning of the *S. miltiorrhiza* pots).

Fig 6. Photographs of harvested *Salvia miltiorrhiza* roots after P and K fertilizer treatments (photo credits: L. Lilan). (A = P1, B = P2, C = P3, D = P4, E = K1, F = K2, G = K3, and H = K4).

from the control and plants grown in sufficient or excess P fertilizer conditions. Secondary metabolites are the products of bioactive compounds that are produced by plants in stressed environments and are affected by several factors (Kirakosyan et al., 2004). In the present study, the roots of *S. miltiorrhiza* in the P-deficient soils of the P2 treatment accumulated higher levels of bioactive compounds than that in the high P available P1, P3, and P4 treatments. The results also showed that the P3 treatment of 0.8 g P per plant applied as 0.4 g P per plant with 0.4 g P per plant overlay during the flowering stage had a more advantageous influence on the root growth and accumulation of bioactive compounds than that in the P1 treatment of 0.8 g P per plant and P4 treatment of 0.4 g P per plant with 0.4 g P per plant overlay applied during the root swelling stage. The results can be associated with the application of P fertilizer during the growth stages of *S. miltiorrhiza*. At the flowering stage of *S. miltiorrhiza*, P fertilizer application simulated root growth and helped to meet the nutritional requirements needed to produce the bioactive compounds. This finding was not in agreement with a previous study by Li et al. (2007), in which the bioactive components increased significantly upon application of the P fertilizer during the late growth stage in field experiments because of the development of the periderm, cortex, and phloem in the roots. One explanation is that a genetic factor in germplasms and differences in the cultivation environment may affect the bioactive compound production in response to external environment changes, thereby resulting in bioactive component concentration differences. Another explanation is that in this experiment, the P overlay was applied in concert with the growth stages of *S. miltiorrhiza*. We compared the effect of the application of P fertilizers at the flowering and root swelling stages on the root growth and bioactive compound concentrations. However, in the field experiment of Li et al. (2007), the effect different concentrations of P fertilizers had on the root growth and bioactive compound concentrations was analyzed. The period, mode, and amounts of fertilizer differed between the 2 experiments, resulting in significant differences in the time and degree of root growth and bioactive compound production in response to P

fertilization. The total bioactive compound yield was determined by multiplying the bioactive component concentration in the roots by the RDW. Similarly, the RDW and bioactive compound concentrations both increased in the P2 treatment, resulting in increased total bioactive compound yield. In contrast, the low root dry weight and bioactive compound concentrations in the P4 treatment resulted in a significant decrease in the total bioactive compound yield.

Effect of K application at different growth stages on growth variables and bioactive compound concentrations and yields

The K4 treatment of 1.25 g K per plant with 1.25 g K per plant overlay applied during the root swelling stages improved the root growth as well as the DDS and SAB concentrations, but negatively affected the accumulations of CTS, TSIIA, and TTS in the roots of *S. miltiorrhiza*. The K1 treatment of 2.5 g K per plant had a positive effect on the accumulations of CTS, TSIIA, and TTS. In the K fertilizer treatments, the plant concentrations of K were highest in K1, followed by K3, K4, and K2 treatments. K deficiency stress resulted in significant root swelling and enhanced the root biomass because shoot growth was inhibited by the low K nutritional supply, and plant growth was mainly concentrated in roots (Aina et al., 2010). The K overlay during the root swelling stages (K4) supplied sufficient K for the plant, and accordingly, the roots grew rapidly. Hydrophilic phenolic acids such as DDS and SAB are not very stable and not prone to accumulate in the roots of *S. miltiorrhiza* (Lin et al., 2006). Thus, the application of K fertilizer at the root swelling or late stage stimulated the secondary metabolite concentration of hydrophilic phenolic acids to increase greatly. These results were consistent with the report of Li et al. (2007), which indicated that the application of K fertilizer at the late stage of *S. miltiorrhiza* improves root growth, root biomass, and SAB concentration. K is an important macronutrient for plants and is needed for vital functions in metabolism, growth, and stress adaptation (Ismail et al., 1994; Rosa et al., 2001). The known functions of K in solute transport, protein synthesis, and enzyme activation indicate a close relationship between K and metabolism. K deficiency affects the metabolite concentrations in crops, with negative consequences for nutritional quality and mechanical stability (Patrick et al., 2009). Several studies have shown that the application of sufficient K fertilizer in plants improves secondary metabolite accumulations, such as protein, carbon metabolites, and stable plant secondary metabolites. For example, Ashley et al. (2006) reported that sufficient K increases the amount of transport proteins in plants. Increased concentrations of soluble sugars are found in the leaves of K-deficient bean (*P. vulgaris*), cotton (*Gossypium hirsutum*), and soybean (*Glycine max*) (Cakmak et al., 1994; Bednarz et al., 1999; Pettigrew, 1999; Huber et al., 1984). Anna et al. (2008) demonstrated that moderate K availability improves the physiological, metabolic, and hormonal processes of *A. thaliana*, and increases the corresponding secondary metabolite concentrations. Patrick et al. (2004) showed a close relationship between K and metabolism (proteins and amino acids), and that sufficient K availability increases the production of proteins, amino acids, and physiological metabolites in plants. Although very low bioactive compound concentrations resulted from the K4 treatment, especially CTS, TSIIA, and TTS, the large RDW in the treatment resulted in a significant increase in the total bioactive compound yield. Despite the high concentration of total bioactive compounds in the K3 treatment, the total yield was low when compared with the K4 treatment. The distinct decrease in the RDW caused a significant decline in the total bioactive compound yield of *S. miltiorrhiza*.

Materials and methods

Plant material

The experiment was performed using a completely randomized block design of 8 treatments with 4 replicates each. The greenhouse experiment was conducted from May 2009 to November 2010 in a flat membrane greenhouse at the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences (CCAMS) and Peking Union Medical College. Germplasm from the production area in Anguo, Hebei province, China, namely the violet flower *S. miltiorrhiza*, was authenticated by Professor Wei Jianhe of IMPLAD, CCAMS. The experimental plastic pots (diameter, 25 cm; height, 40 cm) were filled using a mixture of soils (30 kg per pot) containing basic fertilizers and low-nutrient river-washed sand-soil. The river-washed sand-soil had a soil-to-sand ratio of 3:1 (v/v); the sand-soil was filtered through a 0.5-cm mesh sieve. The chemical properties of the original sand-soil were as follows: 10.16 $g \cdot kg^{-1}$ organic matter concentration; 26.35 $mg \cdot kg^{-1}$ available N; 12.85 $mg \cdot kg^{-1}$ available P; and 65.20 $mg \cdot kg^{-1}$ available K. The germplasm lines were planted in a field at IMPLAD, CAMS, Beijing, China by using conventional cultivation methods on April 28, 2009 and April 28, 2010, respectively. After 1 month, the *S. miltiorrhiza* seedlings were transplanted into the experimental pots on May 28, 2009 and 2010, respectively. Each pot contained 2 seedlings, and the pots were arranged in a flat membrane greenhouse (Fig. 5). The plants were maintained in a rain shelter with sufficient sunlight. The soil moisture was maintained at 60% (w/w) during the experiment. The treatments continued until the plants were harvested on November 20, 2009 and 2010, respectively. The harvested *S. miltiorrhiza* plants subjected to the fertilizer treatments are shown in Figure 6. The root parameters (RL, RD, and RN) were determined; the shoots (leaves and stems) and roots were then separated and dried at 60 °C for 72 h, weighed, and ground and passed through a 0.3-mm sieve.

Fertilizer treatments

Four treatments each for P and K fertilizers were conducted (total, 8 treatments) during this study. The basic P fertilizer treatments were as follows: P1, 0.8 g P per plant; P2, 0.4 g P per plant; P3, 0.4 g P per plant with 0.4 g P per plant overlay applied during the flowering stage (September 1, 2009 and 2010, respectively); and P4, 0.4 g P per plant with 0.4 g P per plant overlay applied during the root swelling stage (October 1, 2009 and 2010, respectively). All P fertilizer treatments were accompanied by 2.5 g K per plant basic K fertilizer treatments. The basic K fertilizer treatments were as follows: K1, 2.5 g K per plant; K2, 1.25 g K per plant; K3, 1.25 g K per plant with 1.25 g K per plant overlay applied during the flowering stage; and K4, 1.25 g K per plant with 1.25 g K per plant overlay applied at the root swelling stage. All K fertilizer treatments were accompanied by 0.8 g P per plant basic P fertilizer treatments. All P and K fertilizer treatments were applied as 3 g N per plant (2/3 (N) as basic fertilizer and 1/3 (N) for the overlay applied a month after transplantation). N was applied as calcium nitrate ($Ca(NO_3) \cdot 4\ H_2O$), P as sodium dihydrogen phosphate (NaH_2PO_4), and K as potassium sulfate (K_2SO_4).

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Fisher Scientific (NJ, USA). Deionized water was purified using a MilliQ system (Millipore, Bedford, MA, USA). Analytical-grade phosphoric acid and ethanol were purchased from Beijing Beihua Fine Chemicals Co., Ltd. (Beijing, China). Authentic standards of DDS, SAB, TSIIA, and CTS were purchased from the National Institute for Control of Biological and Pharmaceutical Products (Beijing, China).

Apparatus and chromatographic conditions

A Waters 1525 HPLC system (Waters Technologies, Milford, MA, USA) equipped with a binary solvent delivery system, online degasser, Waters 717plus autosampler, column temperature controller, and Waters 2457 dual γ-absorbance detector coupled with analytical workstation was used to perform the HPLC analysis. The HPLC fingerprint was carried out on a C18 column by using a Waters Symmetry C18 reverse-phase column (Waters; Symmetry C18, 250 mm × 4.6 mm; pore size, 5 μm), with a sample injection volume of 10 μL. The chromatographic conditions for the hydrophilic and lipophilic components were as follows: detection wavelength at 281 nm, flow rate of 1.0 $mL \cdot min^{-1}$, and column temperature of 30 °C. Gradient elution by using solvents A (acetonitrile) and B (0.05% v/v aqueous phosphoric acid) was performed as follows: 0–10 min, 5%–14% A (v/v); 10–12 min, 14%–17% A (v/v); 12–16 min, 17%–19% A (v/v); 16–20 min, 19%–25% A (v/v); 20–35 min, 25% A (v/v); 35–45 min, 25%–75% A (v/v); 45–55 min, 75%–90% A (v/v); 55–60 min, 90%–5% A (v/v); and 60–70 min, 5% A (v/v). The system was then restored to its initial condition after 10 min. A UV 1100 UV–Vis spectrophotometer from Shanghai Tianmei Instrument Co., Ltd. (Shanghai, China) was used to analyze the TTS concentration. The ultraviolet spectrophotometer was set at a detection wavelength of 270 nm.

Sample preparation for HPLC quantitation

Individual samples (0.500 g each) of DDS, SAB, TSIIA, and CTS were accurately weighed and suspended in 70% methanol, and then extracted by refluxing at 80 ± 2 °C for 1 h. After cooling to room temperature, the methanol lost during the reflux process was restored; the extracted solution was filtered through a 0.45-μm membrane , and 10 μL was injected into the HPLC system. Individual samples (0.500 g) of TTS were accurately weighed and soaked in 5 mL of ethanol at 4 °C overnight, and then extracted in an ultrasonic bath at room temperature for 20 min. After centrifugation at 2856 ×*g* for 20 min, 0.25 mL of the supernatant was diluted using 3.5 mL of ethanol and analyzed using an ultraviolet spectrophotometer at a detection wavelength of 270 nm. The P and K concentrations in the plant tissues were determined by an inductively coupled plasma emission spectroscopy system (ICPE-9000, Japan). Approximately 0.2 g of dried and ground samples were ashed at 600 °C for 4 h in a muffle furnace. The ash was digested by adding 1.0 mL of 6 M HCl for 1 h and 40 mL of a double-acid solution of 0.0125 M H_2SO_4 and 0.05M HCl for an additional 1 h. The digested solution was filtered using a grade 2V Whatman filter paper and analyzed.

Preparation of standard solutions for HPLC quantitation

Separate standard solutions of DDS, SAB, TSIIA, and CTS containing 0.155 $mg \cdot mL^{-1}$ DSS, 0.398 $mg \cdot mL^{-1}$ SAB, 0.201 $mg \cdot mL^{-1}$ TSIIA, and 0.201 $mg \cdot mL^{-1}$ CTS were prepared by dissolving the chemicals in 70% methanol. Approximate aliquots of 0.185-mL DSS, 5-mL SAB, 0.270-mL TSIIA, and 0.100-mL CTS of the stock solutions were separately transferred to 10-mL volumetric flasks, and the final volume was increased to 10 mL by using 70% methanol. The HPLC system was calibrated using DSS, SAB, TSIIA, and CTS standard solutions at injection volumes of 0, 2, 10, 20, 40, 70, and 100 μL. A standard solution of TTS containing 0.120 $mg \cdot mL^{-1}$ TSIIA was prepared by dissolving the chemicals in ethanol. Approximately 0.100, 0.200, 0.400, 0.600, 0.800, and 1.000 mL of TSIIA aliquots of the stock solution were separately transferred to 10-mL volumetric flasks. The final volume was increased to 10 mL by using ethanol and analyzed using an ultraviolet spectrophotometer at a detection wavelength of 270 nm.

Validation of quantitative methods

Method precision and repeatability were evaluated by successive analysis of 5 replicates of the same powder sample. The relative standard deviations were 0.1%–3% for the retention time (t_R). The stability of the sample solutions was determined by analyzing the samples 0, 2, 4, 8, 16, and 24 h after their preparation. The precision was less than 3% for all 5 analytes. The analytical method was accurate and showed overall recoveries from 95.1% to 102.5% for the tested analytes.

Statistical analysis

This experiment was performed using a completely randomized block design of 8 treatments with 4 replications each. The results were analyzed using analysis of variance (ANOVA) followed by the least-significant difference (LSD) test. Significant differences among fertilizer treatments were assessed at the 5% level by using means from the 4 replications over 2 years. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS Institute Inc., USA).

Conclusion

The effects of the applications of P and K fertilizers at different growth stages on the root growth and bioactive compounds were shown to vary greatly. These results have important implications in the recommended amounts of P and K fertilizers applied at the proper growth stages for the cultivation of *S. miltiorrhiza.*

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