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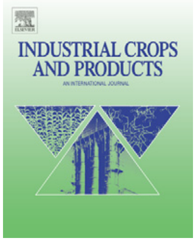

Short communication

Impact of fertilization on drought response in the medicinal herb *Bupleurum chinense* DC.: Growth and saikosaponin production

Zaibiao Zhu[a,b], *Zongsuo Liang*[a,*], *Ruilian Han*[a], *Xin Wang*[c]

[a] *Research Center of Soil and Water Conservation and Ecological Environment, Chinese Academy of Sciences and Ministry of Education, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, PR China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, PR China*
[c] *Hebei Chemical & Pharmaceutical College, Shijiazhuang, Hebei 050026, PR China*

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ABSTRACT

Bupleurum chinense DC. is a plant with high medicinal value. Its roots have been used in Chinese medicine for at least 2000 years. Environmental stress has been used as a strategy to optimize yield of important compounds in other crops. The objective of this pot study was to investigate the combined effects of fertilizer and water-stress on total saikosaponin a (SSa) and saikosaponin d (SSd) yield from *B. chinense*. The 2 × 3 factoral design included two levels of water-stress and three fertilizer amounts. The results showed that mild water-stress significantly increased the SSa and SSd content in *B. chinense* roots, but decreased root biomass. Total SSa and SSd yield were lower in the water-stressed treatment compared to the well-watered treatment. There was significant interaction between the water and fertilizer treatments and the negative effect of water-stress on total SSa and SSd yield could be partly mitigated through the application of N and P fertilizer. In conclusion, results from this study show that the application of proper amounts of fertilizer are important for medicinal plant production in semi-arid and arid regions, and that it is possible to increase total SSa and SSd yield through the combined use of fertilizer and properly timed exposure to water-stress.

1. Introduction

Bupleuri Radix, which are dried roots of *Bupleurum* spp. (Apiaceae), is one of the most common components of Chinese traditional medicine prescriptions for the treatment of chronic hepatitis, kidney syndrome, inflammatory diseases, and ulcers of the digestive system (Pistelli et al., 1996; Guo et al., 2000). It is believed that saikosaponins are responsible for part of the pharmaceutical properties of Bupleuri Radix (Zhu et al., 2006) and saikosaponin content is one of the most important criteria for determining Bupleuri Radix quality (Pan, 2006; S.H. Zhu et al., 2007). Among saikosaponins, saikosaponin a (SSa) and saikosaponin d (SSd) are especially known for their pharmacological activity, including: anti-allergic action, analgesic action and anti-inflammation effects, reduction in blood-cholesterol, hemolytic activity, and

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[*] *Corresponding author at*: Research Center of Soil and Water Conservation and Ecological Environment, Chinese Academy of Sciences and Ministry of Education, No. 26 Xinong Road, Yangling, Shaanxi Province 712100, PR China. Tel.: +86 29 87014582; fax: +86 29 87092262.
E-mail address: liangzs@ms.iswc.ac.cn (Z. Liang).

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protective action against hepatic damage (Aoyagi et al., 2001). Due to its medicinal importance, the demand for Bupleuri Radix has increased steadily in recent years. China uses about eight million kilograms of Bupleuri Radix each year. And gross sales of manufactured prescription medicines containing Bupleuri Radix in Japan amounted to 27 billion yen in 2002 (Pan, 2006). Hence, production protocols for the cultivation of high-yield *Bupleurum* spp. are needed in order to meet the strong market demand for medicinally important compounds.

A significant amount of evidence indicates that environmental stress increases the level of several secondary metabolites in plants (Kirakosyan et al., 2004; Zobayed et al., 2005, 2007). Therefore, it may be possible to stimulate the accumulation of bioactive compounds in medicinal crops by exposing the plants to environmental stress (Murch et al., 2003). For example, Kirakosyan et al. (2004) subjected two hawthorn species (*Crataegus laevigata* and *C. monogyna*) to several stresses, including water-deficiency, cold, flooding, or grazing, and found that drought and cold stress, alone or in combination, enhanced the levels of desired polyphenolics in plant leaves. The authors claimed that the study provided a practical way to obtain consistent and high levels of phenolics from leaves of two hawthorn species year-round in a greenhouse photobioreactor system.

Water-stress is known to increase the amount of secondary metabolites in a wide variety of plant species, including saikosaponin a, saikosaponin c (SSc) and saikosaponin d in *Bupleurum falcatum* L. (Minami et al., 1995), chlorogenic acid, catechin, and (-)-epicatechin in *Crataegus* spp. leaves (Kirakosyan et al., 2004) and hypericin, betulinic acid, pseudohypericin, and rutin in *Hypericum* spp. (Zobayed et al., 2005; Nacif de Abreu and Mazzafera, 2005). Therefore, exposure to drought conditions may improve production from medicinal herb plantations.

It should be noted that water deficit adversely affects plant growth. However, several studies have demonstrated that application of suitable fertilizers mitigated the detrimental effect of drought on biomass yield (Graciano et al., 2005; Ram et al., 2006). Information about the effect of drought-stress and fertilization on medicinal plants is crucial for the development of crop management strategies, especially in arid and semi-arid regions. However, relatively little is known about this topic.

Bupleurum chinense DC. is one of the standard medicinal herbs in the *Pharmacopoeia of the People's Republic of China* (2005). Field culture is its main source for roots and it is generally harvested biannually. Several studies have examined the influence of either fertilization or drought on biomass and quality of *B. falcatum* (Minami and Sugino, 1995; Minami et al., 1995; Kim et al., 1997) and *B. chinense* (Z.B. Zhu et al., 2007; Zhu et al., 2008), however little is known about the interactive effects between water-stress and fertilizer. In a previous paper, we reported the effect of seven combinations of N and P fertilizer on total SSa and SSd yield from *B. chinense* grown under well-watered conditions (Zhu et al., 2008). The objective of this study was to investigate the combined effect of water-stress and fertilizer on SSa and SSd production in *B. chinense* roots.

2. Materials and methods

A pot experiment was conducted from July 2003 to November 2004 at the Research Center of Soil and Water Conservation and Ecological Environment, Chinese Academy of Sciences and Ministry of Education, Yangling, Shaanxi Province, China. We filled 8 L pots with 12 kg of soil and clean sand (2:1, v/v). The soil had been passed through a 0.5 cm mesh sieve. The organic matter content of the original soil was 11.57 $\mathrm{g\,kg^{-1}}$, available-N was 55.6 $\mathrm{g\,kg^{-1}}$, available-P was 21.68 $\mathrm{g\,kg^{-1}}$, and available-K_2O was 160 $\mathrm{g\,kg^{-1}}$. The field water capacity of the soil + sand mixture was 21% (w/w).

The experiment was a 2 × 3 factorial design consisting of two soil water contents (well-watered treatment = soil water content of 15% (w/w) and water-stressed treatment = soil water content of 10%, w/w) and three fertilizer amounts (N0P0 = unfertilized control, N1P1 = 0.15 g N + 0.2 g P_2O_5 $\mathrm{kg^{-1}}$ growth medium, and N2P2 = 0.3 g N + 0.4 g P_2O_5 $\mathrm{kg^{-1}}$ growth medium). The N was applied in the form of urea and P was applied as superphosphate. One third of the N and all of the P were applied basally. The remaining N fertilizer was applied in March 2004, prior to a period of rapid plant growth. Each treatment combination was replicated four times, making a total of 24 pots.

B. chinense seeds were obtained from Shaanxi TASLY Plant Pharmaceutical Co. Ltd., Shangluo, Shaanxi, China. Eight 2-month-old *B. chinense* seedlings raised in the nursery, as morphologically identical as possible, were transplanted into each pot on 8 July 2003. The pots were placed under a rain-shelter in a completely randomized block design. Two weeks later, the seedlings were thinned to four seedlings per pot. The pots were weighed 3–5 times per week and water was added to maintain a soil moisture content of 15% (w/w). On 10 August 2003, water-stress was initiated in half the pots by withholding irrigation until soil moisture reached 10%. Soil water content in the remaining pots was kept at 15% (w/w). The water-stressed and well-watered treatments continued until the plants were harvested in November 2004. Shoots and roots were separated, dried at 60 °C for 72 h, and weighed. Then the roots were ground to pass through a 0.5 mm sieve as described previously (Zhu et al., 2008).

SSa and SSd content was determined using a Waters HPLC system (Milford, MA, USA). The methods and conditions for determination have been reported previously (Zhu et al., 2008). Total SSa and SSd yield was determined by multiplying the saikosaponin content in roots by root dry weight.

Treatment effects were determined by two-way analysis of variance (ANOVA). The difference between treatments was confirmed by Duncan's Multiple Range Test (DMRT) using SAS 8.0 for Windows. Data regarding the interactions were reported when the interactions were statistically significant at $p < 0.05$.

3. Results and discussion

Long-term water-stress resulted in a serious reduction in *B. chinense* growth (Table 1). Averaged across all fertilizer treat-

Table 1 – Growth response of *B. chinense* to fertilizer under well-watered (WW) and water-stressed (WS) conditions

Treatment		Root DW g plant^{-1}	Shoot DW g plant^{-1}	R/S ratio
Water	Fertilizer			
Well-watered	N0P0	1.72 b	9.17 a	0.20 b
Well-watered	N1P1	2.51 a	8.76 a	0.28 a
Well-watered	N2P2	1.26 b	6.75 a	0.18 b
Water-stressed	N0P0	0.25 b	0.72 b	0.32 a
Water-stressed	N1P1	0.46 a	2.14 a	0.26 a
Water-stressed	N2P2	0.35 ab	1.38 ab	0.23 a

N0P0 = unfertilized control, N1P1 = 0.15 g N + 0.2 g P_2O_5 kg^{-1} growth medium, and N2P2 = 0.3 g N + 0.4 g P_2O_5 kg^{-1} growth medium. Potted plants were measured after 16 months of growth. DW, dry weight; R/S ratio, root dry weight to shoot dry weight ratio. Different letters within a water treatment (i.e. WW or WS) indicate significant differences at $p = 0.05$.

ments, shoot and root dry weights of drought-stressed plants were 81–83% lower compared to well-watered plants. However, the decrease in root and shoot dry weight due to water-stress was less in fertilized treatments compared to the unfertilized control. Specifically, root dry weight declined by 86% in the unfertilized treatment, 82% in the N1P1 treatment, and 72% in the N2P2 treatment compared to the same fertilizer amounts in the well-watered treatment. Water-stress resulted in a 92% reduction in shoot dry weight in the unfertilized control treatment (N0P0) compared to a 76% reduction in the N1P1 treatment and an 80% reduction in the N2P2 treatment. In water-stressed plants, root dry weight in the N1P1 treatment (0.46 g plant^{-1}) was 31% greater than in the N2P2 treatment (0.35 g plant^{-1}) and 84% greater than in the unfertilized treatment (0.25 g plant^{-1}) (Table 1). Changes in shoot dry weight followed a similar pattern. These results indicated that fertilizer application mitigated the loss of biomass due to water-stress to some degree. Maximum biomass production occurred when moderate amounts (N1P1) of fertilizer were applied. Similarly, N and P fertilizer increased the growth of *Eucalyptus grandis* (Graciano et al., 2005), *Mentha arvensis* L. (Ram et al., 2006), and maize (Zea mays L.) (Moser et al., 2006) under water-stress conditions. These results can be explained by that good soil fertility increases the ability of plants to maintain relatively high levels of growth, stomatal conductance, and photosynthesis under drought conditions (Kleiner et al., 1992). From a practical point of view, these results highlight the need for appropriate amounts of N and P fertilizer in the cultivation of *B. chinense* in arid and semi-arid regions.

The root to shoot (R/S) ratio of *B. chinense* plants was generally higher in the water-stressed treatments compared to the well-watered treatments (Table 1). In the N0P0 treatment, the R/S ratio was 60% higher in water-stressed plants compared to well-watered plants. In the N2P2 treatment, water-stress resulted in a 28% increase in the R/S ratio. The R/S ratio of water-stressed plants tended to decline as the amount of fertilizer increased, however the change was not significant (Table 1).

Water-stress increased the SSa and SSd content of *B. chinense* roots (Fig. 1). Root SSa content was 15% higher in water-stressed plants compared to well-watered plants when averaged across all fertilizer treatments. Similarly, water-stress increased root SSd content by an average of 22%. The results are similar to those of Minami et al. (1995) who

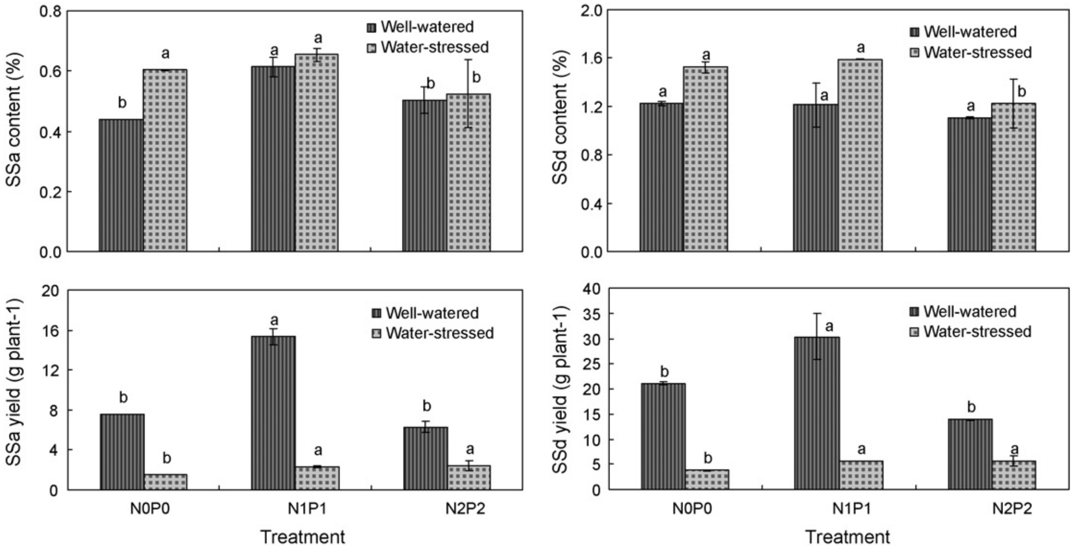

Fig. 1 – Effect of water-stress and fertilizer on saikosaponins content and yield of *B. chinense*. N0P0 = unfertilized control, N1P1 = 0.15 g N + 0.2 g P_2O_5 kg^{-1} growth medium, and N2P2 = 0.3 g N + 0.4 g P_2O_5 kg^{-1} growth medium. Different letters within each water treatment (i.e. well-watered or water-stressed) indicate significant difference at $p = 0.05$.

Table 2 – *F* values from ANOVA showing the main and interactive effects of water and fertilizer on shoot and root dry weight, SSa and SSd content, and total SSa and SSd yield of *B. chinense*

Source of variation	Growth variables			SS content (%)		SS yield (mg plant^{-1})	
	Root DW	Shoot DW	R/S ratio	SSa	SSd	SSa	SSd
Water (W)	147.81^{***}	152.27^{***}	4.45^{*}	6.21^{*}	16.42^{**}	885.32^{***}	235.22^{***}
Fertilization (F)	8.18^{**}	0.48 ns	3.50^{*}	6.56^{*}	5.2^{*}	128.83^{***}	19.68^{**}
W × F	10.48^{***}	4.3^{*}	3.58^{*}	2.21 ns	1.4 ns	114.69^{***}	19.08^{**}

DW, dry weight; R/S ratio, root dry weight to shoot dry weight ratio; SS, saikosaponin; SSa, saikosaponin a; SSd, saikosaponin d. The level of significance between treatments is indicated as follows: n.s. $p > 0.05$; $^{*}0.05 > p > 0.01$; $^{**}0.01 > p > 0.001$; $^{***}0.001 > p$.

reported that one month of water-stress resulted in a significant increase in the SSa, SSc and SSd content of the cork layer and surrounding tissues in *B. falcutum*. Water-stress can alter the oxidative balance of cells and acclimation to drought is generally correlated with keeping the level of active oxygen species (AOS) relatively low through the antioxidant system (Dat et al., 2000). A number of earlier investigations have suggested that oxidative stress plays an important role in the synthesis of secondary metabolites in plants (Shohael et al., 2006). For example, Ali et al. (2005) suggested that inhibition of membrane damage in Asian ginseng and American ginseng may be associated with the induction of ginsenoside production which protects plants from oxidative damage. Similarly, Nacif de Abreu and Mazzafera (2005) suggested that an increase in phenolic compounds and betulinic acid in *Hypericum brasiliense* Choisy plants stressed by drought and hypoxia might represent an antioxidant response to AOS production. The observation that saikosaponins have the ability to eliminate AOS and to prevent peroxidation of biomembranes (Yokozawa et al., 1997; Liu et al., 2005) suggests that the accumulation of saikosaponins under water-stress conditions may be an important part of the complex antioxidant system.

There were significant differences in the SSa and SSd contents among fertilizer treatments. In water-stressed plants, SSa and SSd content tended to be larger in the N1P1 treatment compared to the unfertilized control (N0P0), but the difference between the two treatments was not significant. There was a significant decline in root SSa and SSd content when larger amounts of fertilizer (N2P2) where applied, suggesting the existence of a nutrient threshold for SSa and SSd biosynthesis.

The carbon/nutrient balance (CNB) and growth differentiation balance (GDB) hypotheses predict a trade-off between growth and defense. Because water-stress reduces plant growth, the carbon fixed during photosynthesis can be used for the formation of secondary compounds (Turtola et al., 2003). In this study, water-stress increased the SSa and SSd content of *B. chinense* roots, but reduced root dry matter accumulation by about 80%. Consequently, the overall result was that total SSa and SSd yield was 70–73% lower in water-stressed plants compared to well-watered plants when averaged across fertilizer treatments (Fig. 1). Nevertheless, the application of fertilizer to water-stressed plants increased total SSa and SSd yield in the N1P1 and N2P2 treatments by 49–63% compared to the unfertilized control (N0P0). Total SSa and SSd yield were not significantly different between the N1P1 and N2P2 treatments.

Both watering and fertilization had significant effects on root dry weight, total SSa and SSd yield and there was significant interaction between the water and fertilizer treatments (Table 2). This indicates the importance of coordinating water supply and fertilization in order to obtain maximum saikosaponin yield. Together, it still may be possible to increase total SSa and SSd yield through the combined use of fertilizer and properly timed exposure to water-stress.

Limitations of this study include a lack of information about the effects of water-stress duration and magnitude on SSa and SSd production. Plant growth stage at the onset of water-stress should also be taken into account. Further investigation is needed to develop production protocol for obtaining a balance between *B. chinense* growth and SSa and SSd content. Additionally, the cellular and physiological mechanisms regulating the effect of mineral nutrients and water-stress on saikosaponin biosynthesis and accumulation in *B. chinense* remain to be explored.

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