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Growth and saikosaponin production of the medicinal herb *Bupleurum chinense* DC. under different levels of nitrogen and phosphorus

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ABSTRACT

Dried roots of *Bupleurum* spp. have been used medicinally in China for over 2000 years. The roots, which are called Bupleuri Radix, are used in at least 66% of the formulations/prescriptions in traditional Chinese medicine and Kampo medicine. Demand for Bupleuri Radix is increasing and *B. chinense* is one of the most important *Bupleurum* spp. in China. We conducted a 2-year pot experiment to investigate the effect of nitrogen (N) and phosphorus (P) fertilizer on biomass production and root saikosaponin a (SSa) and saikosaponin d (SSd) content of *B. chinense*. The experiment included seven combinations of N and P fertilizer. The results showed that medium levels of N and P fertilizer significantly increased the biomass and SSa content of *B. chinense* roots, but had no significant effect on root SSd content. The application of moderate amounts of N or P fertilizer increased total SSa and SSd yield significantly compared to the unfertilized control, but the greatest increase in total SSa and SSd yield occurred when N and P were applied together. This suggests that N and P have a synergistic effect on *B. chinense* growth and saikosaponin production. When high amounts of N and P fertilizer were used total SSa and SSd yields declined. This implied that there was a nutrient supply threshold for the biosynthesis of saikosaponins. Total SSa and SSd yields were greater in treatments which received only P fertilizer compared to treatments that received only N fertilizer. Our results reinforce the importance of soil testing and the application of recommended amounts N and P fertilizers for the cultivation of *B. chinense*.

1. Introduction

Bupleuri Radix, which are dried roots of *Bupleurum* spp. (Apiaceae), is one of the most common components of Chinese traditional medicine prescriptions (Pistelli et al., 1996; Sánchez-Contreras et al., 2000). Two *Bupleurum* spp., *B. chinense* and *B. scorzonerifoliu*, were included in the *Chinese Pharmacopoeia*. The two species, along with *B. falcatum* L., have been widely used in Chinese and Japanese herbal medicines for the treatment of chronic hepatitis, kidney syndrome, inflammatory diseases, and ulcers of the digestive system (Zhao et al., 1996; Guo et al., 2000).

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It is believed that saikosaponins are responsible for part of the pharmaceutical properties of Bupleuri Radix (Zhu et al., 2006). Three major oleanane-saponins, saikosaponin a (SSa), saikosaponin c (SSc) and saikosaponin d (SSd), and many minor saponins have been isolated from the roots of *Bupleurum* spp. (Morinaga et al., 2006). Among these saponins, SSa and SSd are especially known for their pharmacological activity (Aoyagi et al., 2001). Both saponins are reported to have anti-cancer and anti-inflammatory effects. In addition, SSa has been shown to increase corticosterone secretion and decrease blood cholesterol, while SSd has immunoregulatory, anti-bacterial, and liver protection effects (Morinaga et al., 2006; Hsu et al., 2004; Leung et al., 2005).

Due to its medicinal importance, the demand for Bupleuri Radix has increased steadily in recent years. About eight million kilograms of Bupleuri Radix are required for prescriptions or exported from China each year. In Japan, the gross sales of manufactured prescription medicines containing Bupleuri Radix amounted to 27 billion yen in 2002 (Pan, 2006).

The application of optimum amounts of fertilizer can increase both biomass yield and active component content of medicinal plants. For example, nitrogen application increased the biomass and essential oil yield of patchouli (Singh et al., 2002), palmarosa (Rajeswara Rao, 2001) and dill (Wander and Bouwmeester, 1998). Phosphorus fertilization had similar effects on fennel (Kapoor et al., 2004) and Japanese mint (Kotheri et al., 1987). Studies in Japan and Korea have investigated the effect of fertilizer on biomass production and terpene content of *B. falcatum* (Kim et al., 1997; Minami and Sugino, 1995), however, little is known about the effect of fertilizer on *B. chinense*, which is one of the standard medicinal plants in *Chinese Pharmacopoeia* 2005 (Zhu et al., 2007b). Therefore, the objective of this study was to determine the effect of N and P fertilizer, alone or in combination, on biomass production and root saikosaponin (a and d) content of *B. chinense*.

2. Materials and methods

2.1 Plant material

The 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 a 12 kg mixture of soil and clean sand (2:1, v/v). The soil had been passed through a 0.5 cm mesh sieve. Chemical properties of the original soil were: organic matter content = 11.57 g kg^{-1}, available-N = 55.6 g kg^{-1}, available-P = 21.68 g kg^{-1}, available-K_2O = 160 g kg^{-1}. The field water capacity of the soil + sand mixture was 21% (w/w). Eight 2-month-old *B. chinense* seedlings were transplanted into each pot on 8 July 2003, then the pots were placed under a rain-shelter. Two weeks later, the seedlings were thinned to four seedlings per pot.

The plants were harvested in November 2004. Shoot 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.

2.2 Fertilizer treatments

The experiment was arranged in a completely randomized block design with seven treatments and four replications. Nitrogen was applied as urea at rates of 0 (N0), 0.15 (N1), 0.3 (N2) g N kg^{-1} growth medium. Phosphorus was applied as superphosphate with rates of 0 (P0), 0.2 (P1), 0.4 (P2) g P_2O_5 kg^{-1} growth medium. The seven fertilizer treatments consisted of different combinations of N and P: N0P0, N0P1, N1P0, N1P1, N1P2, N2P1, and N2P2. The N0P3 and N3P0 fertilizer combinations were not tested in this experiment. 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. The pots were weighed 3–5 times per week and water was added to maintain a soil moisture content of 15% (w/w).

2.3 Assay of saikosaponin a and d

2.3.1 Chemicals and reagents

We purchased HPLC grade acetonitrile from Tedia Co., Inc. (Fairfield, OH, USA). Analytical grade methanol was obtained from the Xi'an Chemical Reagent Plant (Xi'an, China). Saikosaponin a and d were purchased from the National Institute for the Control of Biological and Pharmaceutical Products (Beijing, China). Ultrapure water was generated with a UPW Ultrapure Water System (Shanghai Ultrapure Technology, Shanghai, China).

2.3.2 Apparatus and chromatographic conditions

A Waters HPLC system (Milford, MA, USA) equipped with a 2487 binary pump, manual sample injector, and a Waters 2996 photodiode array detector was used to perform HPLC analysis. The HPLC fingerprint was carried out on a C_{18} column (Waters, SunFire C_{18}, 4.6 mm × 250 mm, 5 μm) at 30 °C with a sample injection volume of 20 μL. Empower2 software was used for data acquisition and processing. Detection wavelength was 252 nm and the flow rate was 1.0 mL min^{-1}. A gradient elution of A (acetonitrile) and B (ultrapure water) was used as follows: 0–5 min, 10–20% A; 5–10 min, 20–35% A; 10–15 min, 35–40% A; 15–25 min, 40–50% A; 25–35 min, 50–75% A; 35–40 min, 75–85% A; 40–50 min, 85–100% A. Then the system was restored to its initial condition after 15 min.

2.3.3 Sample preparation for HPLC quantitation

Samples for HPLC quantitation were prepared using modifications of the methods by Li et al. (2005, 2006). Specifically, 0.5 g powdered root samples were extracted by sonication for 30 min in 25 mL 2% KOH–methanol solution. The solution was cooled to ambient temperature, filtered, and then brought up to a volume of 25 mL with 2% KOH–methanol. A 2.00 mL aliquot of the solution was transferred to a 10 mL volumetric flask to which we added 2 mL 4% HCl–methanol solution. The solution was incubated at ambient temperature for 4 h, then 3 mL of 2% KOH–methanol solution was added to the extract and the final volume was brought up to 10 mL with methanol. The solution was filtered through a 0.45 μm organic membrane filter before injection into the HPLC system.

2.3.4 Preparation of standard solutions for HPLC quantitation

Separate standard solutions containing 1.86 mg mL^{-1} for SSa and 2.00 mg mL^{-1} for SSd were prepared by dissolving the chemicals in methanol. We transferred 0.2 mL aliquots of each stock solution to 10 mL volumetric flasks to which we added 2 mL 4% HCl–methanol solution. The solutions were incubated at ambient temperature for 4 h, then an additional 3 mL 2% KOH–methanol solution was added to the flasks and the final volume was brought up to 10 mL with methanol. The HPLC system was calibrated with the SSa and SSd standards at injection volumes of 0, 2, 4, 6, 8, 10, 15 and 20 μL.

2.3.5 Validation of quantitative methods

Method precision and repeatability were evaluated using the successive analysis of five replicates of the same powder sample. The relative standard deviations (R.S.D.) were 0.1–2.1% for retention time (t_R) and 0.3–1.8% for the peak area (PA) of characteristic peaks. The stability of the sample solutions was determined by analyzing samples at 0, 2, 4, 8, 16, and 24 h after their preparation. Average recoveries were 98.02% for SSa and 99.93% for SSd. The R.S.D. was 1.92% for t_R and 1.14% for PA.

2.4 Statistical analysis

Treatment effects were determined by one-way analysis of variance (ANOVA). The differences between treatments were confirmed by Duncan's Multiple Range Test (DMRT) using SAS 8.0 for Windows.

3. Results

3.1 Effects of N and P fertilizer on growth variables

The application of medium amounts of N and P fertilizer, alone or in combination, tended to increase shoot and root dry weights of *B. chinense* compared to the unfertilized control, however, this increase was not statistically significant (Fig. 1). Larger amounts of N and P fertilizer generally resulted in a decrease in shoot and root dry weight. Among all treatments, shoot dry weight was largest in the N0P1 treatment. It was 75% greater than in the N2P2 treatment and 29% greater than in the unfertilized (control) treatment. Root dry weight in the N1P1 treatment was 46% greater than in the control treatment, 52% greater than in the N1P2 treatment, and 100% greater than in the N2P2 treatment. The lowest shoot and root dry weights were both observed in the N2P2 treatment.

The root to shoot (R/S) ratio was significantly ($p < 0.05$) greater in the N1P1 treatment compared to the other treatments. This is due to the fact that the N1P1 treatment had the highest root dry weight among all treatments and a relatively low shoot dry weight. There were no significant differences in the R/S ratios among the other treatments.

3.2 Effect of N and P fertilizer on saikosaponin content

The SSa content of *B. chinense* was higher in treatments that received medium amounts of N and P fertilizer (Fig. 2). The SSa content was 19% higher in the N1P0 treatment and 20% higher in the N0P1 treatment compared to the unfertilized (control) treatment. The increase in SSa content was even greater in the N1P1 treatment, which suggested that the two nutrients had a synergistic effect. The SSa content of *B. chinense* was larger in the high N and P treatments (N2P1, N1P2, and N2P2) compared to the unfertilized control, but smaller than the medium N and P treatments (N1P0, N0P1, and N1P1).

The SSa content of plants was higher in N1P1 treatment compared to the N1P0 and N1P2 treatments. Similarly, SSa content was higher in the N1P1 treatment compared to the N0P1 and N2P1 treatments. In other words, the data showed that the application of only one nutrient or the application of one nutrient at a high level and the other nutrient at a moderate level resulted in a lower SSa content compared to the combined application of moderate levels on N and P. There was no significant difference in the SSa content of plants in the N2P1 and N2P2 treatments, indicating that P fertilizer had

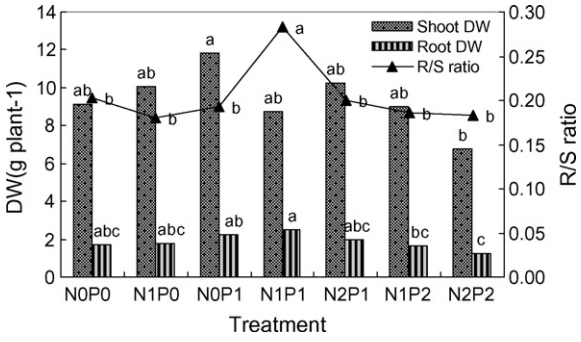

Fig. 1 – Effect of nitrogen and phosphorus fertilizer on plant dry weight and root to shoot (R/S) ratio. N0P0 = unfertilized control, N1P0 = 0.15 g N kg^{-1} growth medium, N0P1 = 0.2 g P kg^{-1} growth medium, N1P1 = 0.15 g N and 0.2 g P kg^{-1} growth medium, N2P1 = 0.3 g N and 0.2 g P kg^{-1} growth medium, N1P2 = 0.15 g N and 0.4 g P kg^{-1} growth medium, and N2P2 = 0.3 g N and 0.4 g P kg^{-1} growth medium. Different letters indicate significant difference at $p = 0.05$.

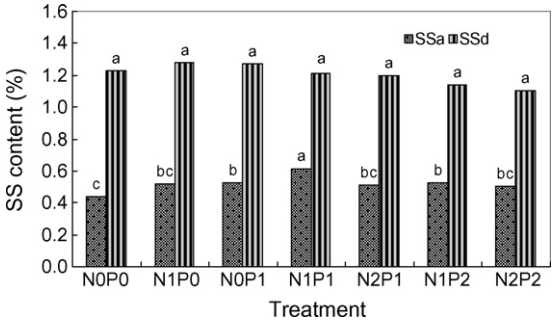

Fig. 2 – Effect of nitrogen and phosphorus fertilizer on saikosaponins content. N0P0 = unfertilized control, N1P0 = 0.15 g N kg^{-1} growth medium, N0P1 = 0.2 g P kg^{-1} growth medium, N1P1 = 0.15 g N and 0.2 g P kg^{-1} growth medium, N2P1 = 0.3 g N and 0.2 g P kg^{-1} growth medium, N1P2 = 0.15 g N and 0.4 g P kg^{-1} growth medium, and N2P2 = 0.3 g N and 0.4 g P kg^{-1} growth medium. Different letters indicate significant difference at $p = 0.05$.

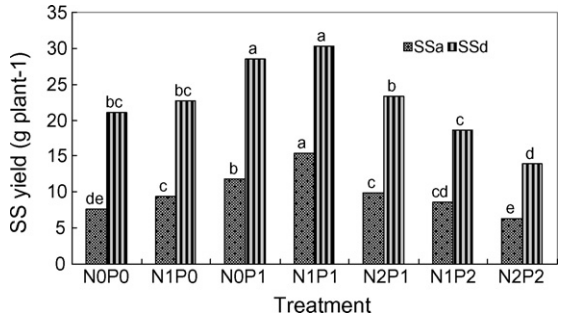

Fig. 3 – Effect of nitrogen and phosphorus fertilizer on saikosaponins yield. N0P0 = unfertilized control, N1P0 = 0.15 g N kg^{-1} growth medium, N0P1 = 0.2 g P kg^{-1} growth medium, N1P1 = 0.15 g N and 0.2 g P kg^{-1} growth medium, N2P1 = 0.3 g N and 0.2 g P kg^{-1} growth medium, N1P2 = 0.15 g N and 0.4 g P kg^{-1} growth medium, and N2P2 = 0.3 g N and 0.4 g P kg^{-1} growth medium. Different letters indicate significant difference at $p = 0.05$.

no significant effect on the SSa content of plants receiving high levels on N fertilizer. Similarly N fertilizer had no effect on plants receiving high amounts of P fertilizer.

The SSd content in *B. chinense* changed in a pattern similar to our observations for SSa, however, these changes were small and the differences between the treatments were not statistically significant ($p > 0.05$).

3.3 Effect of N and P fertilizer on total saikosaponin yield

Total SSa and SSd yield was determined by multiplying the saikosaponin content in roots by root dry weight. The results showed the fertilizer had a significant effect on total SSa and SSd yield in *B. chinense* roots (Fig. 3). Total SSa and SSd yield was largest when moderate amounts of N and P were applied in combination, while it declined at higher levels of N and P fertilizer.

Root dry weight and SSa content both increased in the N1P0, N0P1, and N1P1 treatments, resulting in an increase in total SSa yield. The content of SSd was similar in the N1P0, N0P1, and N1P1 treatments, but total SSd yield increased because root dry weight increased. The N1P1 treatment increased total SSa yield by 103% and total SSd yield by 44% compared to the unfertilized (control) treatment. Total SSa and SSd yield was also greater in the N0P1 and N1P0 compared to the control treatment, but the increase was not as large as for the N1P1 treatment, suggesting synergistic effects between the two treatments. We also noted that total SSa and SSd yield was higher in the N0P1 treatment than in the N1P0 treatment, which indicates that P is more important than N in SSa and SSd production.

Total SSa yield was significantly ($p < 0.05$) larger in the N1P1 treatment than in the N1P0, N1P2, N0P1, and N2P1 treatments. Total SSd yield followed a similar pattern except there was no significant difference in the total SSd yield of the N1P1 and N0P1 treatments. At high levels of N fertilizer, total SSa and SSd yield declined as P fertilizer increased. Specifically, total SSa and SSd yield was significantly ($p < 0.05$) higher in the N2P1 treatment compared to the N2P2 treatment. Similarly, SSa and SSd yield decreased in the high P treatments as N fertilizer increased. The decline in SSa and SSd yield in these treatments can be attributed to a reduction in root dry weight as well as SSa content that occurred when N and P levels were both high. It should be noted that the difference in the SSd content of the high and medium fertilizer treatments was not significant. Overall, the N2P2 treatment resulted in a 16% decrease in SSa and 34% decrease in SSd compared to the control treatment.

4. Discussion

Saikosaponin content is one of the most important criteria for determining the quality of Bupleuri Radix (Zhu et al., 2007a; Pan, 2006). A number of factors affect the saikosaponin content of Bupleuri Radix including: local growing conditions, harvest time, root portion, plant condition, individual plant characteristics, fertilization, and cultivation methods (Zhu et al., 2006; Pan, 2006). Our experiment demonstrated that the application of optimal amounts of N and P fertilizer is an important management tool for increasing *B. chinense* root biomass and SSa and SSd yield. The results are consistent with a previous study which reported increased root yield and total saikosaponin content in *B. falcatum* in response to organic fertilizer (Kim et al., 1997). In contrast, Minami and Sugino (1995) found that whole dried root weight and SSa, SSc and SSd content of *B. falcatum* decreased significantly as the combined application of N, P, and K fertilizer increased. When N, P, or K fertilizers were applied separately, they found that the shoot and root dry weight and saikosaponin content of *B. falcatum* decreased when high levels of N fertilizer were applied, but increased when high levels of P fertilizer were applied. In a field study, Zhu et al. (2007b) reported that the SSa content of *B. chinense* reached a maximum and then declined as the amount of superphosphate fertilizer or pig manure increased. In contrast, the SSa content decreased continuously as N application increased.

The positive effects of fertilizer application on secondary metabolites are in agreement with previous reports for other medicinal plants, such as menthol mint (Ram et al., 2006), palmarosa (Rajeswara Rao, 2001), basil (Sifola and Barbieri, 2006) and fennel (Kapoor et al., 2004). One explanation for these observations is that nitrogen is a major constituent of several important precursors (Ram et al., 2006). Furthermore, P is known to play an important role in the biosynthesis of secondary metabolites (Liu and Zhong, 1998). Because terpenoids are based on an integral C5 unit (isoprenoid), saikosaponin production requires acetyl-CoA, ATP, and NADPH which contain P for biosynthesis (Kapoor et al., 2004).

Our results showed that the application of fertilizers at levels above the N1P1 treatment had detrimental effects on the biosynthesis of SSa and SSd. This implied that there was a nutrient supply threshold for the biosynthesis of saikosaponins. Wander and Bouwmeester (1998) and Zhu et al. (2007b) also reported that overfertilization resulted in decreased concentrations of medicinal compounds. Thus, from a practical point of view, our results show the importance of soil testing and the application of recommended amounts N and P fertilizers for the cultivation of *B. chinense*.

Previous investigations showed that terpene concentration in plants was relatively high when soil fertility was low, but terpene concentrations decreased when N fertilizer was added to the soil (Lee et al., 2005). For example, Barnola and Cedeño (2000) found that the volatile terpene content of pine trees was relatively low when the trees were growing in soil that had high N and P concentrations. It is likely that the effect of soil fertility on terpene concentration and composition in plants varies among plant species, plant growth stage, and environmental conditions (Lee et al., 2005).

SSa and SSd are a pair of stereoisomers (Shyu et al., 2004), differing only in the configuration of the hydroxyl function at C16, i.e. SSa has a β-hydroxyl function and SSd has an α-hydroxyl function (Dobashi et al., 1995). Pharmacological experiments demonstrated that the activity of SSd was greater than for SSa. Therefore it has been suggested that the α-OH group at C16 has more pharmacological activity than β-OH group (Pan, 2006). We found no significant difference in the SSd content among the fertilizer treatments, which suggests that SSd is less responsive to fertilization than SSa. The basic outline of saikosaponin production is fairly well understood, however, the enzymes, genes and biochemical pathways involved in saikosaponin biosynthesis are largely uncharacterized (Chen et al., 2007). Further research elucidating the saikosaponin biosynthesis pathway would provide a better understanding regarding the effect of nutrients on saikosaponin production.

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