Management Effects on Yield and Flavonoid Content in American Skullcap (*Scutellaria lateriflora*)

by

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Abstract

American Skullcap (*Scutellaria lateriflora)* is a member of the mint family (*Labiatae* or *Lamiaceae*), and is a medicinal herb traditionally used for its mild relaxant properties attributed to its content of flavonoids. Field and greenhouse trials were conducted to determine management effects on dry matter yield and flavonoid content in American skullcap. The field experiment was conducted on Marvyn loamy sand (fine-loamy, kaolinitic, Thermic Typic Kanhapludults) with 0-2% slope in central Alabama, to determine the effect of timing and frequency of harvest on shoot yield and flavonoid content. The experimental design was 2X2 split plot factorial in a randomized complete block design with four replications of each treatment. The main factors were number of harvests in the first season (2008) - one harvest per season and two harvests per season. The sub factors were timing of harvests in the second season (2009) - early harvest and late harvest. In the first year (2008), harvesting twice gave 36 % higher yield than harvesting once. Baicalein had higher concentration and yield than other flavonoids in 2008. In the second year, there was no difference in yield between early or late harvesting but all the parameters considered in the study were significantly higher in first harvest than in the second harvest. Baicalin was higher in concentration and yield than other flavonoids in 2009.Flavonoid yield was 58% higher in the first harvest than in the second harvest in year 2 (2009). No residual effect from first year treatment was observed on yield in second year.

Greenhouse trials were conducted (September 2010 and January 2011) to determine the effects of nitrogen (N), phosphorus (P) and potassium (K) fertilizer on biomass yield and flavonoid content of American skullcap. Plants were grown in fritted clay in plastic pots in the greenhouse. Separate experiments were carried out for N, P and K. Each experiment was carried out two times and consisted of six levels of each nutrient. The levels of treatment for N experiment were: 0 kg N ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 400 kg ha^{-1} and 800 kg ha^{-1}, for P experiment were: 0 kg ha^{-1}, 20 kg ha^{-1}, 40 kg ha^{-1}, 80 kg ha^{-1}, 160 kg ha^{-1} and 320 kg ha^{-1} and for K experiment were: 0 kg ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 300 kg ha^{-1} and 400 kg ha^{-1}. Each treatment received a standard rate of micronutrient solution as needed as well as the highest level of the other two major elements. The nutrients were supplied as solution. Regression analysis gave maxima for dry matter, baicalein and chrysin yield at 446 kg N ha^{-1}, 412 kg N ha^{-1} and 351 kg N ha^{-1} for N fertilizer respectively in greenhouse 2. Dry matter yield exhibited a linear response to P application. The yield of scutellarein, baicalin, baicalein and chrysin increased with addition of P. Regression analysis gave maximum dry matter yield at 208 kg K ha^{-1} for potassium fertilizer. A linear response to K fertilization was observed for scutellarein concentration.

American skullcap may be harvested twice in the first year and at least twice in second year or cultivation. N, P and K increased dry matter and analyzed flavonoids yield in the greenhouse experiment. Field experiments are required to validate the finding of the greenhouse experiment and to determine if three harvests may be carried out in second and subsequent years.

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Chapter 1. Introduction and Literature Review

Plant products have long been considered as potential drugs for many diseases. About 80% of the world population depends on herbal medicine (Rodrigues and Casali 2002); two-thirds of the species used for medicine are collected directly from nature, especially in tropical countries (Chlodwig 1993). Use of herbal therapies has declined considerably with the arrival of synthetic drugs (Mannfried, 1993). However, the use of herbal medicine has been growing in the recent years (Azaizeh et al., 2005). Wills et al (2000) reported that according to World Health Organization about 70 percent of the world population makes use of herbs as their main form of therapy.

Skullcap (*Scutellaria spp.*) is a member of the mint family (Labiatae or Lamiaceae).The genus Scutellaria includes 300 species (Joshee et al. 2002). American skullcap (*Scutellaria lateriflora*) is the most commonly grown and marketed skullcap species (Wills and Stuart 2004). American skullcap is indigenous to North America, growing in wet places from Canada to Florida and westward to British Columbia, Oregon and New Mexico (Bergeron et al. 2005). It is also known as mad-dog skullcap, mad dog weed, mad weed, hoodwort, helmet flower, Virginia skullcap, blue skullcap, and Quaker bonnet (Joshee et al. 2002, Wills and Stuart 2004). American skullcap is a perennial plant that grows about 0.5 meter high with blue colored flower and helmet shaped fruit (Bergeron et al. 2005).

Medicinal Uses

Scutellaria species have been used in China, Korea, India, Japan, many European Countries and North America in traditional medical systems (Joshee et al. 2007). *Scutellaria baicalensis* is the species most extensively used. Skullcaps have been used as a sedative, nervine, antispasmodic and anticonvulsant (Millspaugh 1974) but large doses can cause dizziness, erratic pulse, mental confusion, twitching of the limbs and other symptoms indicative of epilepsy (Newall et al. 1990).

The aqueous extract of the flowering parts of American skullcap has been traditionally used as a nerve tonic and for its sedative and diuretic properties (Burlage 1968), epilepsy, cholera, nervous tension state (Newall et al. 1996), insomnia, anxiety, neuralgia (Foster and Duke, 2000), rabies, diarrhea, digestive problem (Greenfield and Davis, 2004). American skullcap is used by Cherokee women to maintain healthy menstrual cycles (Joshee et al. 2007). The aerial parts of American skullcap are used as herbal tea (Lininger et al. 2000). Skullcap was sometimes used in mixtures with different substances such as moistened roots and bear grease for dressing for sores, inflammation and other types of wounds (Hamel and Chitoskey 1975). Commercially, *S.lateriflora* is available in the form of herbal teas, tablets, capsules and oral liquid preparations (Wills and Stuart 2004).

Flavonoid Content

Flavonoids, volatile oils, iridoids, diterpenoids, waxes and tannins are the chemical constituents found in American skullcap which makes it pharmacologically important (Wren 1998). *Scutellaria* flavonoids can be used in adjuvant therapy for malignant tumors, including gliomas (Parajuli et al, 2010). According to Parajuli et al

(2011), *Scutellaria* flavonoids could inhibit proliferation of gliomas by specifically targeting molecules involved in regulation of malignant phenotype. Flavonoids anchor to the polar heads of membrane phospholipids forming reversible physiochemical complexes and this is helpful in treatments of cerebral ischemial injuries where blood supply is restricted in brain (Zhang et al. 2006).

Different types of flavonoids have been identified in American skullcap. They include glycosides baicalin, dihydrobaicalin, ikonnikoside I, lateriflorin, scutellarin and oroxylin A-7-O-glucuronide and the aglycones baicalein, oroxylin A, wogonin, and 5,6,7trihydroxy-2”-methoxyflavone (Bergeron et al. 2005). Baicalin is one of the most efficient antioxidant and most prevalent flavones in *Scutellaria* species (Boyle et al. 2011). Wogonin could potentially have very high anticancer activity among the flavonoids (Parajuli et al, 2009). Among the flavones found in *Scutellaria* species, the relative antioxidant capacity of baicalin is the highest followed by baicalein, wogonin, scutellarein and chrysin respectively which was assessed by the ferric reducing antioxidant power (FRAP) assay (Boyle et al. 2011).

Cultivation of American Skullcap

American skullcaps are naturally found in meadows, sunny edges, grassy slopes, and light woodland (Crop Development Branch, Saskatchewan Agriculture and Food, 2005). Cold stratification period and light is required for the American skullcap seed to germinate (Greenfield and Davis, 2004). American skullcap generally may be grown from seed or transplants. American skullcap is generally planted in spring. Transplants are set out in the field after danger of frost. In Alabama, American skullcap may be

transplanted in April (Similien 2009). During germination entire bed should be kept evenly moist by misting or spraying (Walker 2004). Cold stratification period and light is required for the American skullcap seed to germinate (Greenfield and Davis, 2004). Greenfield and Davis (2004) recommended shallow sowing of seed into flats containing soil mix, which should then be moistened and refrigerated at 4-10 C for seven days. The flats must be transferred in the green house for germination after the cold stratification period (Greenfield and Davis 2004).

As the plants are perennial, a site should be chosen where the plants can be grown for three or four years. Suggested field spacing is 15-30 cm between plants with rows spacing up to 60 cm .An alternative is to grow the plants in beds if the beds are up-to 90 cm wide (Crop Development Branch, Saskatchewan Agriculture and Food, 2005). Greenfield and Davis (2004) suggested spacings of 20-30 cm between plants in rows spaced 45-90 cm apart. In Alabama, Similien (2009) used a spacing of 30 cm X 30 cm spacing in paired rows on beds, which gave a population density of 53,000 plants per hectare.

Field Site and Soil Requirement

Light shade and ample moisture may be desirable for American skullcap for best production (Wills and Stuart 2004). Dry matter yield can be expected to be 40% higher in shade than in full sun (Similien, 2009). Similien (2009) reported that dry matter yield of American skullcap was highest with irrigation and added nutrients whereas the lowest yield was obtained with the control and fertilized, non-irrigated plots. Similien (2009) reported low survival in second year under conditions of full sun and no irrigation, which

suggests that American skullcap is less tolerant to direct sunlight under hot, dry conditions .According to Similien (2009), low moisture and hot temperature may result in the lower mineral uptake in full sun cultivation of American skullcap.

The fertility requirements for American skullcap are not well known, but according to the Crop Development Branch, Saskatchewan Agriculture and Food (2005), fertilizer is desirable once production begins. Similien (2009) reported that chemical fertilizers for nitrogen, phosphorus and potassium elements with adequate moisture gave significantly higher dry matter yield than without adequate moisture. However, manure surpassed chemical fertilizer for dry matter yield when moisture was lacking. According to the Carbon Nutrient Balance (CNB) hypothesis (Matthew et al., 2006), increased nutrients, especially nitrogen, increase alkaloid concentrations but decrease phenolics such as flavonoids. According to Similien (2009), irrigation did not have a significant effect on flavonoid concentration under shade but increased the concentration significantly in full sun. However, Alexievia et al, (2001); Zobayed et al (2007); and Khalid, (2006) reported higher concentrations of flavonoids in plants grown under water stress than when moisture was adequate. Treatment with CO_2 increased total biomass in American skullcap by 89% which suggests that significant improvements in growth and productivity of American skullcap can be achieved by CO_2 enhancement (Stutte et al. 2007).

Disease and Pest Control

Skullcaps are susceptible to tomato spotted wilt virus or impatiens necrotic spot virus (Joshee et al. 2002). Leaf beetles have been noted in a few countries (Crop

Development Branch, Saskatchewan Agriculture and Food, 2005) .Some diseases of American skullcap documented in the Index of Plant diseases in the United States are *Cercospora scutellariae*; the stem rot, *Botrytis cinerea*; the powdery mildews, *Phymatotrichum omnivorum* and *Rhizoctonia solani galeopsidis* and *Microsphaera sp* (Greenfield et al, 2004). Similien (2009) reported higher occurrence of powdery mildew in shade than in full sun.

Harvesting and Storage

American Skullcap is cut when it begins to flower or in the late flowering period when seed pods are present (Crop Development Branch, Saskatchewan Agriculture and Food, 2005, Greenfield and Davis, 2004). American skullcap blooms from May to August in southeastern USA (Joshee et al. 2007). Greenfield and Davis (2004) recommended for a single cutting in first year and two cuttings in the second year. Similien (2009) reported four harvests in two years: two in first year and two in second year. As the plants are cut, the tops should be piled thinly in a shaded location to avoid compaction (Wills and Stuart 2004). According to research in Australia, compaction of product can reduce the flavonoid levels (Wills and Stuart 2004).

Good care is needed during storage. Soon after harvest, American skullcap needs special care of handling till it reaches the drying room for minimal physical damage because fresh plant is still metabolically active and such damage could result in enzymatic or chemical changes to the flavonoids (Wills and Stuart, 2004). Wills and Stuart (2004) reported that when the dried plant material is cut into sections there is no significant effect but when dried material is mechanically stressed there will be

significantly loss of flavonoids. High temperature drying (40-70 C) is feasible because it reduces drying time without significant loss in chemical composition (Wills and Stuart 2004). However, the resulting color, due to high temperature, might not be desirable to the buyer. Porter (2000) recommended that full color be retained after drying. The dried materials need to be stored in a dark place under temperature from 5 to 30 C (Porter, 2000). The loss of flavonoids is not directly related to temperature but dried ground skullcap stored at any temperature up to 30 degree centigrade will lose about 0.1 % of flavonoid per day and will be more serious losses if the product reabsorbs moisture (Wills and Stuart 2004). Wills and Stuart (2004) also reported that if plants are stored in bins or sacks without adequate ventilation, there is a considerable danger of mold growth. Harvested material shouldn't be allowed to heat up after harvesting (Crop Development Branch, Saskatchewan Agriculture and Food, 2005). Little information is available on storage limitation.

Yields

There is very little data on skullcap yield. In the USA, yields of over 2246 kg ha^{-1} of dried American skullcap have been reported (Crop Development Branch, Saskatchewan Agriculture and Food, 2005). Similien (2009) reported that the highest dry matter yield for an individual harvest was 1280 kg ha^{-1} and the highest total dry matter yield in four harvests over two years was 2662 kg ha^{-1} .The lowest total yields for the 4 harvests were 724.8 kg ha^{-1} and 771.4 kg ha^{-1} (Similien 2009). According to Similien (2009), individual harvest yield of American skullcap can be increased from 283 kg ha^{-1} to 1280 kg ha^{-1} with proper treatments like integrating shade with manures and

irrigation. Under optimum growing conditions, yields up to 2,275 kg of dry matter per hectare are possible (Janke, 2004; Porter, 2000). According to Wills and Stuart (2004), yield of flavonoids varies with the plant section harvested. They reported flovonoid concentrations, 52.9 mg g^{-1} in leaves, 22.9 mg g^{-1} in stem and 32.4 mg g^{-1} in roots in their experiment.

Research on optimum timing and frequency of harvest of American Skullcap for yield is lacking. Hayden (2006) reported that multiple harvests per year of American skullcap are possible. Nutrient responses of American skullcap have not been well documented. Similien (2009) have reported effect of added nutrients to dry matter and flavonoids of American skullcap under field condition.

Research Goal and Objectives

The objectives of the experiments are to determine the effect of timing and frequency of harvest on yield and flavonoid content in organically grown American skullcap and to find out the NPK response on dry matter yield and flavonoid concentration of American skullcap.

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Chapter 2. Harvesting Number and Timing Effects on Shoot Yield and Flavonoid Content in Organically Grown American Skullcap (*Scutellaria lateriflora*)

Abstract

Increased interest in alternative medicine has increased demand for cultivation of medicinal herbs, some of which are traditionally harvested in the wild. *Scutellaria lateriflora* (Lamiaceae) has been used as a mild relaxant in the traditional medical system in North America. Information on optimal management practices for high dry matter and flavonoid yield is lacking. A field experiment was conducted on a Marvyn loamy sand (fine-loamy, kaolinitic, Thermic Typic Kanhapludults) with 0-2% slope in central Alabama, to determine the effect of timing and frequency of harvest on shoot yield and flavonoid content of *Scutellaria lateriflora* (American skullcap). The experimental design was 2X2 split plot factorial in randomized complete block design with four replications of each treatment. The main factor was number of harvests in first season (2008): one harvest per season and two harvests per season, and sub factors were timing of harvests in second season (2009): early harvest and late harvest. Only organically approved inputs were utilized. In the first year (2008), harvesting twice gave 36 % higher yield than harvesting once. In the second year, there was no difference in yield between early or late harvesting but all the parameters considered in the study were significantly higher in first harvest than second harvest. The flavonoid baicalein was at higher concentration and yield followed by baicalin, apigenin and chrysin; scutellarein and wogonin were found in very low concentration and yield in first year of

harvest (2008). In the second year (2009), baicalin was at higher concentration and yield followed by baicalein and apigenin; scutellarein, wogonin and chrysnin were found at low concentration and yield. Flavonoid concentration and yield averaged 32% and 50% higher, respectively, in 1 harvest per season than in 2 harvests per season in 2008. There were no differences in flavonoid yield between early and late harvest. However, the flavonoid yield was 58% higher in the first harvest than in second harvest in second year harvest (2009). Biomass and flavonoid yield data suggest that American skullcap may be harvested twice in the first season and at least twice in the second season.

Introduction

American skullcap is one of two species of the *Scutellaria* genus commonly marketed as medicinal herbs (Wills and Stuart, 2004). In 2001, 85% of marketed American skullcap came from cultivated sources in North America (Greenfield and Davis, 2004). American skullcap is indigenous to North America, growing in wet places from Canada to Florida and westward to British Columbia, Oregon and New Mexico (Bergeron et al. 2005). It is also known as Virginia Skullcap, Mad Dog Skullcap or Blue Skullcap. American Skullcap is a perennial plant that grows about 0.5 meter tall with blue colored flowers and helmet shaped fruit (Bergeron et al. 2005). Flavonoids, volatile oils, iridoids, diterpenoids, waxes and tannins are the chemical constituents found in American Skullcap that make them pharmacologically important (Wren 1998). Skullcaps have been used as a sedative, nervine, antispasmodic and anticonvulsant (Millspaugh 1974). The aqueous extract of the flowering parts of American skullcap was traditionally

used by Native Americans as a nerve tonic and for its sedative and diuretic properties (Burlage 1968).

The herb is used mainly for sedative purpose today. High demand for American skullcap is expected because of its sedative properties. Similien (2009) demonstrated that American skullcap can be successfully grown in Alabama. Highest yields were obtained with partial shade, irrigation and fertilization.

Greenfield and Davis (2004) reported that light cutting of the American skullcap in the first year is possible, followed by two cuttings each consecutive year (Greenfield and Davis, 2004). Similien (2009) harvested twice in both the first and second years in his study. Documentation on the effect of harvesting at different times on dry matter yield and on flavonoid concentration and yield is lacking.

A field experiment was carried out to evaluate the effect of number and timing of harvests on dry matter and flavonoid yield of American skullcap.

Materials and Methods

Site Description and Land Preparation

The field experiment was conducted in 2008 and 2009 in the organic plot at the Auburn University Horticulture Unit of the E.V.Smith Research Center, Shorter, 26 miles east of Montgomery, Alabama (latitude 32^0 30'N, longitude 85^0 40' W).The soil type was Marvyn loamy sand (fine-loamy, kaolinitic, Thermic Typic Kanhapludults) with 0-2% slope. The experimental site was planted with a cover crop of rye, which was rolled on April 23rd, 2008, before planting (Table 2.1). A four inch thick layer of composted cotton gin, wood chips and cattle manure was applied to plots on May 15, 2008.

Experimental Design and Treatments

The experimental design was a randomized complete block with four replications in year 1 (2008). Treatments consisted of 1 harvest per season and 2 harvests per season. In year 2, the plots were each split in two to form two subplots in a 2 x 2 split plot factorial in randomized complete block design. The main factor was number of harvests in first season (2008). The sub factor was planned to consist of number of harvests in second season (2 harvests per season vs. 3 harvests per season), but an unanticipated disease infestation prevented a third harvest, so the sub factor treatments consisted of early harvest and late harvest. The size of the main plots was 1.21 m wide by 3.35 m long and sub plots were 1.21 m wide by 1.67m long (Fig. 2.1). The four rows were spaced 0.304 m apart and the plants were spaced 0.304 m apart in a row. Each main plot consisted of 4 rows and 44 plants.

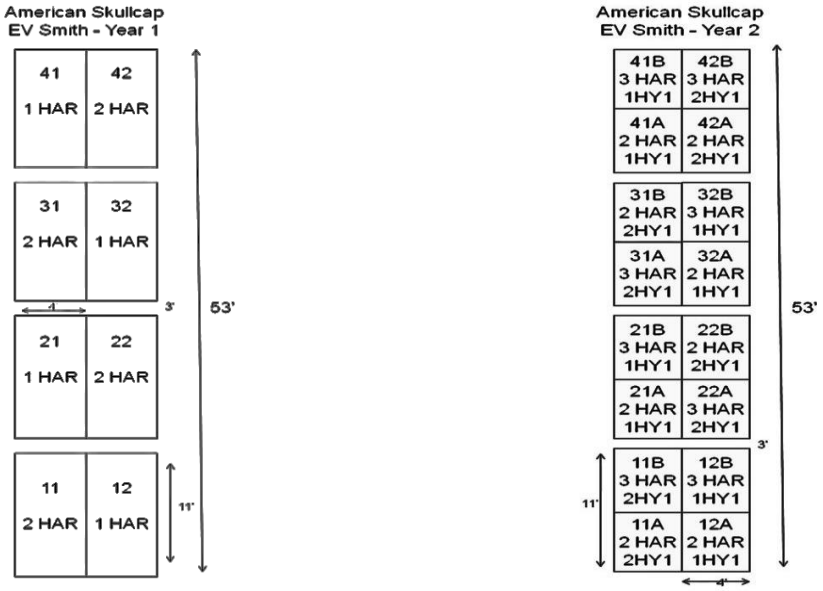

Fig. 2.1 Plot diagram showing main plots and sub plots.

(HAR= Harvest, 1HY1=1 Harvest/season in first year, 2HY1=2 Harvest/season in first year)

Shade cloth was placed on stakes at 1 m height in 2008 over the plots during the extreme heat of June 2008 and was removed in September after temperatures cooled.

A Sun Blocker Commercial Shade House measuring 4.26x17.98 m² was erected over the plots on June 2, 2009 to provide 40% shade during the heat of the summer. Initially, the shade was opened from the East - West sides only, but on July 4th, 2009, the shade cloth was removed from all sides except the top for better aeration and to counteract the fungal infection.

Only Organic Materials Review Institute (OMRI) approved organic fertilizers and fungicides were used. Hand weeding was carried out periodically as needed throughout the growing season.

Fertilization

An organic fertilizer (Nature Safe 8-5-5 Agriculture Fertilizer, Griffin Industries) was applied via irrigation on May 14, 2008 to supply 67kg N ha^{-1}, 37.5kg P ha^{-1} and 37.5kg K ha^{-1}. The ingredients of 8-5-5 Nature Safe fertilizer were feather, meat, bone and blood meals, sulfate of potash, yeast, sugars, carbohydrates and humus.

Between June 2, and October 13, 2008, an organic fertilizer (3-1-1 Pinnacle, Daniels Plant Food) was applied to the plots through drip irrigation at 3-4 days intervals (Table 2.1) to supply 34 kg N ha^{-1}, 10 kg P ha^{-1} and 10 kg K ha^{-1} at the rate of 1130 kg pinnacle ha^{-1} per application. The total of 44070 kg ha^{-1} of 3-1-1 Pinnacle was used in fertilization for entire season for which nitrogen, phosphorus and potassium accounted for 1320 kg ha^{-1}, 240 kg ha^{-1} and 240 kg ha^{-1} respectively. The fertilizer, 3-1-1 Pinnacle, is the derivative of oil seed extract. A total of 340 kg of Organic fertilizer (8-5-5 Nature Safe Fertilizer) was applied in rows only on May 29, 2009, which supplied 67kg N ha^{-1}, 37.5kg P ha^{-1} and 37.5kg K ha^{-1} per year via irrigation. 3-1-1 Pinnacle was not applied in second year.

Seedling Establishment and Husbandry

Seeds of *Scutellaria lateriflora* were obtained from Horizon Herbs LLC. William, OR 91544. The seeds were sown in Fafard 52 potting mix (Conrad Fafard Inc.) in 18 flats of deep 606 liners at the Plant Sciences Research Center greenhouse at Auburn University on March 11, 2008. The Fafard 52 mix was made from processed pine bark, Canadian sphagnum peat moss, vermiculite, perlite, dolomitic limestone, starter nutrients and wetting agent. Then the seeds were cold stratified for 7 days at 5 C in the cold room at Patterson Greenhouse Complex. On March 17, 2008, the seeds were transferred to the greenhouse of the Plant Research Science Research Center. All flats were watered as needed. Prior to transplanting in the field, the seedlings were kept outside next to the greenhouse for seven days to harden under alternating sun and shade. The seedlings were transplanted on May 26 of 2008, after the seedlings reached 5 cm height. Drip irrigation pipes with a capacity of 340 Liter per Hour /100m @ 55,000 Pascal were laid 30cm apart from each other between the rows.

Disease Management

In 2008, powdery mildew was controlled as needed with a broad spectrum neem oil extract fungicide (Trilogy, Certis U.S.A. L.L.C.). In 2009, occurrence of powdery mildew was observed for the first time on June 21and *Erysiphe spp.* was suspected as a casual organism. Trilogy was sprayed on June 22, 2009 at the rate of 15ml L^{-1} in a solution. After that, spraying was done in four weekly intervals on June 28th, July 3rd, July 10th and July 17th in 2009 (Table 2.2). On July 17, 2009, a severe infestation with *Pythium spp.*, causing root rot and plant die-off was first observed. As a result, fewer plantd were present for the second harvest in second season (2009). By the time of the

scheduled third harvest, there were no plants left to harvest. As a result, it was not possible to compare number of harvests in the second year; the only comparison possible was early vs. late harvest or the two harvests in year 2. Post-harvest handling was the same as in the first season in 2008.

Observations

The one harvest per season treatment was harvested at full bloom stage on July 16, 2008 (Table 2.2). The two harvests per season treatment was harvested on July 3, 2008 at full bloom stage and October 8, 2008 at late bloom stage (Table 2.2). The inner two rows from each plot were harvested, leaving 30 cm border at the ends of the plots. Plants were harvested with secateurs at about 10 cm height, removing one half to two thirds of top growth. In the second season, the late harvest season treatment was harvested at late bloom and active vegetative growth stages, respectively, on July 8, 2009 and September 11, 2009 (Table 2.2). The early harvests season treatment was harvested at full bloom stage on June 12, 2009 and August 6, 2009, respectively (Table 2.2). Plant height was measured before each harvest in second season and the plants stand were counted after each harvest in both the seasons. In the second year, it was impossible to distinguish individual plants because of the proliferation of shoots within the bed, so the numbers of stems were counted in the harvest area. The harvested leaves and stems were weighed and dried for 3-5 days in the drier along with the weighed samples (250 g) from each plot. Skullcaps were thinly piled in the drier room in which the temperature and humidity were maintained at approximately 35 C and 58% respectively. The dry weight of the plants was calculated by multiplying the sample percent dry matter content by the fresh weight.

Flavonoid Determination

Plant samples were ground and passed through a 1mm mesh screen using the Thomas Wiley Laboratory mill, Model 4, Thomas Scientific, USA. Ground samples were packed in Whirl-Pac air proof bag and stored at 25 C for chemical analysis.

Flavonoid content was determined by the reversed phase high performance liquid chromatography (RP-HPLC) procedure at the National Center for Natural Products Research at the University of Mississippi. Flavonoid content was established using finely ground samples. High- performance liquid chromatography grade solvents methanol (MeOH) and acetonitrile (HPLC grade) were purchased from VWR International L.L.C (Suwanee, GA). Flavone standards baicalin (95%), baicalein (98%), scutellarein (pure), apigenin (98%), 6- hydroxyflavone (97%), wogonin (98%), chyrsin (pure) and luteolin (pure) were used.

Ground samples of American skullcap (5 g) were mixed with standard Ottawa sand (VWR International L.L.C.) in order to prevent sample compaction and facilitate extraction and loaded in 22 ml extraction cartridges. Extraction of plant materials was performed using Accelerated Solvent Extraction (ASE®) apparatus (Dionex Corp., Sunnyvale, CA) at the USDA-Agricultural Research Service Natural Products Utilization Research Unit (USDA, ARS, NPURU). Extraction parameters applied were 1000 psi pressure, 40 C temperature, 10 min static time, 90 sec purge time, 100% flush volume, 4 cycles, 0 min pre heat, 5 min heat and extraction solvent MeOH: H_2O (80:20). The ASE extracts were then transferred to 20 ml tared vials and were concentrated under vacuum using a Savant SpeedVac (Model SPD121P; Savant Instruments, Inc., Holbrook, NY). After speed vacuum, 20-30 mg of dried extracts was transferred to 2 ml

capacity universal screw top vials (Micro Solv Technology Corporation, NJ, USA). The contents of each vial were then mixed with 0.1% acetic acid in MeOH and then sonicated for 30 minutes. Internal standard of 600ug/ml 6-Hydroxyflavone was added to the solution and put in a vortex machine. The solutions were transferred to other 2 ml vials by filtering with syringe filter (4 mm, nylon, 0.2 um pore size filter) using Nalgene (VWR International L.L.C., GA) and were ready to be analyzed by high pressure liquid chromatography (HPLC) for levels of flavonoids.

Samples and standards were analyzed on a Hewlett-Packard 1100 HPLC equipped with evaporation light scattering detector (ELSD 2000) using an Inertsil ODS-2 5 µ column. The mobile phase consisted of 0.005% phosphoric acid (solvent A) and acetonitrile (solvent B). All samples and standard injections were analyzed at room temperature using a nonlinear gradient from 70%: 30% (H_2O with 0.005% H_3PO_4 : acetonitrile) to 30%:70% (H_2O with 0.005% H_3PO_4 : acetonitrile) over 30 min run at a flow rate of 1 $mL \cdot min^{-1}$. Analytes were detected at 270 nm with a reference of 550 nm by the evaporation light scattering detector (ELSD 2000). The flavonoids were quantified from chromatograms of the standards with 6hydroxyflavone as internal standard.

Flavonoid yield is obtained by the product of flavonoid concentration (mg g^{-1}) and the total dry matter yield (kg ha^{-1}) and expressed in grams per hectare.

Data analysis

All data were analyzed using the PROC GLM procedure of SAS Version 9.2 (SAS Institute, Cary, NC). Differences between treatment means were tested by Tukey's method. Blocks and main error residuals are maintained as random effects.

Results

The total rainfall for the first year of the experiment (2008) was 436 mm for the first harvest of both the treatment period (May 26 - July 3, 2008), and 387 mm for the second harvest of both the treatment period (July 3- September 3, 2008) (Table 2.3). The total rainfall that the one harvest per season plot received from the time of transplanting to harvest (May 26, 2008 – July 16, 2008), was 129 mm while that for the two harvests per season plot was 194 mm. Total rainfall for the second year, starting April 3, 2009 at emergence, was 765 mm (407 mm for the first harvest of second season (April 3 – June 12, 2009), and 358 mm for the remaining all harvests (June 12-September 11, 2009). The total rainfall during the dormancy period from October 1, 2008 to April 3, 2009 was 669 mm (Table 2.3). Average air temperature, soil temperature and relative humidity for the growing period in 2008 was 26 C, 30C and 68% respectively and for 2009 was 24C, 27C and 70% respectively.

In 2009, the plots with higher stands were first to be infected with powdery mildew and were devoid of dense leaves. The average density of plant stands in second year was 305,000 shoots ha^{-1} after the first harvest and 106,000 shoots ha^{-1} after the second harvest. The plants were grown under shade where higher humidity prevails than in open sun. American skullcap has weak stems which touch the ground when the density of plant population is high, which makes it vulnerable to *Pythium* infection when accompanied by rain. *Pythium* infection may be attributed to lack of proper ventilation, high rainfall and high humidity. There were 14 rain days in July, 16 in August and 16 in September. The relative humidity during July and August of 2009 was high enough to provoke diseases (Fig. 2.2) High density of plants and high humidity are conducive to

diseases such as powdery mildew and *Pythium* infection. Allen et. al (2004) reported that *Pythium* infection is severe in hot, humid, rainy or cloudy weather. As a consequence, our plots were infested with powdery mildew and *Pythium* and there were significant stand losses.

Plant density and dry matter in first season (2008)

No significant differences among treatments were observed for plant density (Table 2.4). However, significant differences in plant density were observed were observed between first and second harvest in the two harvests per season treatment. First harvest yielded 19% higher plant density than second harvest in two harvests per season treatment (Table 2.4).

Average percent dry matter was significantly higher in two harvests per season than one harvest per season ($p= 0.021$). The second harvest in the two harvests per season treatment had significantly higher percent dry matter than did the first harvest ($p=0.048$) (Table 2.4).

Significant differences were observed in the first year (2008) for total dry weight yield. The two harvest per season treatment yielded 1708 kg ha^{-1} and one harvest per season yielded 1256 kg ha^{-1} (Table 2.4). In two harvests per season (2008), first harvest gave significantly higher yield (971 kg ha^{-1}) than second harvest (737 kg ha^{-1}) (Table 2.4).

Flavonoids in first season (2008)

No significant differences in concentrations of flavonoids were found in the first year between the one harvest per season and the two harvests per season treatment (Table 2.5 and Fig. 2.5). However, significant differences were observed for average

yield of baicalin, baicalein and chrysin in first year (Table 2.5). The yield of baicalin and chrysin was 18% and 20% higher in two harvests per season treatment than one harvest per season treatment, respectively (Table 2.5). Baicalein yield was higher with one harvest per season than with two harvests per season. In the two harvests per season treatment, average concentration and yield of baicalein and chrysin was significantly higher in first harvest than in second harvest (Table 2.5, Fig. 2.7 and Fig. 2.8). Similarly, significantly higher yield of wogonin was observed in first harvest than in second harvest in two harvests per season treatment (Table 2.5, Fig. 2.7 and Fig. 2.8).

Plant height, density and dry matter in second season (2009)

In the second year (2009), timing of harvest had no significant effect on plant height (Table 2.6 and 2.7). Average plant height at first harvest (32 cm) was significantly higher than that at second harvest (20 cm) (Table 2.6 and 2.7). First season treatments had no effect on plant height in the second year.

Timing of harvest had no significant effect on plant density (Table 2.6 and 2.7). However, harvest was significant for plant density (Table 2.6). Average plant density at first harvest was 305,000 shoots ha^{-1}, which was 65 % greater than the density at second harvest (Table 2.6 and 2.7).

Timing of harvest and harvest sequence had significant effects on percent dry matter content (Table 2.6 and 2.7). Significant interaction was observed in timing by Year 1 residual treatment effects for percent dry matter content but differences were only in magnitude and not in direction (Table 2.6 and Table 2.8). There was a significant difference between harvests of the late harvest season treatment (Table 2.6 and Table 2.9).Harvest 1 had higher percent dry matter than harvest 2 in late harvest treatment

(Table 2.6 and Table 2.9). At first harvest, harvesting late gave significantly higher percent dry matter than harvesting early (Tables 2.6 and 2.9).

Harvest sequence was significant for dry matter yield (Table 2.6 and Table 2.7). The total dry matter yield of the first harvest was 823 kg ha^{-1} whereas for the second harvest, the yield was only 363 kg ha^{-1} (Table 2.7). Significant interaction in dry matter yield was observed for timing by harvest sequence (Table 2.6, Table 2 .9).The effect of timing on dry matter yield was also significant at first harvest (Table 2.6 and 2.9). At first harvest in 2009, harvesting late gave significantly higher dry matter yield than did harvesting early (Table 2.6 and 2.9). However, at the second harvest in 2009, significantly higher dry matter yield was observed by harvesting early than by harvesting late (Table 2.6 and 2.9). Highest yield of a single harvest was obtained in the first harvest by harvesting late (Table 2.9). The reason for lower yield in second harvest may be attributed to the lower plant stands remaining following plant die-off.

Interestingly, higher dry matter yield and percent dry matter content was associated with harvest at the late bloom stage than with harvest and full bloom stage (Table 2.10) over two years of cultivation (2008-2009).

Flavonoids in second season (2009)

In the second year (2009), time of harvest and the residual effects from year one treatments had no significant effects on flavonoid concentration and yield (Table 2.11). Significantly higher concentration was observed for scutellarein, baicalin and wogonin in second harvest than first harvest, whereas apigenin and baicalein concentration was higher in first harvest than in second harvest (Table 2.12). Interestingly, yields of baicalin, apigenin, baicalein, wogonin and chrysin were significantly higher in first

harvest than in second harvest (Table 2.12). Timing X year 1 residual was significant for wogonin concentration (Table 2.11). Significant timing X harvest interactions were observed for concentration and yield of scutellarein and wogonin, for concentration of baicalin, and for yield of apigenin, baicalein and chrysin (Table 2.14).Late harvest increased concentration of scutellarein at harvest 2 but had no effect at harvest 1, early harvest increased baicalin concentration at harvest 1, but late harvest increased baicalin concentration at harvest 2 and late harvest increased the concentration of wogonin at harvest 1 but had no effect on harvest 2 (Table 2.14). Late harvest increased the yield of apigenin, baicalein, wogonin and chrysin at harvest 1 but early harvest increased the yield at harvest 2 (Table 2.14).

Discussion

Harvesting twice in the first year gave higher dry matter yield, percent dry matter and yield of baicalin and chrysin than harvesting once, and had no effect on these parameters in the second year. This is consistent with Crop Development Branch, Saskatchewan (2005), who reported high yield with two harvests per season in the first year. Harvesting once in the first season gave a higher yield of baicalein in the first year than harvesting twice because the concentration of baicalein was 55% higher with one harvest per season than two harvests per season. Baicalein had higher concentration and yield than other analyzed flavonoids in 2008 whereas baicalin was found to be the highest in concentration and yield in 2009 (Table 2.12 and 2.14). This result is contrary to Similien (2009), who reported that baicalin was found to be the highest in concentration and yield in all harvests followed by baicalein. These differences may be

due to differences in the cultivation method with that of Similien (2009). The concentration of scutellarein, wogonin and chrysin were very low which is in accordance with the results reported by Wills and Stuart (2004), Awad et al. (2003) and Similien (2009). In this experiment, a higher concentration and yield of baicalein, wogonin and chrysin was found than was reported by Similien (2009). However, baicalin concentration and yield was much lower in this experiment than in Similien's (2009) experiment. Higher concentration of baicalein was found in first harvest of each year (Table 2.5 and Table 2.12) which is in accordance with Similien's finding who reported seasonal differences in flavonoid concentration. Scutellarein and apigenin concentration was higher in the second harvest than in the first harvest of each year. Total yield of the flavonoids measured were higher in two harvests per season than one harvest per season in first year and in harvest 1 than in harvest 2 in second year, which may be due to higher dry matter yield for the respective harvest.

Conclusion

At least two harvests of American skullcap per season may be attained, including the first year, if diseases are controlled. Timing of harvest and stage of plant at harvest have an effect on yield of dry matter and flavonoid content of American skullcap. Harvesting twice in the first year was not harmful to second year yield because there were no residual effects from first year treatment in second year yield. Dry matter yield was found to be higher in the late bloom stage of plant at harvesting than full bloom stage. Two harvests per season, including the first year, and harvesting in the late bloom stage in both years is advisable in order to maximize dry matter and yield of

those flavonoids measured (except for baicalein). The experiment should be repeated to determine if three harvests per year in second and subsequent years are feasible.

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Table 2.1 Main field operation from March 23, 2008 to September 11, 2009.

Date(mo/day/year)	Activities	Amount
4/23/2008	Rolled down Rye as cover crops	
5/14/2008	Applied 8-5-5 Nature safe Fertilizer	340 kg
5/15/2008	Applied 4" thick layer of compost in row	
5/20/2008	Planted American skullcap	
6/2/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/5/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/9/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/12/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/16/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/19/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/23/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/26/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
6/30/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/3/2008	1st harvest of 2har/season, year 1	
7/3/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/7/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/10/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/14/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/16/2008	1st harvest of 1har/season, year 1	
7/17/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/21/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/24/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/28/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
7/31/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/4/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/7/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/11/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/14/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/18/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/21/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/25/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
8/28/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/2/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/5/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/8/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/11/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg

9/15/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/18/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/22/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/25/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
9/29/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
10/2/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
10/3/2008	2nd harvest of 2har/season,year1	
10/6/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
10/9/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
10/13/2008	Applied 3-1-1 Pinnacle organic food through drip irrigation	4.52 kg
5/29/2009	Applied 8-5-5 Nature safe Fertilizer	340 kg
6/2/2009	Erected shade structure	
6/12/2009	1st harvest of 3har/season,year2	
6/28/2009	Applied Trilogy to American skullcap	1% solution
7/3/2009	Applied trilogy to American skullcap	1% solution
7/8/2009	1st harvest of 2har/season,year2	
7/10/2009	Applied Trilogy to American skullcap	1% solution
7/17/2009	Applied Trilogy to American skullcap	1% solution
8/6/2009	2nd harvest of 3har/season,year2	
9/11/2009	2nd harvest of 2har/season,year2	

Table 2.2 Harvest dates, stage of growth and plant stand density at harvest in first year (2008) and second year (2009) of trial

Year	Treatments	Date of harvest	Growth stage	Plant density ha^{-1}
2008	2 harvests/season	7/3/2008	Full bloom	108642
2008	1 harvest/season	7/16/2008	Late bloom	77093
2008	2 harvests/season	10/3/2008	Late bloom	91279
2009	Early harvest	6/12/2009	Full bloom	295071*
2009	Late harvest	7/8/2009	Late bloom	183104*
2009	Early harvest	8/6/2009	Full bloom	150937*
2009	Late harvest	9/11/2009	Vegetative stage	192383*

* Shoots density ha^{-1}

Table 2.3 Rainfall Record for E.V Smith Research Center and Education Center, Shorter, AL. May 2008- September 2009

	2008								2009								
Date	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	March	April	May	June	July	August	Sep.
1	X	0	0	0	8.38	0	0	0.25	0	0	22.86	24.89	0	0	0	4.32	0
2	X	0	0	0	0.25	0	0	0	0	0	4.57	1.52	0	0	0	1.78	0
3	X	0	0	6.6	0	0	0	0	1.27	7.87	0	7.62	2.79	0	0	35.81	7.62
4	X	0	0	0	0	0	0	0	9.4	0	0	0	34.8	1.78	0	0	0.25
5	X	0	0	0	0	0	0	5.59	0	0	0	17.27	4.83	60.2	0	7.37	0.51
6	X	0	0	0	0	0	0	0	1.78	0	0	11.94	0	0	18.03	3.81	0.51
7	X	0.51	6.35	0	0	0	0	0	16.76	0	0	0	99.31	0	4.32	0	0
8	X	0	0	10.41	0	0	17.02	0	0	0	0	0	34.29	0	0	0	0.76
9	X	0	42.67	3.3	0	16.51	0	0	0	0	0	0	0	0	5.08	0	0
10	X	0	8.64	35.31	5.59	0	0	5.08	0	0	0	0	0	0	0.25	0	0
11	X	0	1.02	0	2.29	1.02	0	42.42	10.67	0	0	0.76	3.56	0	0.76	0	42.93
12	X	0	19.81	0	0.25	1.02	0	1.78	0	10.41	0	0	0	0	0	12.45	0
13	X	9.91	0	0	0.51	1.02	0	0	0	0	0	40.89	7.87	3.3	13.21	0	0
14	X	14.22	0	0	0.76	1.02	0	0	0	9.14	1.27	0.76	3.56	10.16	4.57	0	0.76
15	X	1.52	0	0	0	1.02	4.32	0	0	0	53.85	0	0	12.19	0	0	32.26
16	X	3.3	0	0	0	1.02	0	6.86	0	0.25	36.32	0	0	0	4.83	0	4.83
17	X	0	0	0	0.25	1.02	0	5.84	0	0	13.72	0	0.76	0	5.59	4.06	24.38
18	X	4.32	0	0	0	8.38	0	1.27	5.08	1.02	0	0	0.51	0	0	6.35	1.52
19	X	0	0	0	0.25	0	0	0	1.27	26.16	0	0	28.19	0	0	0.51	1.02
20	X	0	0	0	0	0	0	0	0	0	0	3.05	0	0	0	0	1.78
21	X	0	9.14	0	0	0	0	0	0	0	0	0	0	0	0	41.15	7.87
22	X	0	19.05	0	0	0	0	0	0	0	0	0.51	0	0	0	12.7	5.59
23	X	0	15.24	3.05	0	0	0	0	0	0	0	0	6.6	0	0.25	0	0
24	X	0	0	116.08	0	56.64	0	0	0	0	0	0	5.59	11.68	0	0	0
25	X	0	0	4.32	0	0.25	0.76	11.18	3.05	0	0	0	7.62	0.25	0	0	0
26	0	0	0	72.64	0	0.25	0	0	0	0	18.29	0	20.83	0	0	0	0
27	0	0	0	0	0	0.25	0	1.02	0	0	65.02	0	1.27	0	0.25	19.56	15.49
28	0	0	0	0.25	0	0.25	0	0.25	2.03	50.29	27.18	0	0	0	0	14.48	0
29	0	8.64	0	0	0	0.25	37.85	0.76	0.76	0	0.51	0	0	0	7.37	9.14	0
30	0	6.35	0	0	0	0.25	32.77	0	0	0	0	0	0	0	7.37	12.45	0
31	0	0	4.32	0	0	0.25	0	0	0	0	0	0	0	0	3.3	5.84	0
Total	**0**	**48.77**	**126.24**	**251.97**	**18.54**	**90.42**	**92.71**	**82.3**	**52.07**	**105.16**	**243.59**	**109.22**	**262.38**	**99.57**	**75.18**	**191.77**	**148.08**

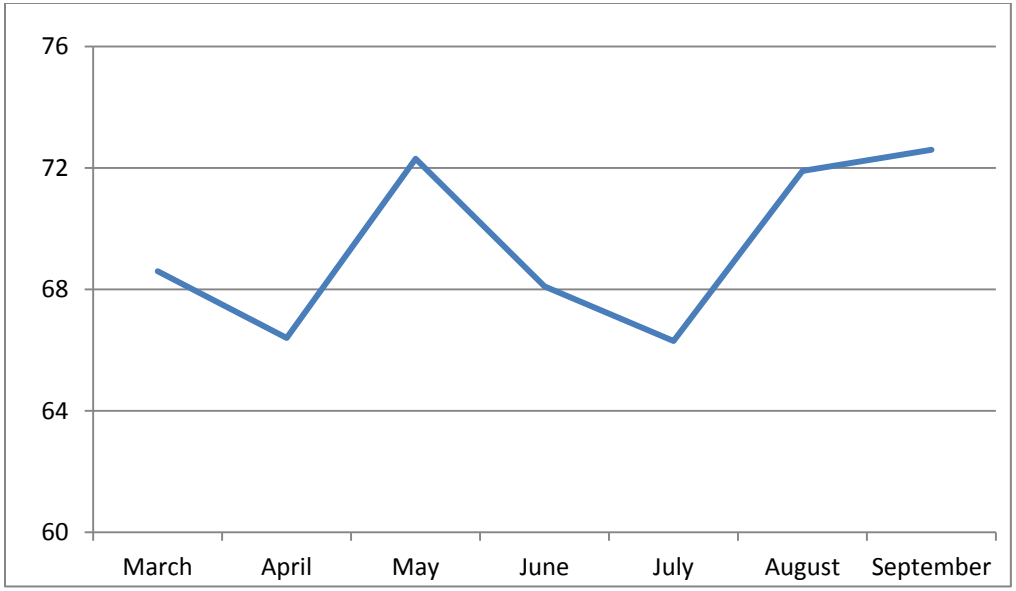

Fig. 2.2 Average relative humidity during the months of second season (2009)

Table 2.4 The effects of number of harvests on plant height, average plant stand, % dry matter and dry matter yield of American skullcap in 2008

Treatments	Harvest	Density (1000 Plants ha^{-1})	% Dry matter	Yield (kg ha^{-1})
2 harvest/season	1	109	19	971
	2	91	27	737
	Combined	100	23	1708
1 harvest/season	1	77	19	1256
1 vs 2 Harvests (Pr>F)		0.256	**0.021**	**0.01**
Harvest 1 vs Harvest 2 (2 Harvest Treatment)		**0.033**	**0.048**	**0.043**

Note: Means were compared using Tukey's method

Bold numbers represent significant difference

Table 2.5 Harvest number and timing effects on concentration and yield of scutellarein, baicalin, apigenin, baicalein, wogonin and chrysin in American Skullcap in 2008

Treatments	**Harvest**	**Concentration (mg g^{-1})**					
		Scutellarein	Baicalin	Apigenin	Baicalein	Wogonin	Chrysin
2 harvest/season	1	0.05	0.23	0.13	1.07	0.11	0.15
	2	0.09	0.26	0.16	0.21	0.08	0.07
	Average	0.07	0.24	0.15	0.64	0.09	0.11
1 harvest/season	1	0.06	0.28	0.12	1.19	0.1	0.14
		<----------Pr>F----------					
1 Harvest vs 2 Harvests		0.524	0.63	0.534	0.117	0.119	0.64
Harvest 1 vs Harvest 2 (2 Harvest Treatment)		0.194	0.662	0.297	**0.007**	0.479	**0.058**
Treatments	**Harvest**	**Yield (g ha^{-1})**					
		Scutellarein	Baicalin	Apigenin	Baicalein	Wogonin	Chrysin
2 harvest/season	1	49	223	126	1038	107	146
	2	66	192	118	155	59	52
	Total	115	415	244	1193	168	198
1 harvest/season	1	75	352	151	1495	126	176
		<----------Pr>F----------					
1 Harvest vs 2 Harvests		0.524	0.508	**0.09**	0.254	**0.017**	0.119
Harvest 1 vs Harvest 2 (2 Harvest Treatment)		0.194	0.401	0.606	0.48	**0.002**	**0.055**

Note: Means were compared using Tukey's method

Bold numbers represent significant difference

Table 2.6 Statistical summary of second year (2009) harvests of American Skullcap

Source of Variation	Height	Density	% dry matter	Yield
	**<----------Pr>F----------> **			
Timing	0.604	0.604	**0.065**	0.909
Year 1Residual	0.184	0.325	0.259	0.987
Timing*Year 1 Residual	0.81	0.265	**0.023**	0.206
Harvests	**<.0001**	**0.006**	**0.046**	**<.0001**
Timing*Harvest	0.496	0.496	0.166	**0.004**
Year 1 Residual *Harvest	0.355	0.92	0.401	0.181
Timing*Year 1 Residual *Harvest	0.897	0.107	0.63	0.957
Year 1 Residual (Early Harvest)‡	0.42	0.258	0.337	0.465
Year 1 Residual (Late Harvest)	0.622	0.415	0.576	0.441
Timing (Harvest 1)	0.053	0.515	**0.011**	**0.005**
Timing (Harvest 2)	0.302	0.877	0.798	**0.02**
Harvests (Early)	0.067	**0.027**	0.61	0.241
Harvests (Late)†	0.028	0.134	**0.08**	**0.012**

‡ Year 1 treatment in early harvest in year 2. †First and second harvests at Late harvest.

Note: Means were compared using Tukey's method

Bold numbers represent significant differences

Table 2.7 Main effects of timing, harvest and year 1 residual on plant height, plant stand, % dry matter and yield of American skullcap in each harvest in year 2 (2009)

Source	Treatment	Height (cm)	Density (1000 shoots ha^{-1})	PDM † (%)	Yield (kg ha^{-1})
Timing	Early harvest	26	223	18	588
	Late Harvest	27	188	21	598
Harvests	Harvest 1	32	305	21	823
	Harvest 2	20	106	18	363
Year 1 residual	1 harvest in 2008	25	239	19	592
	2 harvest in 2008	27	172	20	594
Contrast		**<----------Pr>F---------->			
Early vs Late		0.604	0.604	**0.065**	0.909
Harvest 1 vs Harvest 2		**<.0001**	**0.006**	**0.046**	**<.0001**
Year 1 residual		0.184	0.325	0.259	0.987

† Percent Dry Matter.

Note: Means were compared using Tukey's method.

Bold numbers represent significant difference

Table 2.8 Interaction effects of timing and year 1 residual on plant height, plant stand, % dry matter and dry matter yield of American skullcap in 2009

Year 1 residual (Harvest of 2008)								
	Height (cm)		Density(1000 shoots ha^{-1})		PDM (%)		Yield(kg ha^{-1})	
Treatment	1 harvest	2 harvest	1 harvest	2 harvest	1 harvest	2 harvest	1 harvest	2 harvest
Early harvest	25	27	295	151	18	19	532	644
Late harvest	25	28	183	192	20	22	652	544
	<----------(Pr>F)---------->							
Timing* Year 1 residual	0.810		0.265		**0.023**		0.206	
Year 1 residual (early harvest)	0.420		0.258		0.337		0.465	
Year 1 residual (late harvest)	0.622		0.415		0.576		0.441	

Table 2.9 Interaction effects of timing and harvests on plant height, plant stand, % dry matter and dry matter yield of American skullcap in 2009

Harvest								
	Height (cm)		Density(1000 shoots ha^{-1})		PDM (%)		Yield(kg ha^{-1})	
Treatment	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2
Early harvest	30	23	346	100	19	18	645	532
Late harvest	35	18	264	111	23	19	1002	193
	<----------(Pr>F)---------->							
Timing*harvest	0.496		0.496		0.166		**0.004**	
Timing (harvest 1)	**0.053**		0.515		**0.011**		**0.005**	
Timing (harvest 2)	0.302		0.877		0.798		**0.020**	

Note: Means were compared using Tukey's method

Bold numbers represent significant difference

Table 2.10 Effect of growth stage at harvest on yield and percent dry matter of American skullcap over two years (2008-2009)

Effect	PDM (%)	Yield (kg ha^{-1})
Late bloom	23	999
Full bloom	19	665
Pr>F	**0.0012**	**0.0013**

Table 2.11 Significance levels (Pr>F) for main effect and interactions for scutellarein, baicalin, apigenin, baicalein, wogonin and chrysin concentration and yield of American skullcap in 2009

Scutellarein	Concentration	Yield
Timing	0.185	0.662
Year 1 Residual	0.698	0.101
Timing*Year 1 Residual	0.132	0.183
Harvests	**0.002**	0.183
Timing*Harvest	**0.029**	**0.041**
Timing*Year 1 Residual *Harvest	0.347	**0.078**

Baicalin	Concentration	Yield
Timing	0.122	0.471
Year 1 Residual	0.739	0.505
Timing*Year 1 Residual	0.874	0.768
Harvests	**0.012**	**0.001**
Timing*Harvest	**0.001**	0.364
Timing*Year 1 Residual *Harvest	0.147	0.651

Apigenin	Concentration	Yield
Timing	0.429	0.347
Year 1 Residual	0.533	0.152
Timing*Year 1 Residual	0.123	0.989
Harvests	**0.004**	**<.0001**
Timing*Harvest	0.339	**0.002**
Timing*Year 1 Residual *Harvest	0.455	0.414

Baicalein	Concentration	Yield
Timing	0.966	0.61
Year 1 Residual	0.835	0.147
Timing*Year 1 Residual	0.648	0.359
Harvests	**0.08**	**<.0001**
Timing*Harvest	0.803	0.005
Timing*Year 1 Residual *Harvest	**0.045**	**0.042**

Wogonin	Concentration	Yield
Timing	0.169	0.803
Year 1 Residual	0.311	0.237
Timing*Year 1 Residual	**0.013**	0.194
Harvests	**0.031**	**0.009**
Timing*Harvest	**0.023**	**0.038**
Timing*Year 1 Residual *Harvest	**0.049**	0.429

Chrysin	Concentration	Yield
Timing	0.939	0.459
Year 1 Residual	0.207	0.551
Timing*Year 1 Residual	0.161	0.674
Harvests	0.705	**0.001**
Timing*Harvest	0.161	**0.029**
Timing*Year 1 Residual *Harvest	**0.026**	0.149

Table 2.12 Harvest effects on concentration and yield of scutellarein, baicalin, apigenin, baicalein, wogonin and chrysin in American Skullcap in 2009

		Concentration (mg g^{-1})					
Source	Treatments	Scutellarein	Baicalin	Apigenin	Baicalein	Wogonin	Chrysin
Timing	Early Harvest	0.028	0.321	0.181	0.23	0.062	0.068
	Late Harvest	0.038	0.378	0.198	0.228	0.075	0.067
Harvests	Harvest 1	0.022	0.301	0.224	0.256	0.057	0.066
	Harvest 2	0.044	0.398	0.154	0.201	0.080	0.069
Year 1 residual	1 harvest	0.032	0.355	0.182	0.232	0.073	0.073
	2 harvest	0.034	0.343	0.196	0.226	0.063	0.062
		**<----------(Pr>F)----------> **					
Early vs Late		0.185	0.122	0.429	0.966	0.169	0.939
Harvest 1 vs Harvest 2		**0.002**	**0.012**	**0.004**	**0.080**	**0.031**	0.705
Year 1 residual		0.698	0.739	0.533	0.835	0.311	0.207
		Yield (g ha^{-1})					
Source	Treatments	Scutellarein	Baicalin	Apigenin	Baicalein	Wogonin	Chrysin
Timing	Early Harvest	16	189	106	135	37	40
	Late Harvest	23	226	118	136	45	40
Harvests	Harvest 1	18	248	184	211	47	54
	Harvest 2	16	144	56	73	29	25
Year 1 residual	1 harvest	19	210	108	137	43	43
	2 harvest	20	204	116	134	37	37
		**<----------(Pr>F)----------> **					
Early vs Late		0.662	0.471	0.247	0.61	0.803	0.459
Harvest 1 vs Harvest 2		0.183	**0.001**	**<.0001**	**<.0001**	**0.009**	**0.001**
Year 1 residual		0.101	0.505	0.152	0.147	0.237	0.551

Note: Means were compared using Tukey's method

Bold numbers represent significant difference

Table 2.13 Harvest timing and year 1 residual treatment effects on concentration of scutellarein, baicalin, apigenin, baicalein, wogonin and chrysin in 2009

Source	year 1 residual	Scutellarein	Baicalin	Apigenin	Baicalein	Wogonin	Chrysin	TFC‡
		<----------Concentration (mg g^{-1})---------->						
Early harvest	1 harvest	0.022	0.330	0.157	0.226	0.053	0.066	**0.854**
	2 harvest	0.035	0.311	0.205	0.233	0.070	0.068	**0.922**
		<----------Pr>F---------->						
		0.096	0.832	0.251	0.893	0.225	0.923	
Late harvest	1 harvest	0.041	0.382	0.208	0.239	0.093	0.078	**1.041**
	2 harvest	0.033	0.374	0.187	0.219	0.057	0.055	**0.925**
		<----------Pr>F---------->						
		0.608	0.941	0.550	0.628	0.128	0.1156	

‡Total Measured Flavonoid Concentration

Means were compared using Tukey's method

Table 2.14 Interaction effect of timing X harvest on concentration and yield of scutellarein, baicalin, apigenin, baicalein, wogonin and chrysin in 2009

Harvests					
Flavonoids		Concentration(mg g^{-1})		Yield (g ha^{-1})	
	Treatments	Harvest 1	Harvest 2	Harvest 1	Harvest 2
Scutellarein	Early harvest	0.02	0.03	15	17
	Late harvest	0.02	0.05	19	10
	<----------(Pr>F)---------->				
	Timing * Harvest	**0.029**		**0.041**	
Baicalin	Early harvest	0.35	0.28	234	141
	Late harvest	0.24	0.51	239	95
	<----------(Pr>F)---------->				
	Timing * Harvest	**0.001**		0.364	
Apigenin	Early harvest	0.21	0.16	141	89
	Late harvest	0.24	0.15	245	30
	<----------(Pr>F)---------->				
	Timing * Harvest	0.339		**0.002**	
Baicalein	Early harvest	0.26	0.19	169	106
	Late harvest	0.25	0.21	259	41
	<----------(Pr>F)---------->				
	Timing * Harvest	0.803		**0.004**	
Wogonin	Early harvest	0.06	0.06	39	35
	Late harvest	0.09	0.05	52	18
	<----------(Pr>F)---------->				
	Timing * Harvest	**0.023**		**0.038**	
Chrysin	Early harvest	0.07	0.06	48	35
	Late harvest	0.06	0.07	58	14
	<----------(Pr>F)---------->				
	Timing * Harvest	0.161		**0.029**	

Chapter 3. Nitrogen, Phosphorus and Potassium Effects on Biomass Yield and Flavonoid Content of American Skullcap (*Scutellaria lateriflora)*

Abstract

American Skullcap (*Scutellaria lateriflora)* is a member of the mint family (*Labiatae* or *Lamiaceae*). *S. lateriflora* is a medicinal herb of North America traditionally used for its mild relaxant properties attributed to its content of flavonoids. Information on optimum dosage of nitrogen (N), phosphorus (P) and potassium (K) fertilizer for high dry matter yield and flavonoid yield is lacking. Greenhouse experiments were conducted (September 2010 and January, 2011) to determine the effects of N, P and K fertilizer on biomass yield and flavonoid content of American skullcap. Plants were grown in fritted clay in plastic pots sized 29 cm diameter and 28 cm height. Separate experiments were carried out for N, P and K. Each experiment was carried out two times and consisted of six levels of each nutrient. The N treatment levels for the N experiment were: 0 kg N ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 400 kg ha^{-1} and 800 kg ha^{-1}, P levels for the P experiment were: 0 kg ha^{-1}, 20 kg ha^{-1}, 40 kg ha^{-1}, 80 kg ha^{-1}, 160 kg ha^{-1} and 320 kg ha^{-1} and K levels for the K experiment were: 0 kg ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 300 kg ha^{-1} and 400 kg ha^{-1}. Each treatment received a standard rate of micronutrient solution as needed as well as the highest level of the other two major elements. Dry matter yield and uptake of nutrients increased with addition of fertilizers. The regressions gave maxima for dry matter, baicalein yield and chrysin yield at 446 kg N ha^{-1}, 412 kg N ha^{-1} and 351 kg N ha^{-1} for N fertilizer respectively. Dry matter yield

exhibited linear response to P application. The yield of scutellarein, baicalin, baicalein and chrysin increased with addition of P. The regression gave maximum for dry matter at 208 kg K ha^{-1} for K fertilizer. A linear response to K fertilization was observed for scutellarein concentration.

Fertilization of American skullcap with N, P and K increased shoot yield, flavonoid content and N and P uptake in above-ground parts. Phosphorus application had the greatest effect on the flavonoids analyzed, whereas K had least, which may be attributed in part to the presence of K in the fritted clay medium.

Introduction

American skullcap (*Scutellaria lateriflora)* is a perennial herb in the Lamiaceae family indigenous to North America, growing in wet places from Canada to Florida and westward to British Columbia, Oregon and New Mexico (Gafner et al. 2003, Bergeron et al. 2005). American skullcap has been used for over 200 years as a mild relaxant and has long been hailed as an effective therapy for anxiety, nervous tension, convulsions Foster, 1996), epilepsy, cholera (Newall et al. 1996), rabies, diarrhea, digestive problems (Greenfield and Davis, 2004), promotion of menstruation, elimination of after birth (Wohlmuth, 2007), anxiety, sleeplessness and various types of spasms in Europe and North America (King, 1866; Mills and Bone, 2000; Rafinesque, 1830; Sarris, 2007; Wojcikowski et al., 2007). Extracts of American skullcap and the isolated flavonoids from the extracts have antioxidant, anticancer, and antiviral properties (Awad et al., 2003).

There is increasing demand for American skullcap as a complementary and alternative medical treatment for anxiety (Greenfield and Davis, 2004). Despite the long-term and widespread use of these medicinal plants, there is limited information on the horticultural production of *Scutellaria* species (Stutte et al.2008). Wills and Stuart (2004) provided a general overview of the production of American skullcap in Australia. Greenfield and Davis (2004) established general guidelines for field production of American skullcap in North Carolina and Janke et al. (2005) gave production recommendations for small farmers in Kansas. Similien (2009) demonstrated that application of a combination of nutrients increased yield but the response to individual nutrients was not determined. Three experiments were carried out in the greenhouse to determine the effect of major elements (N, p and K) on biomass yield and flavonoid content of American skullcap.

Materials and Methods

Growing medium preparation

The experiment was conducted in the greenhouse of Plant Science Research Center, Auburn University, AL. The growing medium used was fritted clay (Xtrasorb Plus Absorbent, Moltan Company, Memphis, TN). Due to low pH and high electrical conductivity of fritted clay, the clay was leached with tap water until the pH and electrical conductivity (EC) reached acceptance levels of pH 6- 6.5 and EC of 100-250 µS/cm (microSiemens/centimeter). The initial pH was near 4 and electrical conductivity was around 2000 µS/cm which was brought to around 6.5 and 150 µS/cm respectively. The average nutrients content of sample fritted clay was 1265 mg N kg^{-1}, 33 mg P kg^{-1} and

377 mg K kg^{-1} after washing. The fritted clay was put into the 144 black plastic pots (Nurseries Supplies Inc., Tustin, CA) sized 29 cm diameter and 28 cm height. Garden fabric was placed in the bottom of the pots before adding the potting medium. The experiment was conducted in two lots in two different greenhouses, zones 5 and 11, hereafter referred to as greenhouses 1 and 2, respectively.

Seedling establishment and transplantation

Seeds of *Scutellaria lateriflora* were obtained from Horizon Herbs LLC. William, OR 91544. The seeds for the first set of trials (Lot # 5637) were wrapped in a moist paper towel and cold stratified for 7 days at 5°C starting on August 15, 2010 for seven days .The seeds were then sown in moist potting mix and transferred to an Adaptis Multi-Application Chamber (Conviron, East Greenbush, NY) for germination on August 22, 2010. The temperature in the growth chamber was maintained at 25 C and 12 hours of light per day. Watering was done on a daily basis. The potting mix (Sunshine Professional Peat-Lite Mixes # 8 / LC 8, Sun Gro Horticulture Canada Ltd) was formulated with Canadian sphagnum peat moss, coarse grade perlite, coarse grade vermiculite, dolomitic limestone, gypsum and a long lasting wetting agent. The seedlings were transplanted in the greenhouse after the seedlings reached 5cm height on September 20, 2010. A second batch of seeds was obtained from Horizon Herbs (Lot # 5637) were cold stratified as described previously on January 14, 2011. The seeds were transferred to another growth chamber (Pro- Grow, Propagating Chamber, Model PC- 70- Dual zone, Pro-Grow Supply Corporation, Brookfield, WI) on January 21, 2011. The temperature and the light hours were maintained at 25 C and 12 hours per day respectively in the growth chamber. Adequate moisture was maintained

automatically by the chamber. The seedlings were transplanted to pots in a second greenhouse area on January 21, 2011, after the seedlings reached 5cm height.

Experimental design and agronomic management

Three experiments were carried out, one each for N, P and K in both the greenhouse areas. These experiments followed guidelines published by Tennessee Valley Authority National Fertilizer Development Center, Muscle Shoals, Alabama (Allen et al., 1976). The experimental design was completely randomized design with 4 replications. Each experiment consisted of six different levels of one nutrient. The N levels of treatment for the N experiment were: 0 kg ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 400 kg ha^{-1} and 800 kg ha^{-1} ; P levels for the P experiment were: 0 kg ha^{-1}, 20 kg ha^{-1}, 40 kg ha^{-1}, 80 kg ha^{-1}, 160 kg ha^{-1} and 320 kg ha^{-1} ; and K levels for the K experiment were: 0 kg ha^{-1}, 50 kg ha^{-1}, 100 kg ha^{-1}, 200 kg ha^{-1}, 300 kg ha^{-1} and 400 kg ha^{-1}. Laboratory grade ammonium nitrate (NH_4NO_3), monocalcium phosphate ($Ca(H_2PO_4)_2$) and potassium chloride (KCl) were used as a source of nitrogen, phosphorus and potassium respectively. The area was calculated based upon diameter at the top of the pot. In each experiment, the highest levels of other two nutrients used in the other experiments were applied as non- limiting elements. All levels of experimental nutrients were applied in two split doses - one before planting and another after 30 days of transplanting, that is in the vegetative growth stage. For the N experiment, a 31.25 ml aliquot of solution was equal to a rate of 50 kg N ha^{-1} while 500 ml of solution was equal to 800 kg N ha^{-1}. For the P experiment, a 31.25 ml aliquot of solution was equal to 20 kg P ha^{-1} while 500 ml of solution was equal to 320 kg P ha^{-1}. For the K experiment, a 31.25 ml aliquot of solution equaled 50 kg K ha^{-1} rate while 500

ml of solution was equal to 400 kg K ha^{-1}. Secondary and micronutrient solutions were prepared with reagent grade chemicals and applied to all pots at the rate of 14mg Mg per pot as magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 14mg Fe per pot as iron sulfate ($FeSO_4.7H_2O$), 11mg Mn per pot as manganese sulphate ($MnSO_4.H_2O$), 12 mg Zn per pot as zinc sulphate ($ZnSO_4.7H_2O$), 4 mg Cu per pot as copper sulphate ($CuSO_4.5H_2O$) and 1.4mg B per pot as sodium borate ($Na_2B_4O_7.10H_2O$), respectively, per 100ml of solution were applied to all pots along with the first dose of experimental levels. Pots were rotated after each irrigation to minimize the experimental error caused by uneven sunlight and other variables within greenhouse.

Table 3.1 Micronutrients applied in the nutrient response trials.

Compound	Formula	Rate (mg)
Magnesium sulphate	$MgSO_4.7H_2O$	14
Iron sulphate	$FeSO_4.7H_2O$	14
Manganese suphate	$MnSO_4.H_2O$	11
Zinc sulphate	$ZnSO_4.7H_2O$	12
Copper sulphate	$CuSO_4.5H_2O$	4
Sodium borate	$Na_2B_4O_7.10H_2O$	1.4

Harvesting and Analysis

American skullcap in greenhouse 1 was harvested on January 12, 2011 after the majority of the plants started wilting and dying due to unexpected problems. Before harvesting, height of the plants was taken. The plants were cut 2 cm above the soil using scissors and immediately weighed to determine the fresh weight. The plants were dried at 50 C for 3 days and dry matter yield weight was taken. On April 7, 2011, American skullcap of experimental unit 2 was harvested after the flowering of more than

50% plants was observed. Heights of each plant were noted before harvesting. The plants were dried at 50 C for 3 days and dry matter yield weight was taken.

Only the plants from greenhouse 2 were used for analysis of flavonoids and nutrient uptake. The plants from greenhouse 1 were not used for analysis due to inadequate dry matter yield. The samples were ground and passed through a 1mm mesh screen using the Thomas Wiley Laboratory mill, Model 4, Thomas Scientific, USA. The samples were then analyzed for total N concentration by dry combustion using a LECO TruSpec CN (Leco Corp, St. Joseph, MI). P and K concentrations were determined according to by inductively coupled argon plasma spectrophotometry (ICAP), (SPECTROCIROS CCD, Side-on plasma. Germany). Nutrient uptake was calculated as the product of nutrient concentration and dry matter yield (DMY) of *Scutellaria lateriflora* tops.

Flavonoid Determination

Tissue samples were ground and passed through a 1mm mesh screen using a Thomas Wiley Laboratory mill, Model 4, Thomas Scientific, USA. After that ground samples were packed in Whirl-Pac air proof bag and stored at 25 C for chemical analysis.

Flavonoid content was determined by the reversed phase high performance liquid chromatography (RP-HPLC) procedure at the National Center for Natural Products Research at the University of Mississippi. Flavonoid content was established using finely ground samples. High performance liquid chromatography grade solvents methanol (MeOH) and acetonitrile (HPLC grade) purchased from VWR International L.L.C (Suwanee, GA). Flavone standards baicalin (95%), baicalein (98%), scutellarein

(pure), apigenin (98%), 6- hydroxyflavone (97%), wogonin (98%), Chrysin (pure) and luteolin (pure) were used.

Powdered samples weighing 5 g were mixed with standard Ottawa sand (VWR International L.L.C.) to prevent sample compaction and facilitate extraction which was loaded in extraction cartridges. Extraction of plant materials was performed using Accelerated Solvent Extraction (ASE®) apparatus (Dionex Corp., Sunnyvale, CA) at the USDA-Agricultural Research Service Natural Products Utilization Research Unit (USDA, ARS, NPURU). Extraction parameters applied were 1000 psi pressure, 40 C temperature, 10 min static time, 90 sec purge time, 100% flush volume, 4 cycles, 0 min pre heat, 5 min heat and extraction solvent MeOH: H_2O (80:20). The ASE extract were then transferred to 20 ml tared vial and were concentrated under vacuum using a Savant SpeedVac (Model SPD121P; Savant Instruments, Inc., Holbrook, NY). After speed vacuum, 20-30 mg of dried extracts was transferred to 2 ml capacity universal screw top vials (Micro Solv Technology Corporation, NJ, USA). Contents of each vial were mixed with 0.1% acetic acid in MeOH and sonicated for 30 minutes. An internal standard (IS) of 600ug/ml 6-Hydroxyflavone was added to the solution and put in a vortex machine. Solutions were transferred to another 2 ml vial by filtering with syringe filter (4 mm, nylon, 0.2 um pore size filter) using Nalgene (VWR International L.L.C., GA) and were ready to be analyzed by high pressure liquid chromatography (HPLC) for levels of flavonoids.

Samples and standards were analyzed on a Hewlett-Packard 1100 HPLC equipped with evaporation light scattering detector (ELSD 2000) using an Inertsil ODS-2 5 µ column. The mobile phase consisted of 0.005% phosphoric acid (solvent A) and

acetonitrile (solvent B). All samples and standard injections were analyzed at room temperature using a nonlinear gradient from 70%: 30% (H_2O with 0.005% H_3 PO_4: acetonitrile) to 30%:70% (H_2O with 0.005% H_3 PO_4 : acetonitrile) over 30 min run at a flow rate of 1 $mL \cdot min^{-1}$. Analytes were detected at 270 nm with a reference of 550 nm by the evaporation light scattering detector (ELSD 2000). The flavonoids were quantified from chromatograms of the standards with 6-hydroxyflavone as internal standard. Flavonoid yield was obtained by the product of flavonoid concentration (mg g^{-1}) and the total dry matter yield (g pot^{-1}) and expressed in milligram per pot.

Data analysis

All data were analyzed using linear regression in the PROC Mixed procedure of SAS Version 9.2 (SAS Institute, Cary, NC). Effects and interactions were determined using F-tests.

Results and Discussions

Due to die-back, poor growth and meager harvestable plant material in greenhouse 1, statistical analysis was carried out only on dry matter and height. Flowering was first observed mostly on the treatments that gave the highest dry matter yield in all of the experiments, suggesting the direct relationship between flowering and dry matter in American skullcap.

Baicalin was highest in average concentration and yield (Table 3.3) which is in accordance with results reported by Wills and Stuart (2004), Bergeron and Gafner (2005), Awad (2003) and Similien (2009). Chrysin was found in very low concentration

and yield (Table 3.3) which supports the results reported by Wills and Stuart (2004) and Bergeron and Gafner (2005). Apigenin and luteolin were not detected in most of the samples and were not analyzed statistically.

Nitrogen

Nitrogen fertilizer application significantly increased dry matter yield in both the greenhouses and height in greenhouse 1(Table 3.2), although only the quadratic coefficients were significant, indicating a curvilinear effect. In greenhouse 1, the regression curve gave a maximum predicted shoot dry matter yield of 2.74 g pot^{-1} at 425 kg N ha^{-1} and gave maximum predicted height of 7 cm at 450 kg N ha^{-1} (Fig. 3.1and 3.2). In greenhouse 2, regression analysis gave a maximum predicted shoot yield of 22.74 gram pot^{-1} at 446 kg N ha^{-1} for dry matter (Fig.3.3). This indicates a strong relationship between N fertilization rate and shoot biomass yield. Dry matter yield decreased gradually with the addition of N fertilizer beyond 446 kg N ha^{-1}. Zhang et al, (2007) developed a model of optimum N fertilizer rate for *Scutellaria baicalensis* which gave highest predicted yield at between 226 Kg N ha^{-1} to 200kg N ha^{-1}.The optimum fertilizer rate for growth and yield of American skullcap has not been well documented or researched.

No significant effect of N was observed for scutellarein concentration and yield (Table 3.4). Baicalin was detected in the nitrogen trial but only in one replication of each level, which is in contrast to the findings of Similien (2009), who reported that baicalin was the most abundant flavonoid in American skullcap. Nitrogen application had significant effects on concentration and yield of baicalein (Table 3.4). Baicalein concentration and yield exhibited quadratic responses to N application (Fig. 3.4 and

3.5). Regression analysis gave maximum predicted yield of 1622 mg baicalein pot^{-1} at 412 kg N ha^{-1}. Nitrogen application had no effect on chrysin concentration but gave a quadratic response in chrysin yield (Table 3.4 and Fig.3.6), which may be attributed to the increased dry matter yield with N application. Regression analysis gave a maximum predicted yield of 0.0076 mg chrysin pot^{-1} at 351 kg N ha^{-1}.

Phosphorus

Above-ground yield and height of American skullcap exhibited linear responses to phosphorus fertilizer application in the greenhouse 1 and quadratic responses in greenhouse 2 (Table 3.2; Fig.3.7, 3.8, 3.9 and 3.10). Dry matter yield in greenhouse 1 increased with every increment rate of P fertilizer (Fig 3.7). Zhang et al, (2007) determined that the optimum phosphorus fertilizer rate for highest yield of *Scutellaria baicalensis* was between 306 Kg P ha^{-1} and 446 kg P ha^{-1}. The dry matter yield for American skullcap for phosphorus ranged from 0.78 gram pot^{-1} without P application to 16.22 gram pot^{-1} at the high rate of P application (320 kg P ha^{-1}).

Phosphorus application had a quadratic effect on scutellarein yield but had no significant effect on scutellarein concentration (Table 3.4 and Fig 3.12). Phosphorus application did not have a significant linear effect on baicalin concentration, but the quadratic effect was significant (Table 3.4). Phosphorus had a linear effect on baicalin yield (Table 3.4and Fig. 3.13). The means of scutellarein and baicalin yields were 1890 mg pot^{-1} and 2452 mg pot^{-1} respectively. A quadratic effect was observed for baicalein concentration but the yield of baicalein was linearly significant (Fig 3.13 and 3.14). Phosphorus application had no effect on chrysin concentration (Table 3.4), but there was a linear effect on chrysin yield (Fig 3.15), reflecting the increase in biological yield

with P application. The average yields of baicalein and chrysin were 155 mg pot^{-1} and 0.0022 g pot^{-1}, respectively.

Potassium

Application of K had no significant effect on dry matter yield and height in the greenhouse 1 (Table 3.2). However, in greenhouse 2, above-ground yield showed a significant curvilinear response to potassium fertilizer application (Table 3.2 and Fig.3.16). Regression analysis gave a maximum predicted value of 19.34 gram pot^{-1} at 208 kg K ha^{-1} for dry matter. Dry matter yield decreased gradually beyond from 208 kg K ha^{-1} with the addition of K fertilizer. This is in accord with the findings of Zhang et al, (2007) who documented the optimum potassium fertilization dosage for *Scutellaria baicalensis* above ground yield to be 214 kg ha^{-1}. The limited response to K application may be attributed to the high content of K in the fritted clay.

Potassium application exhibited a linear relation for scutellarein concentration but had no effect on scutellarein yield (Table 3.4). Potassium application had no effect on scutellarein, baicalin, baicalein and chrysin concentration or yield (Table 3.4).

Nutrient Uptake

Nitrogen uptake in above-ground parts of American skullcap exhibited a linear response to N fertilizer application (Table 3.5 and Fig. 3.17). Nitrogen uptake increased from 118 mg N pot^{-1} to 691 mg N pot^{-1} with increased nitrogen fertilization rate. Phosphorus uptake exhibited a linear response to P fertilizer application (Table 3.5 and and Fig. 3.20) and phosphorus uptake increased from 4 mg P pot^{-1} with no P fertilizer to 222 mg P pot^{-1} at the 320 kg P ha^{-1}rate. Potassium uptake in above-ground skullcap

exhibited a curvilinear response to K fertilizer application (Table 3.5 and Fig.3.23). Regression analysis gave a maximum predicted uptake of 213 kg K ha^{-1}.

Significant but modest effects of N application on P and K uptake were observed - both exhibited quadratic responses (Table.3.5, Fig.3.18 and 3.19). Regression analysis gave maximum predicted values of 199 mg P pot^{-1} uptake at 392 kg N ha^{-1} and 1283 mg K pot^{-1} at 406 kg N ha^{-1}. Phosphorus fertilizer application resulted in a linear increase in nitrogen and potassium uptake (Table 3.5, Fig 3.21 and 3.22). Fertilization with K had no effect on nitrogen and phosphorus uptake (Table 3.5).

Conclusion

American skullcap responded well to application of N, P and K fertilizer. Nitrogen and phosphorus application increased N and P uptake in above-ground parts as well as biological yield. Dry matter yield increased to a maximum nitrogen fertilization rate (425 kg N ha^{-1} in greenhouse 1 and 446 kg N ha^{-1} in greenhouse 2) and decreased beyond that rate. Highest predicted yield of baicalein and chrysin were obtained with 412 kg N ha^{-1} and 351 kg N ha^{-1}, respectively. The optimum rate of nitrogen for shoot biomass yield and flavonoid yield are within the range of 350 kg ha^{-1} to 450 kg ha^{-1}. Further research, under field conditions, is required to validate the optimum rate of nitrogen found in this experiment.

Dry matter yield increased with every increment of phosphorus fertilizer applied in both greenhouses. Phosphorus fertilization increased shoot biomass yield, N and P uptake in above-ground plant parts and the yield of each flavonoid analyzed. In this experiment, phosphorus had the greatest effect on dry matter, flavonoids yield and

nutrient efficiency. Higher rates of phosphorus must be included in future experiments to determine optimum fertilizer rate for P.

Fertilization with K resulted in curvilinear responses in above-ground dry matter yield and K uptake, with maxima at 208 kg K ha^{-1} and 213 kg K ha^{-1}, respectively. No other parameters in potassium experiment were significant. The limited response to K application may be due to the presence of a high amount of potassium in the leached fritted clay.

Multi-locational field trials are needed to validate these findings under field conditions.

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Table 3.2 Significance levels for N, P and K application rate effects on dry matter yield, height and uptake of American skullcap in greenhouse 1 and greenhouse 2

		DMY†	Height	DMY†	Height
		Greenhouse 1		Greenhouse 2	
Element	Effect	<------Pr>F------>		<-------Pr>F------->	
Nitrogen	Rate	0.5808	0.3385	0.2862	0.3594
	Rate*Rate	**0.0156**	**0.033**	**0.0115**	0.2319
Phosphorus	Rate	**0.0007**	**0.0047**	**<.0001**	**<.0001**
	Rate*Rate	0.1229	0.2495	**0.0506**	**0.0104**
Potassium	Rate	0.3402	0.7005	0.3401	0.9354
	Rate*Rate	0.7342	0.8802	**<.0001**	0.7448

† DMY= Dry Matter Yield.
Bold numbers represent significance difference

Table 3.3 Mean concentration and yield of flavonoids of American skullcap on application rate of N, P and K

	Scutellarein	Baicalin	Baicalein	Chrysin		Scutellarein	Baicalin	Baicalein	Chrysin	
	<------Mean Concentration (ug/g)--------->				TFC†	<----------Yield (ug/pot)---------->				TFY‡
Nitrogen	87.08	NA	22.31	0.0004	109.39	1181	NA	379	0.0058	1560
Phosphorus	225.29	229.61	26.48	0.0004	481.38	1250	1730	155	0.0022	3135
Potassium	109.25	153.16	17.18	0.0003	279.59	3014	4327	272	0.0047	7613
Average	140.54	191.39	21.99	0.0003	290.12	1815	3029	269	0.0042	4103

†Total Measured Flavonoid Concentration, ‡ Total Measured Flavonoid Yield

Table 3.4 Significance levels for nitrogen, phosphorus and potassium application rate effects on concentration and yield of flavonoids of American skullcap

		Scutellarein	Baicalin	Baicalein	Chrysin	Scutellarein	Baicalin	Baicalein	Chrysin
		Concentration				Yield			
Element	Effect	<----------Pr>F---------->							
Nitrogen	Rate	0.3222	NA	0.8003	0.2828	0.3323	NA	0.7604	0.3289
	Rate*Rate	0.8374	NA	**0.0165**	0.2817	0.6947	NA	**0.0022**	**0.0528**
Phosphorus	Rate	0.3837	0.5351	0.2315	0.1876	**0.0040**	**0.0098**	**<.0001**	**<.0001**
	Rate*Rate	0.2159	0.6769	**0.0551**	0.5662	**0.0252**	0.9239	**0.0955**	0.3546
Potassium	Rate	**0.0735**	0.2530	0.3056	0.4545	0.7155	0.1463	0.3295	0.9444
	Rate*Rate	0.6820	0.4687	0.1580	0.1265	0.8377	0.6430	0.3288	0.5383

Bold numbers represent significance difference

Table 3.5 Significance levels for nitrogen, phosphorus and potassium application rate effects on nutrient uptake in American skullcap in greenhouse 2

Nitrogen			Phosphorus			Potassium		
Element	Effect	Pr>F	Element	Effect	Pr>F	Element	Effect	Pr>F
Nitrogen Uptake	Rate	**<.0001**	Phosphorus Uptake	Rate	**<.0001**	Potassium Uptake	Rate	0.3375
	Rate*Rate	**0.0164**		Rate*Rate	0.1036		Rate*Rate	**0.0013**
Phosphorus Uptake	Rate	0.8237	Nitrogen Uptake	Rate	**<.0001**	Nitrogen Uptake	Rate	0.5455
	Rate*Rate	**0.0334**		Rate*Rate	0.2479		Rate*Rate	0.1892
Potassium Uptake	Rate	0.9423	Potassium Uptake	Rate	**<.0001**	Phosphorus Uptake	Rate	0.5901
	Rate*Rate	**0.0145**		Rate*Rate	0.321		Rate*Rate	0.1956

Bold numbers represent significance difference

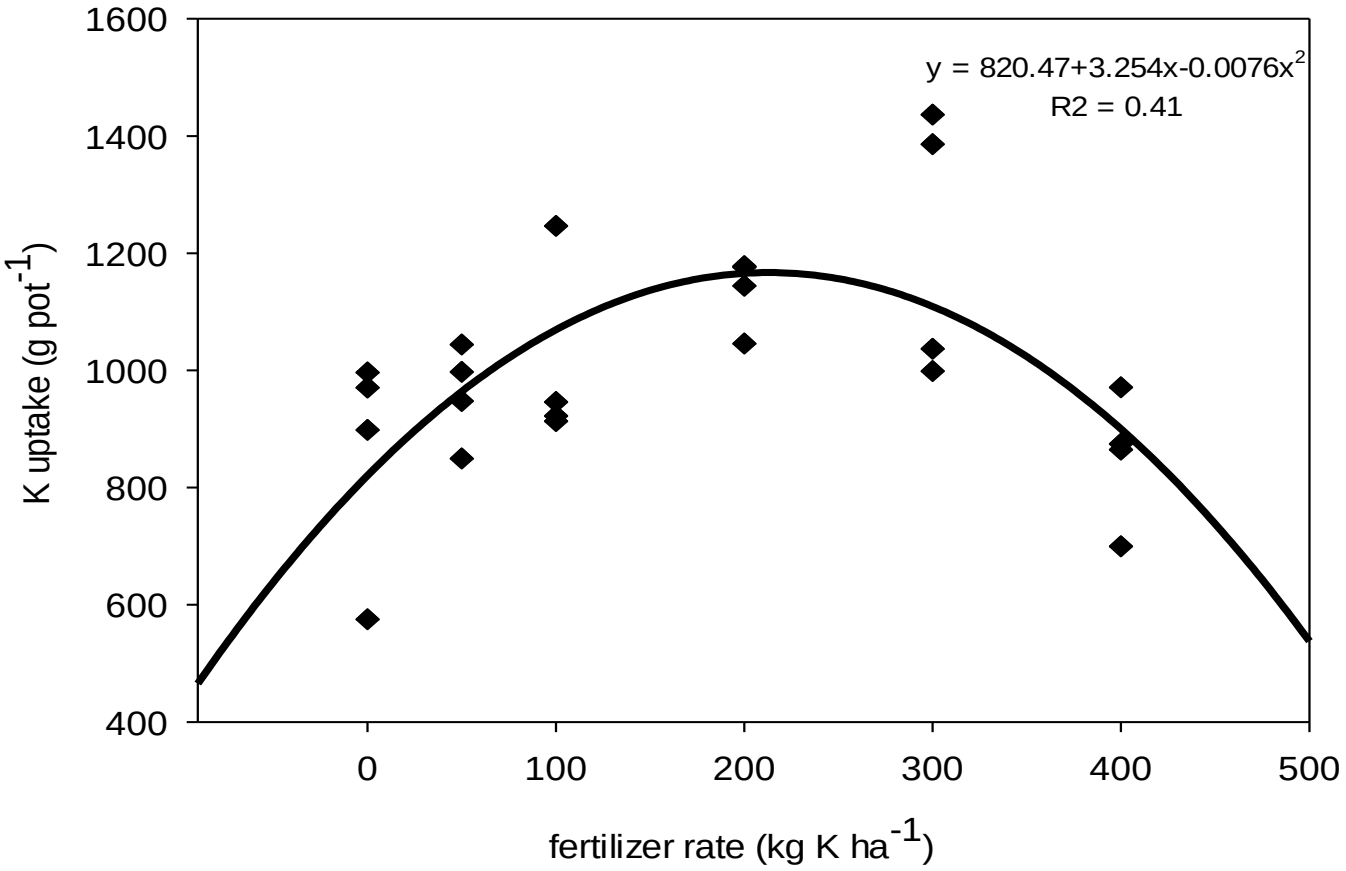

Fig. 3.1 Nitrogen fertilizer effects on above-ground dry matter in American skullcap in greenhouse 1

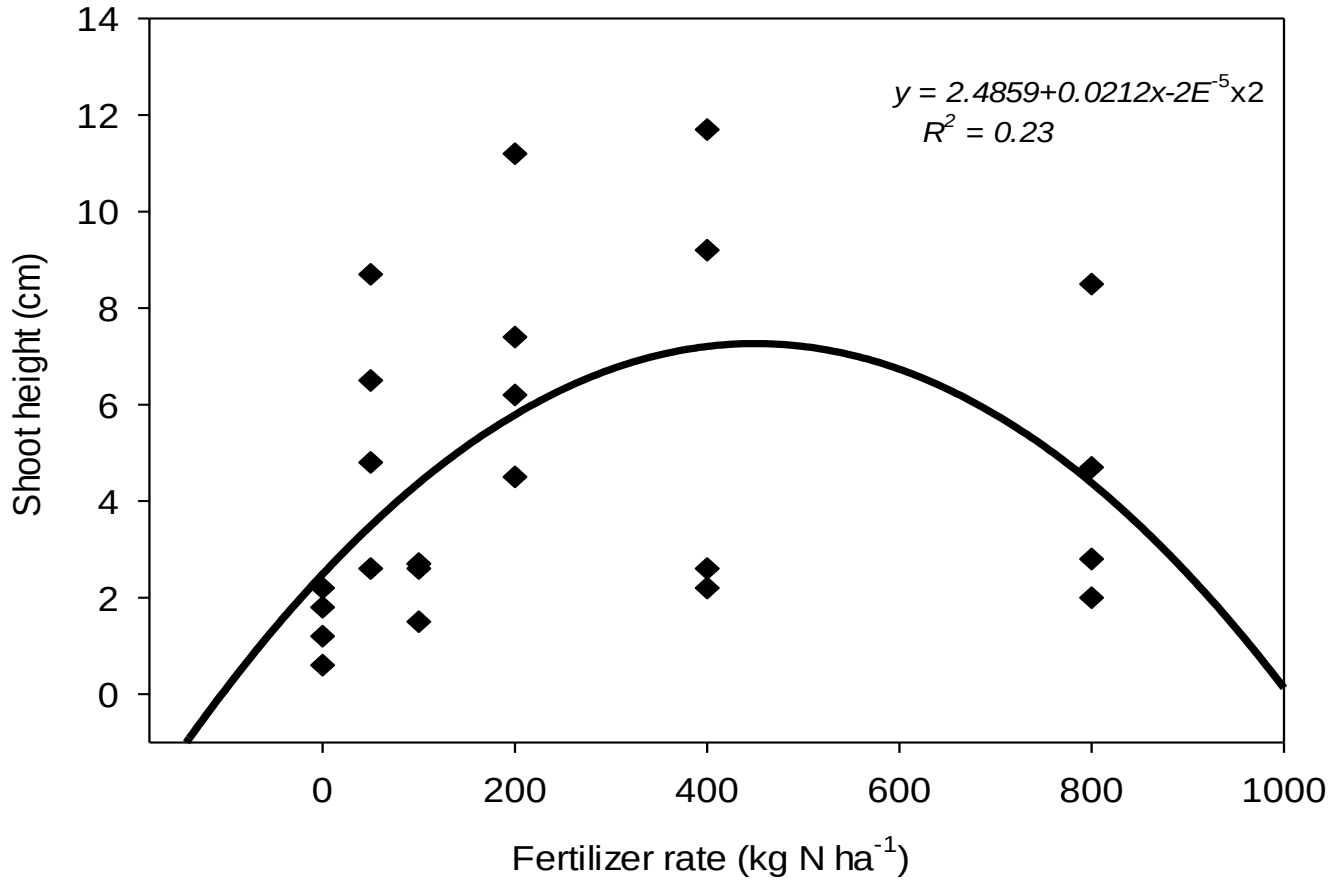

Fig. 3.2 Nitrogen fertilizer effects on plant height in American skullcap in greenhouse 1

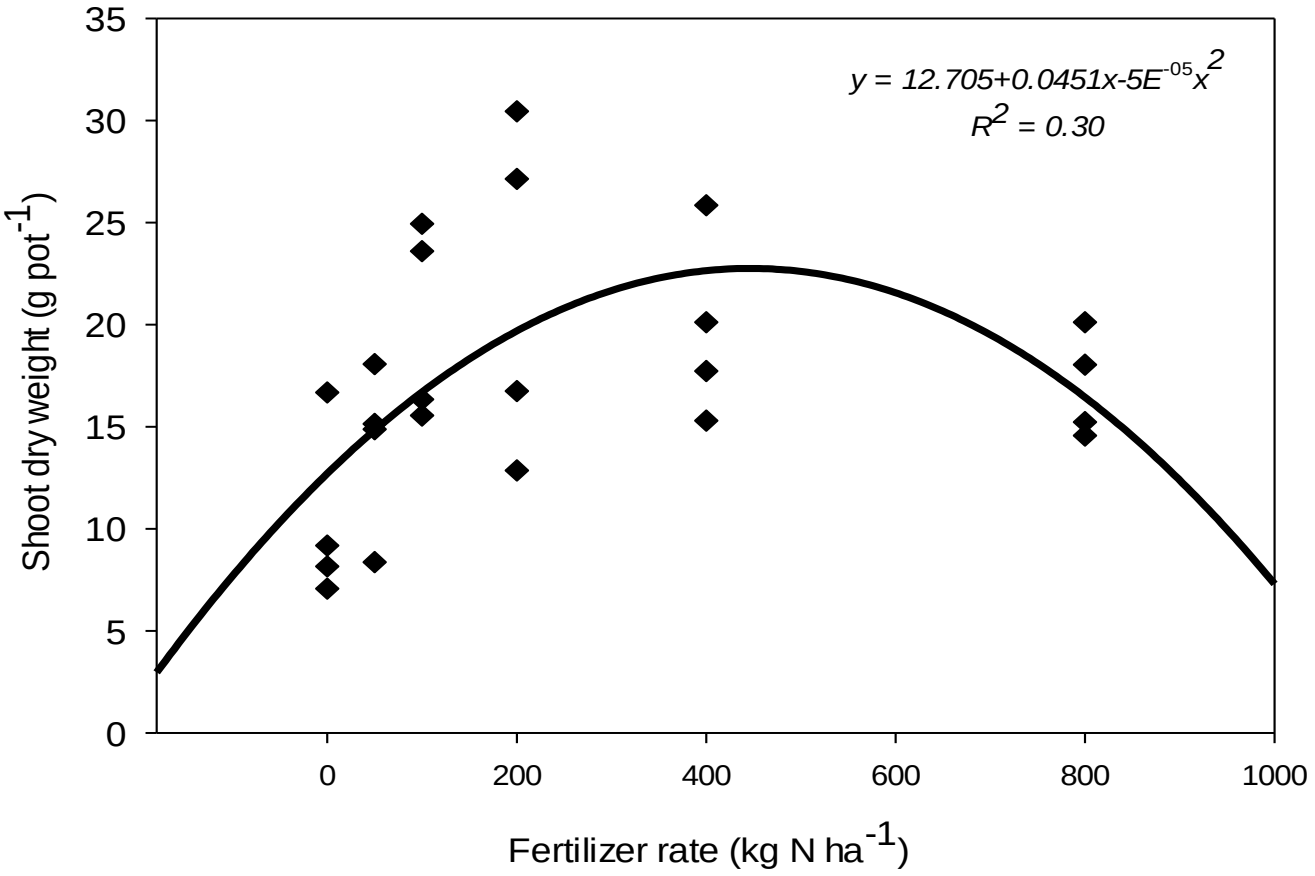

Fig. 3.3 Nitrogen fertilizer effects on above-ground dry matter in American skullcap in greenhouse 2

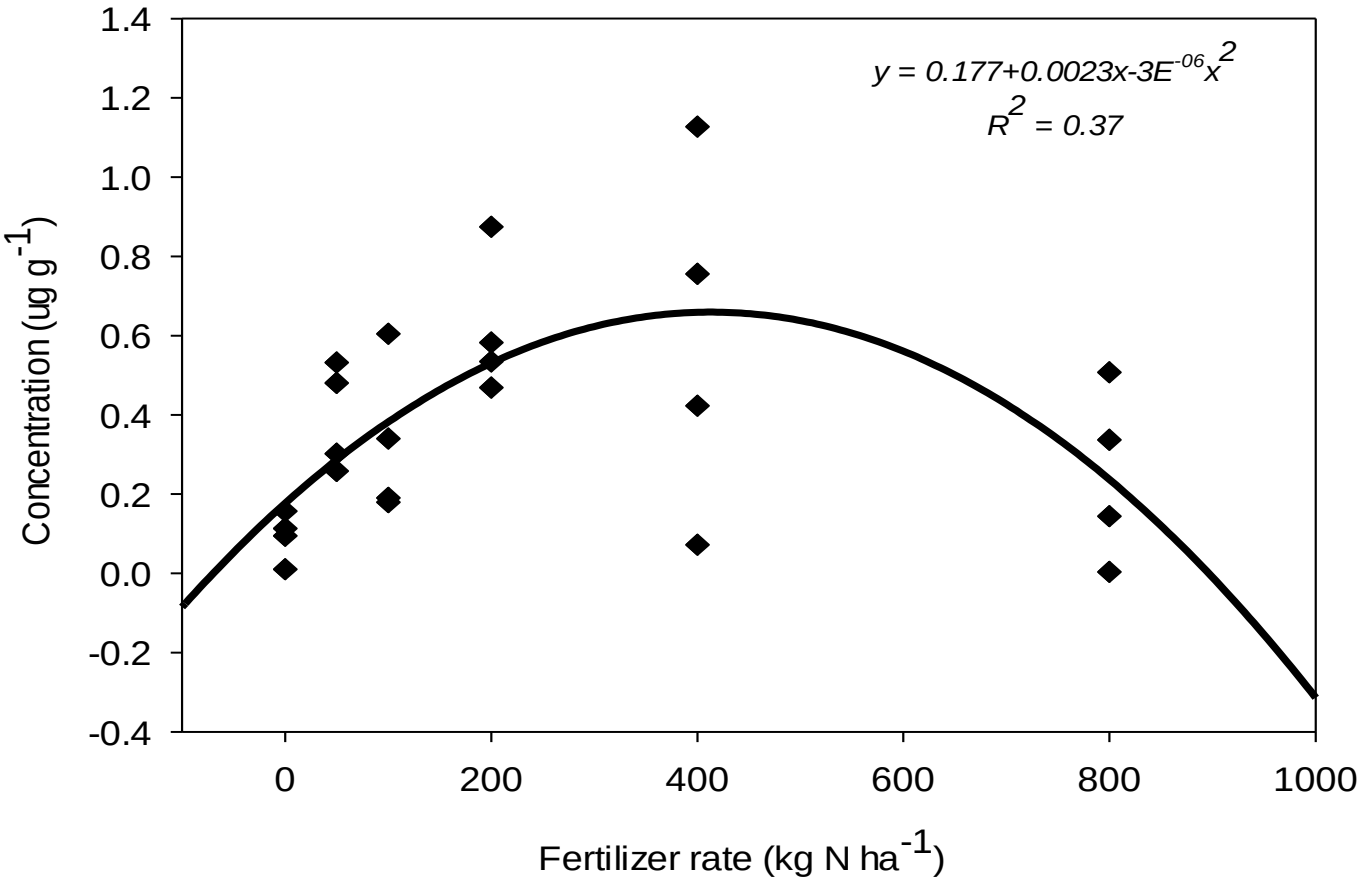

Fig. 3.4 Nitrogen fertilizer effects on baicalein concentration in American skullcap in greenhouse 2

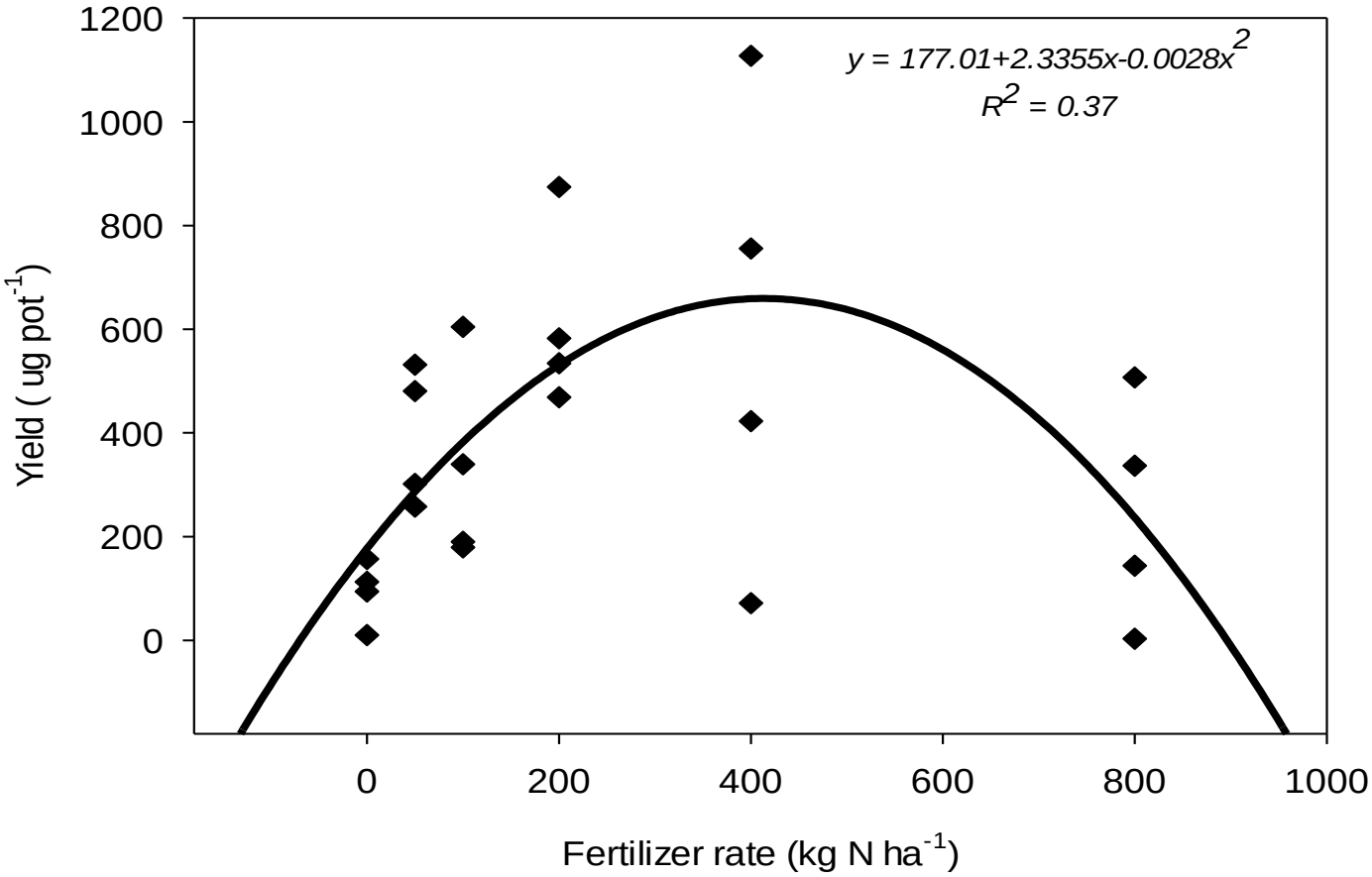

Fig. 3.5 Nitrogen fertilizer effects on baicalein yield in American skullcap in greenhouse 2

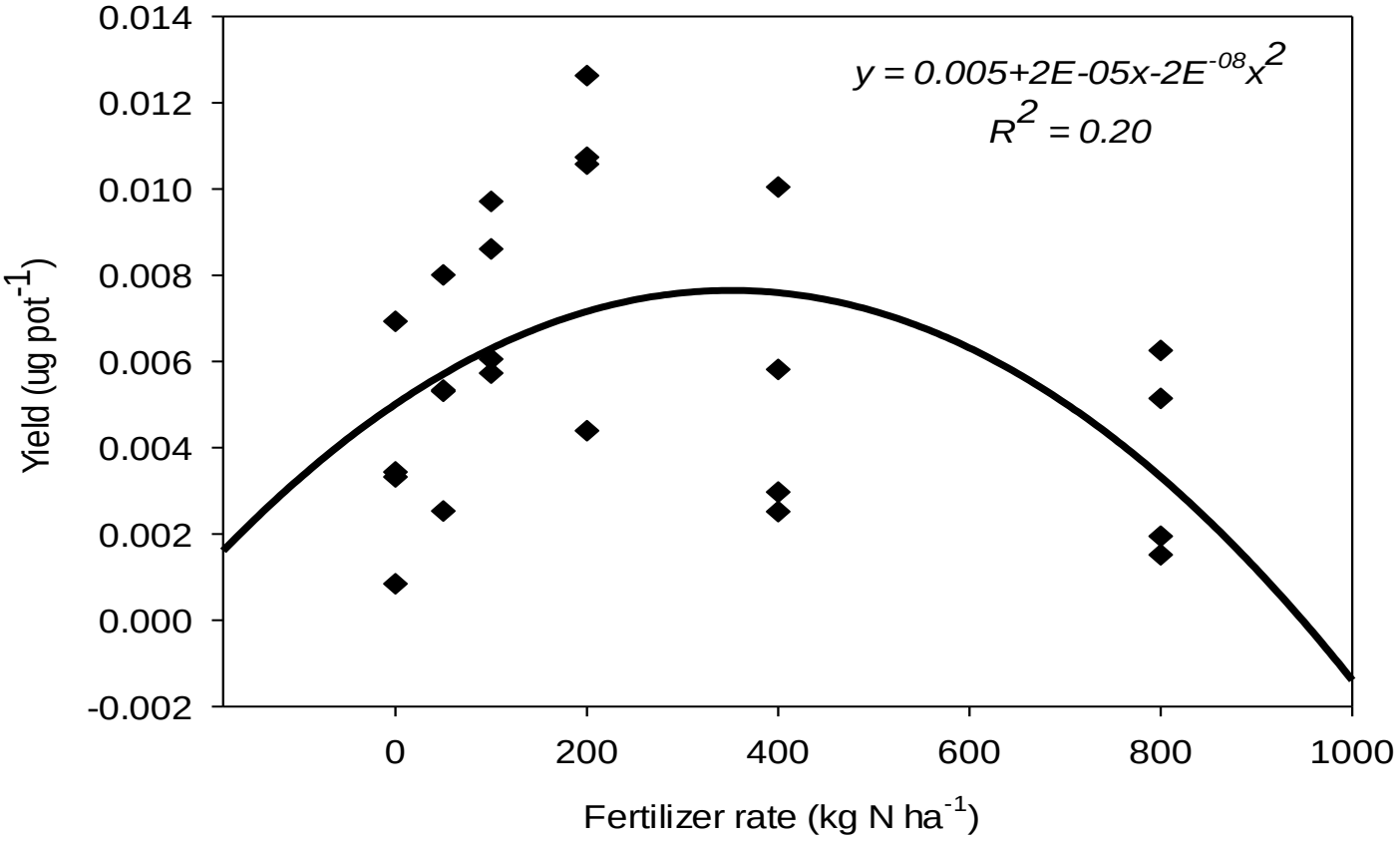

Fig. 3.6 Nitrogen fertilizer effects on chrysin yield in American skullcap in greenhouse 2

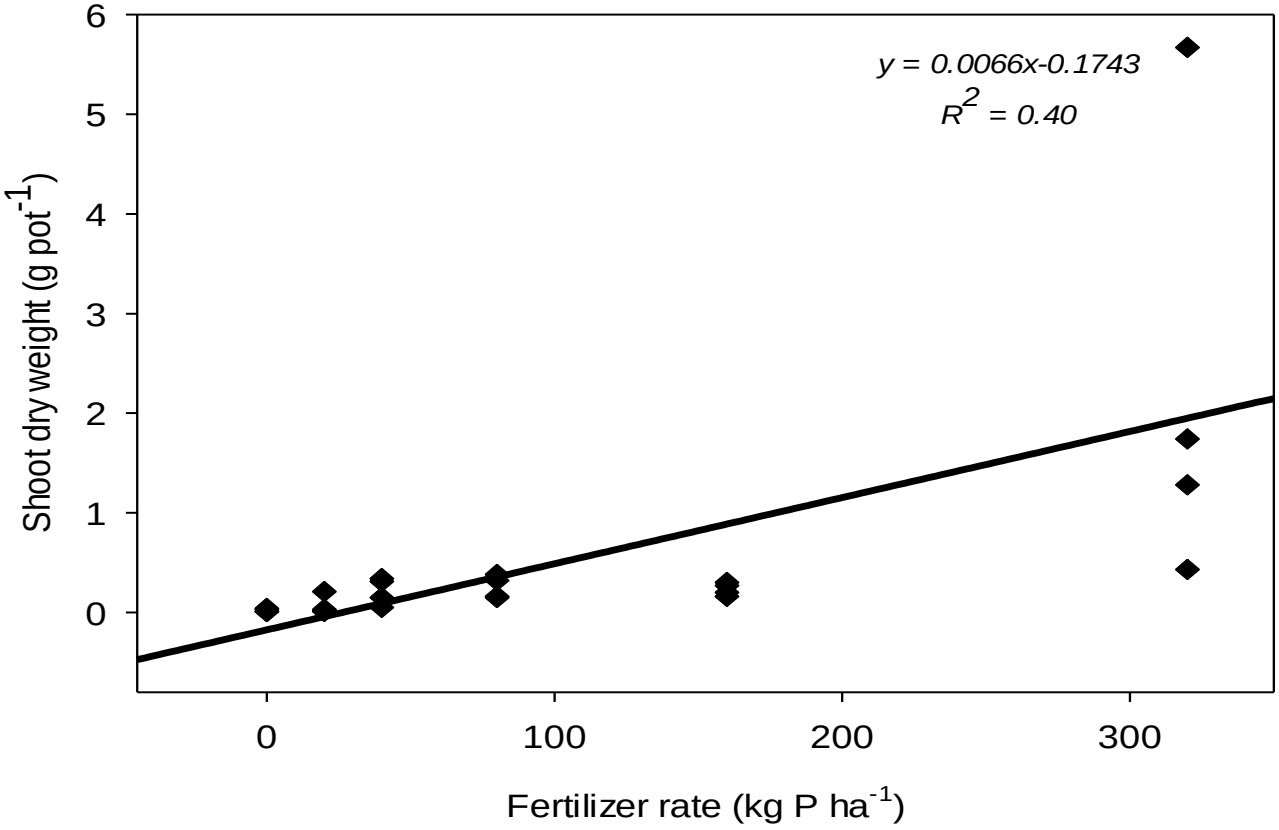

Fig. 3.7 Phosphorus fertilizer effects on above-ground dry matter in American skullcap in greenhouse 1

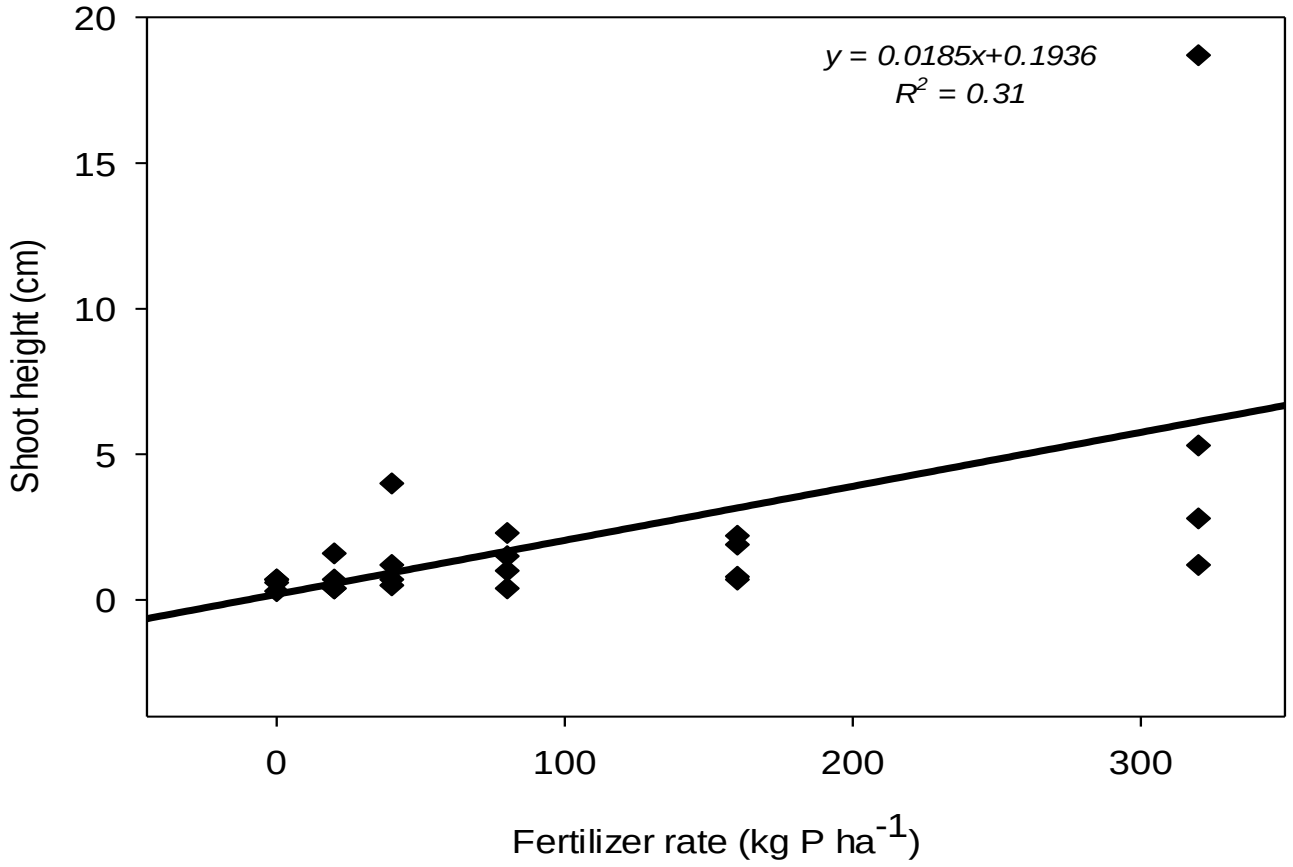

Fig. 3.8 Phosphorus fertilizer effects on plant height in American skullcap in greenhouse 1

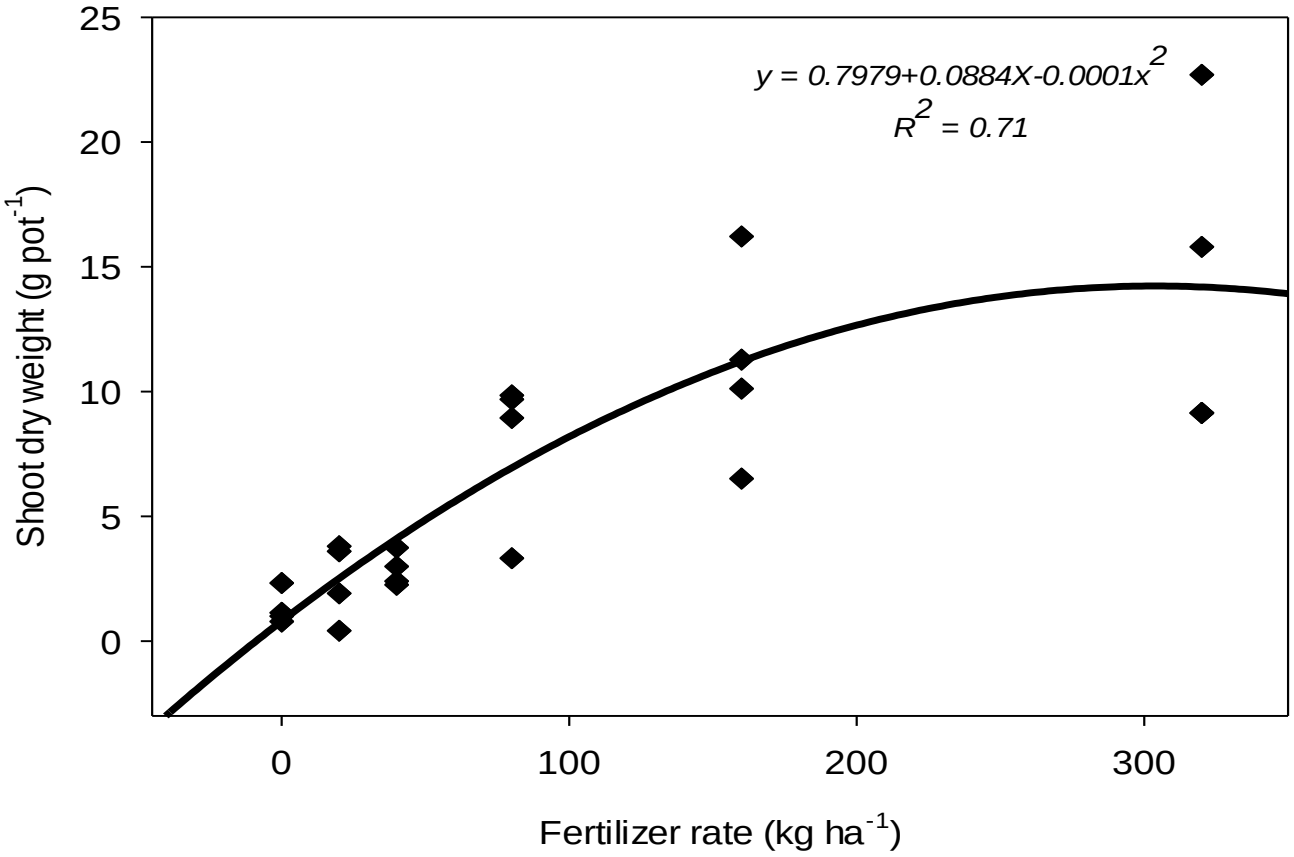

Fig. 3.9 Phosphorus fertilizer effects on above ground dry matter in American skullcap in greenhouse 2

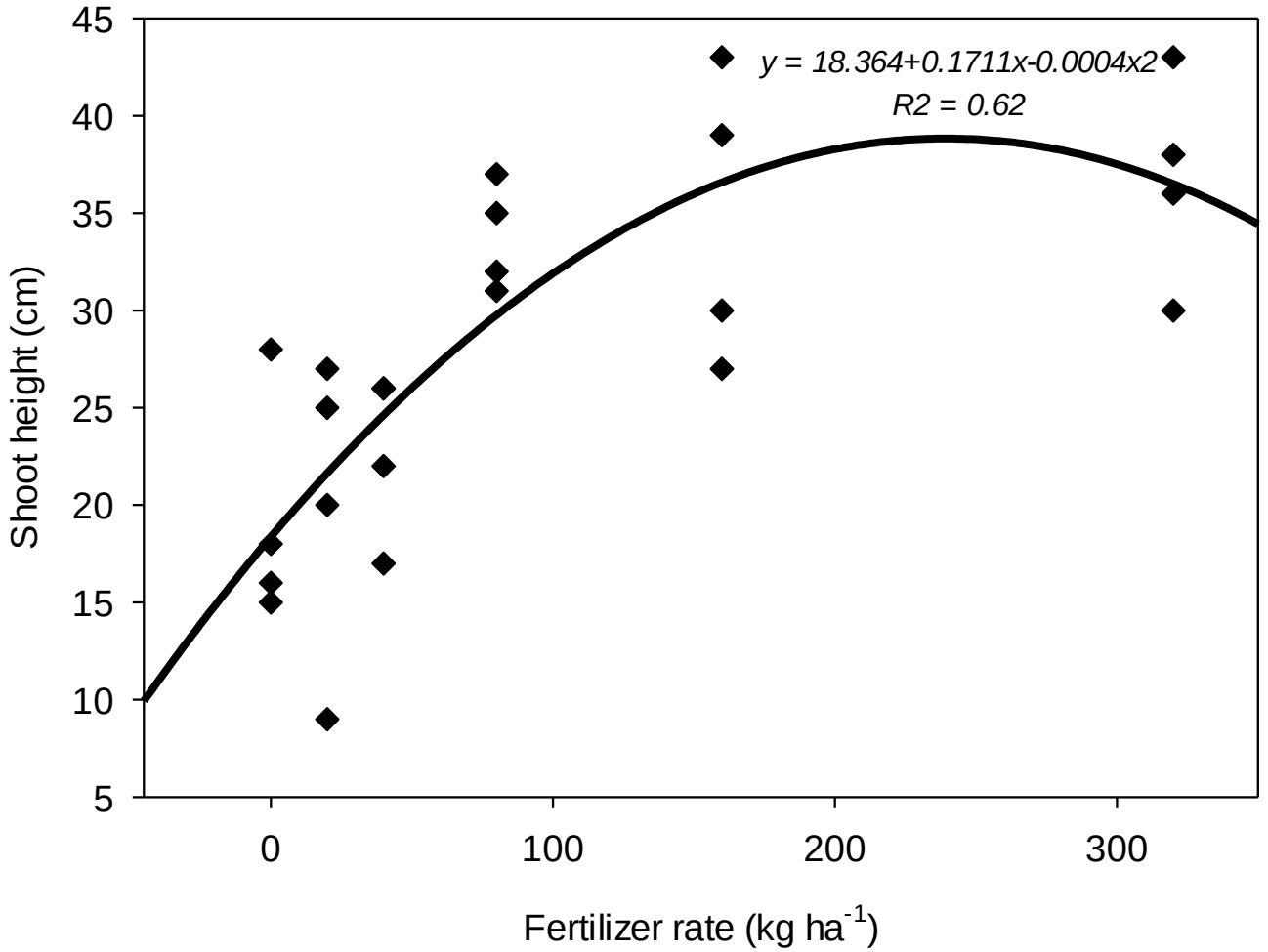

Fig. 3.10 Phosphorus fertilizer effects on plant height in American skullcap in greenhouse 2

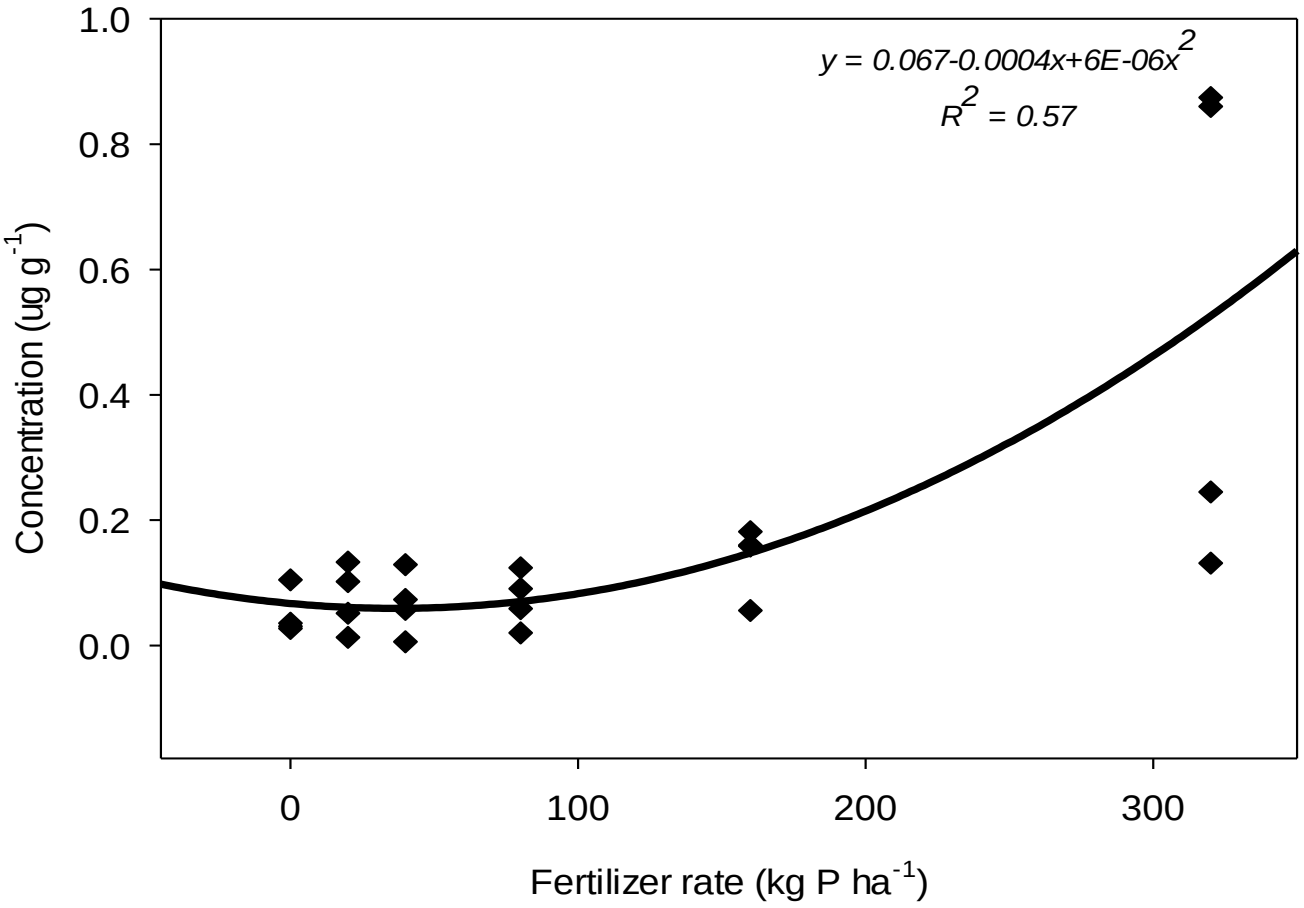

Fig. 3.11 Phosphorus fertilizer effects on baicalein concentration in American skullcap in greenhouse 2

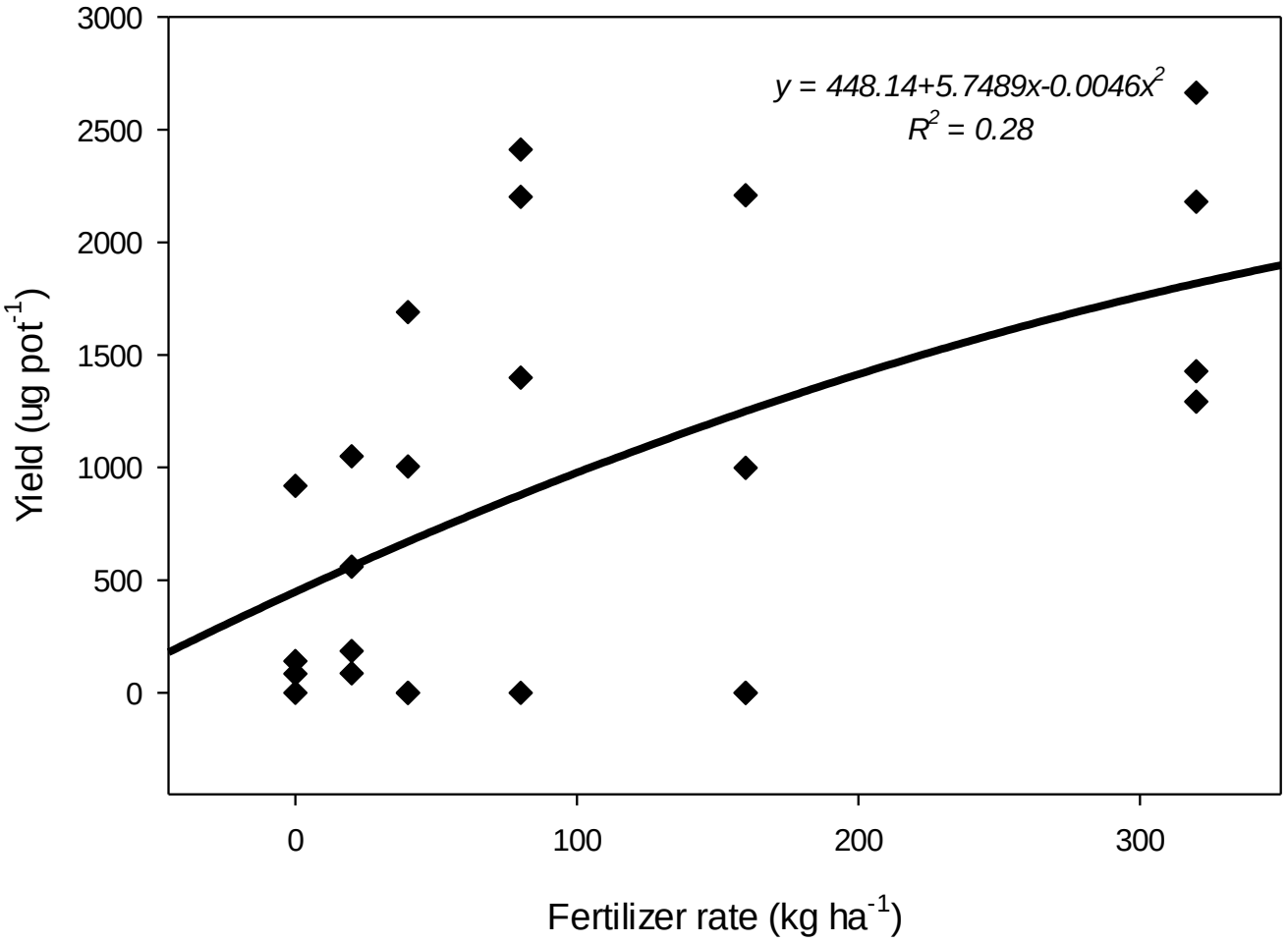

Fig. 3.12 Phosphorus fertilizer effects on scutellarein yield in American skullcap in greenhouse 2

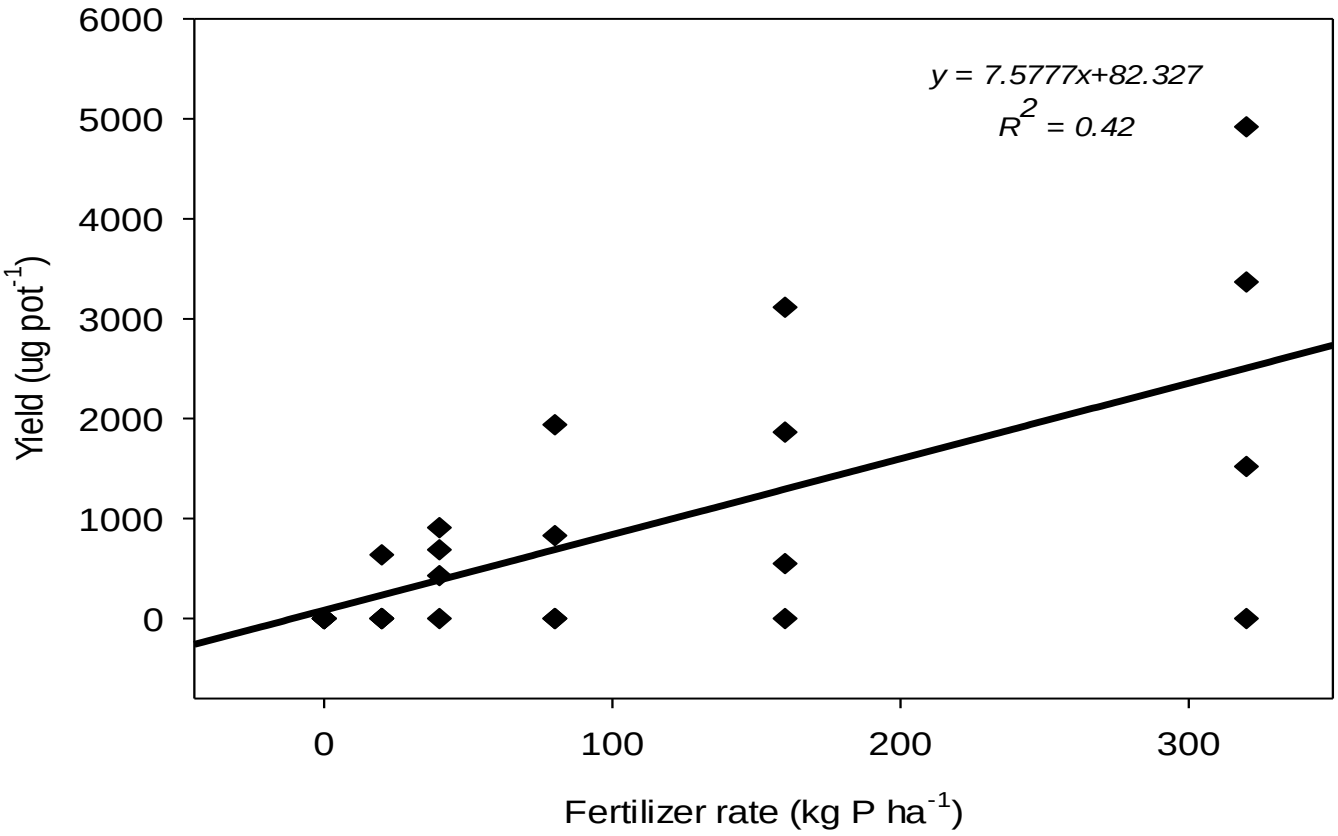

Fig. 3.13 Phosphorus fertilizer effects on baicalin yield in American skullcap in greenhouse 2

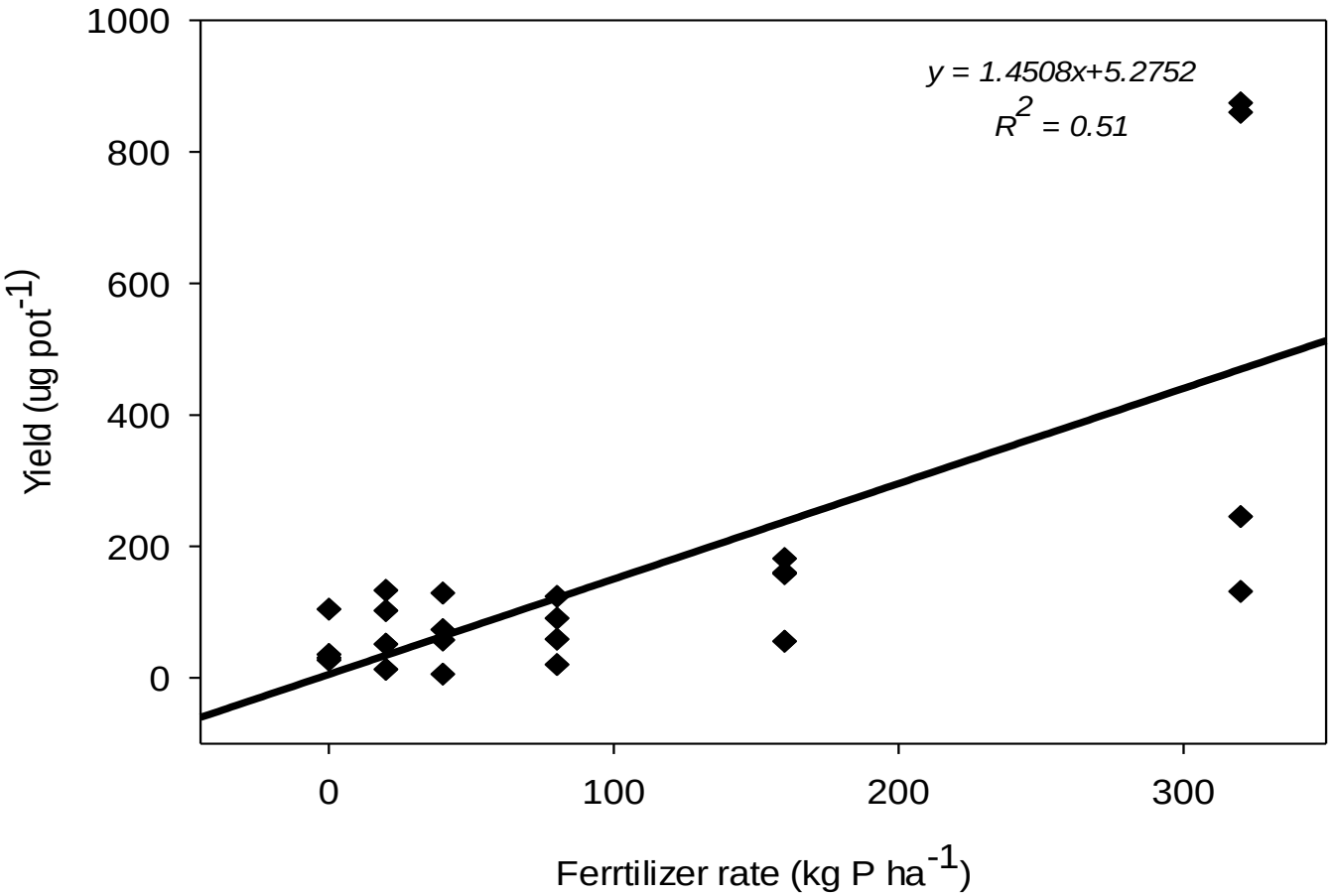

Fig. 3.14 Phosphorus fertilizer effects on baicalein yield in American skullcap in greenhouse 2

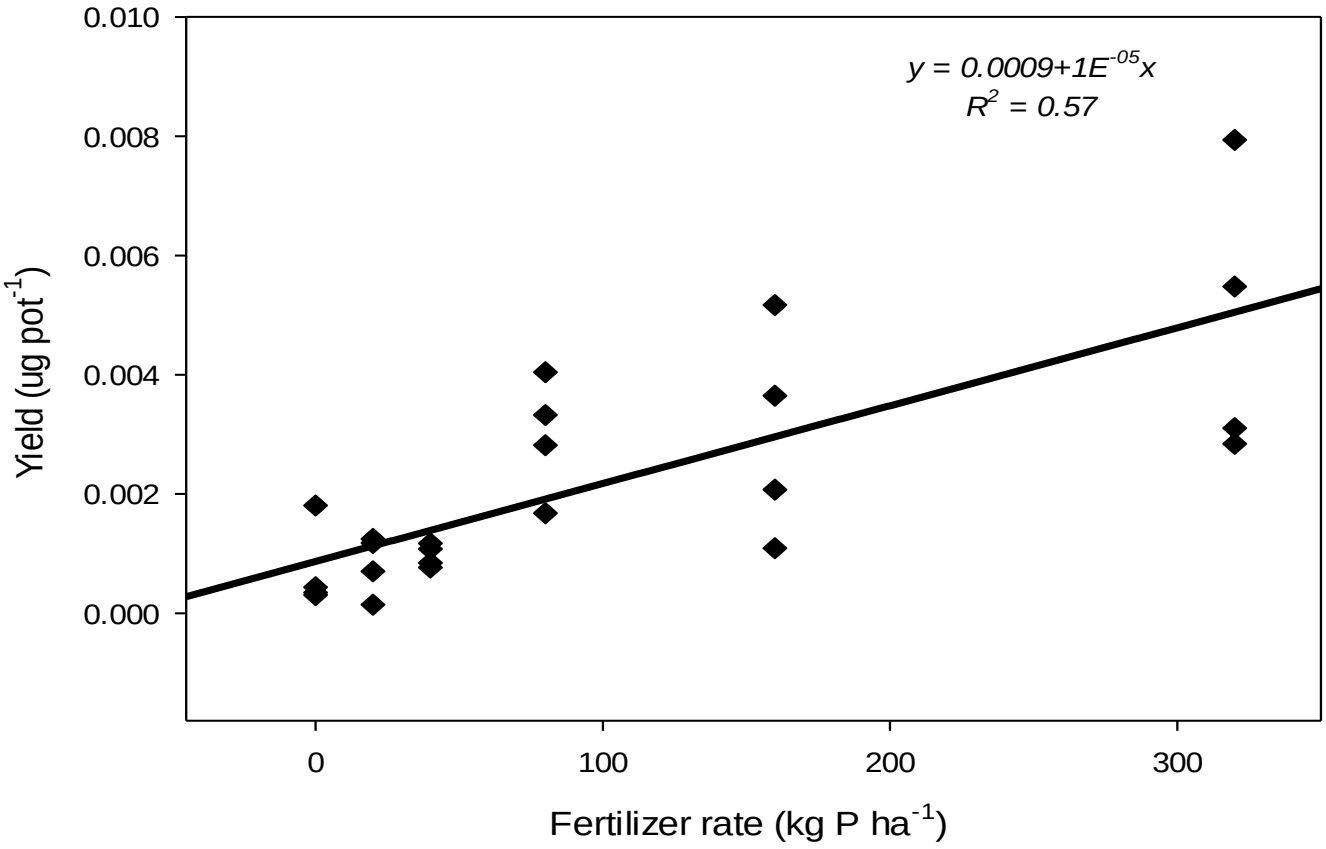

Fig. 3.15 Phosphorus fertilizer effects on chrysin yield in American skullcap in greenhouse 2

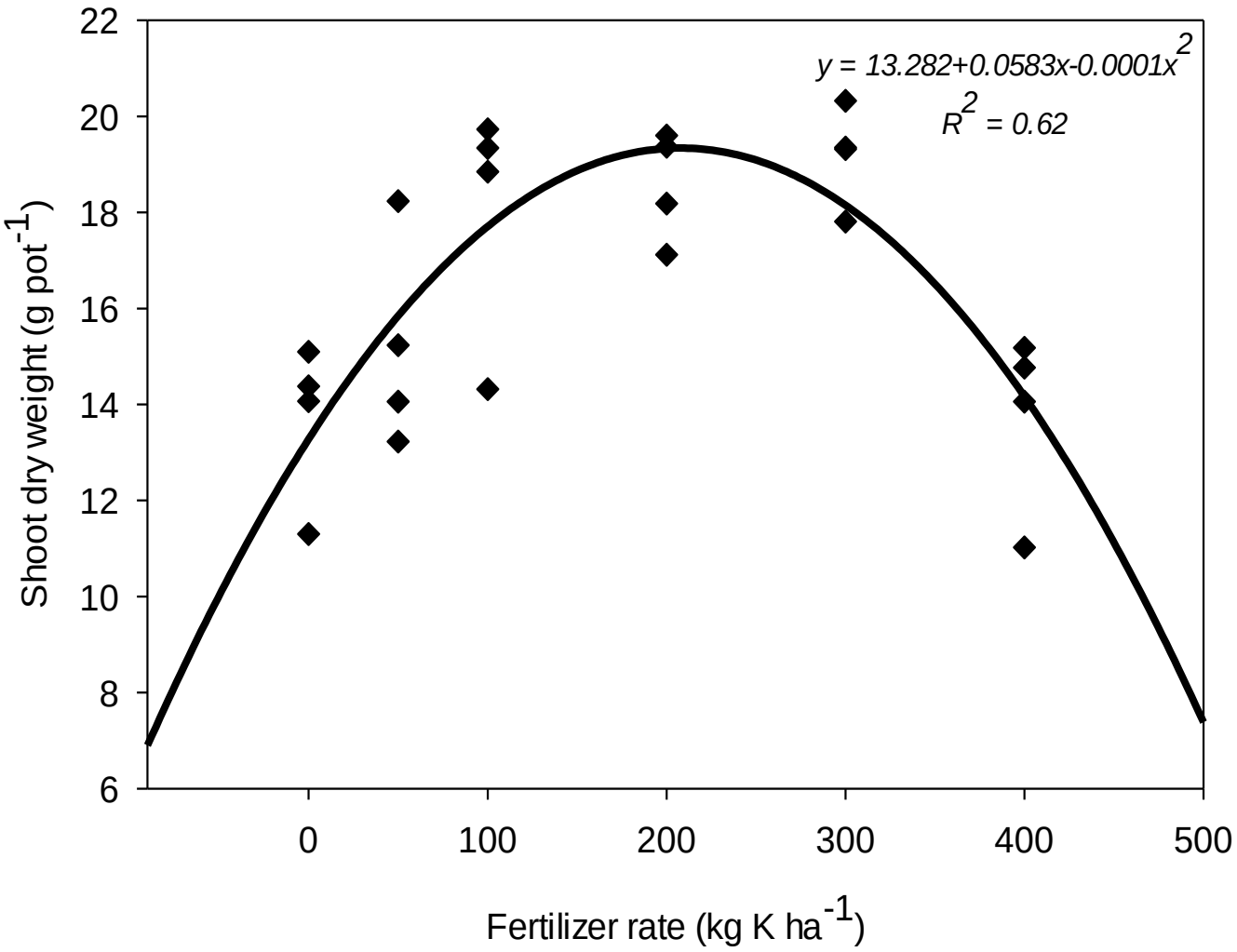

Fig. 3.16 Pottasium fertilizer effects on above ground dry matter in American skullcap in greenhouse 2

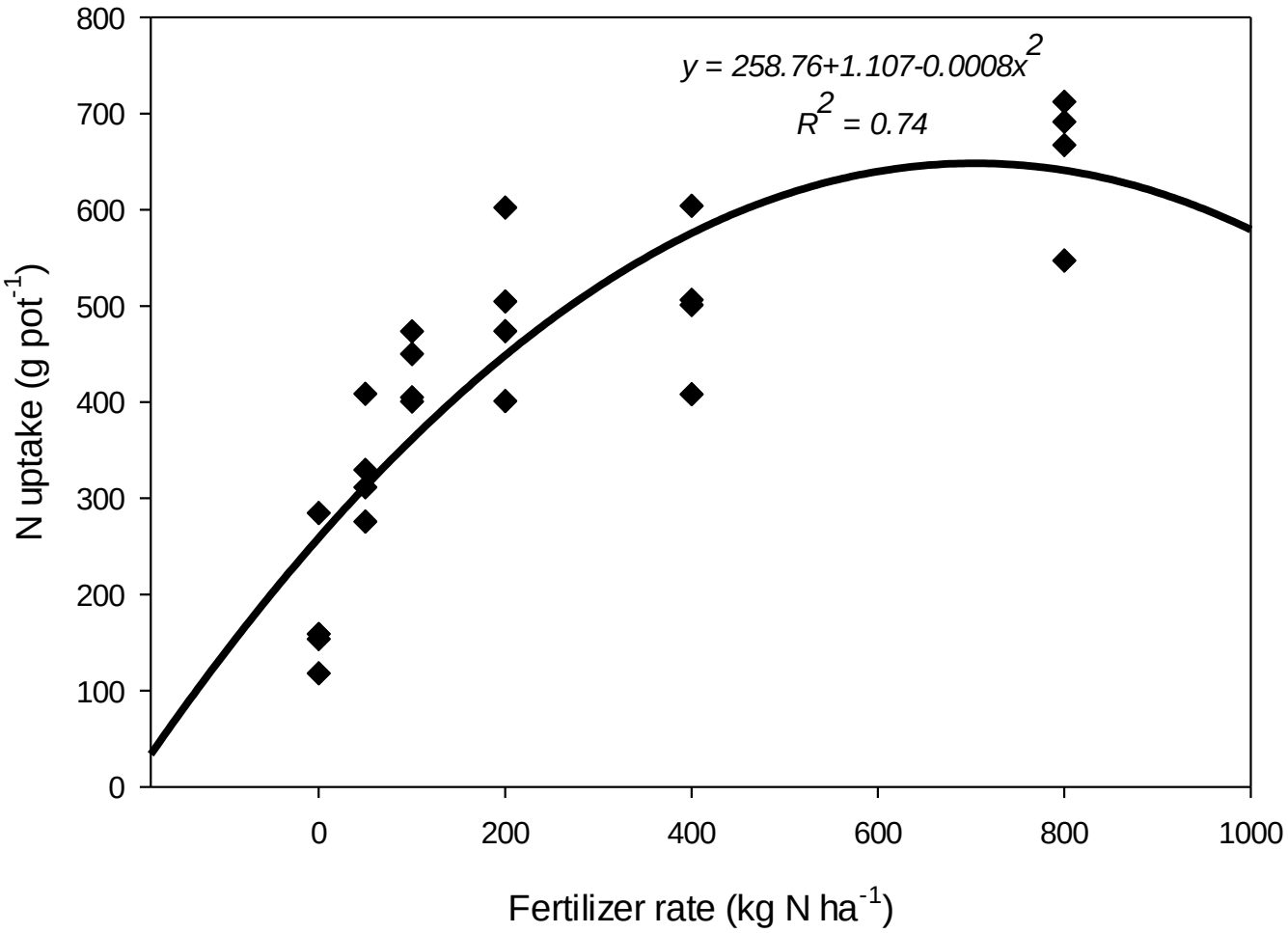

Fig. 3.17 Nitrogen fertilizer effect on nitrogen uptake in American skullcap in greenhouse 2

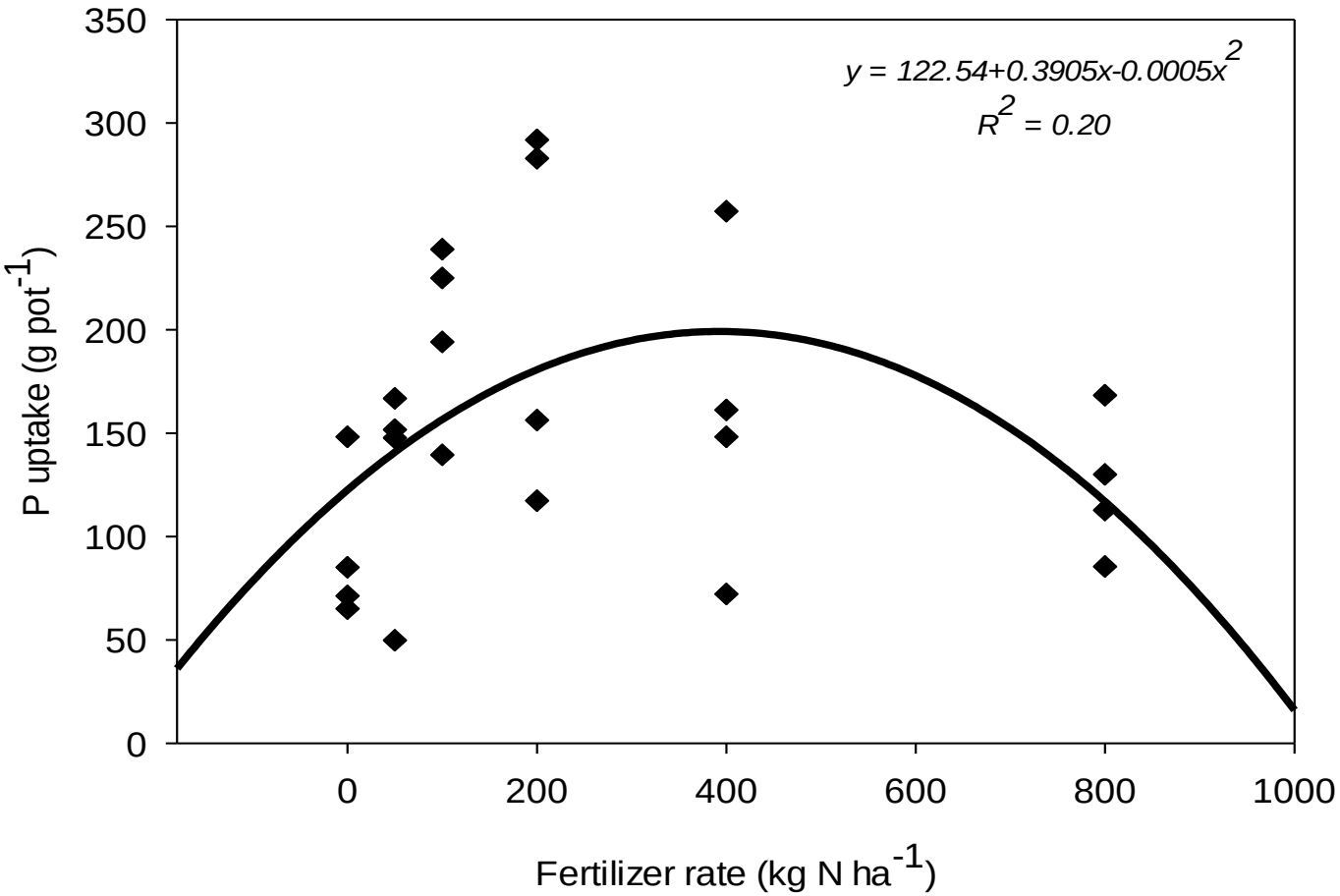

Fig. 3.18 Nitrogen fertilizer effect on phosphorus uptake in American skullcap in greenhouse 2

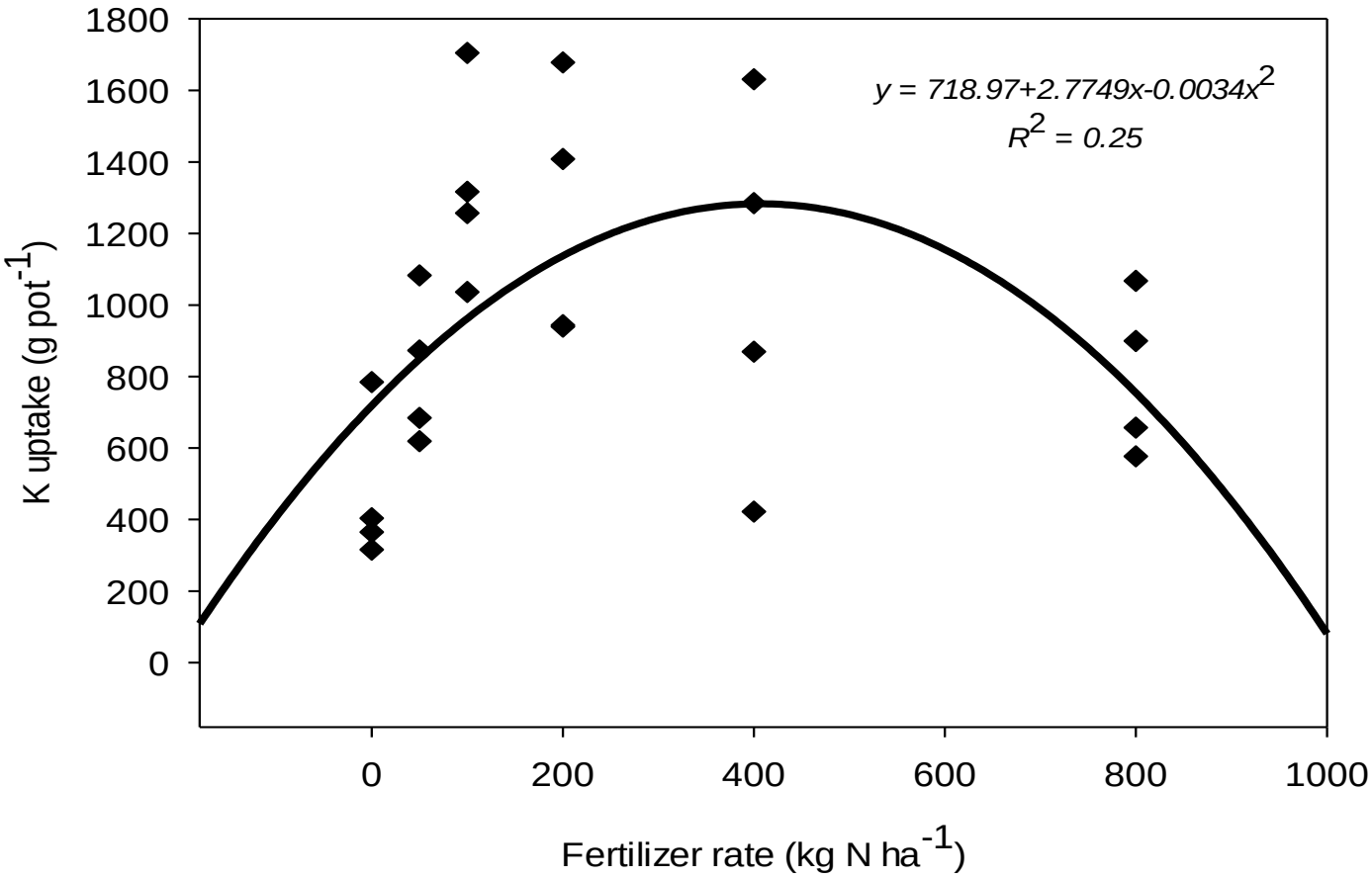

Fig. 3.19 Nitrogen fertilizer effect on potassium uptake in American skullcap in greenhouse 2

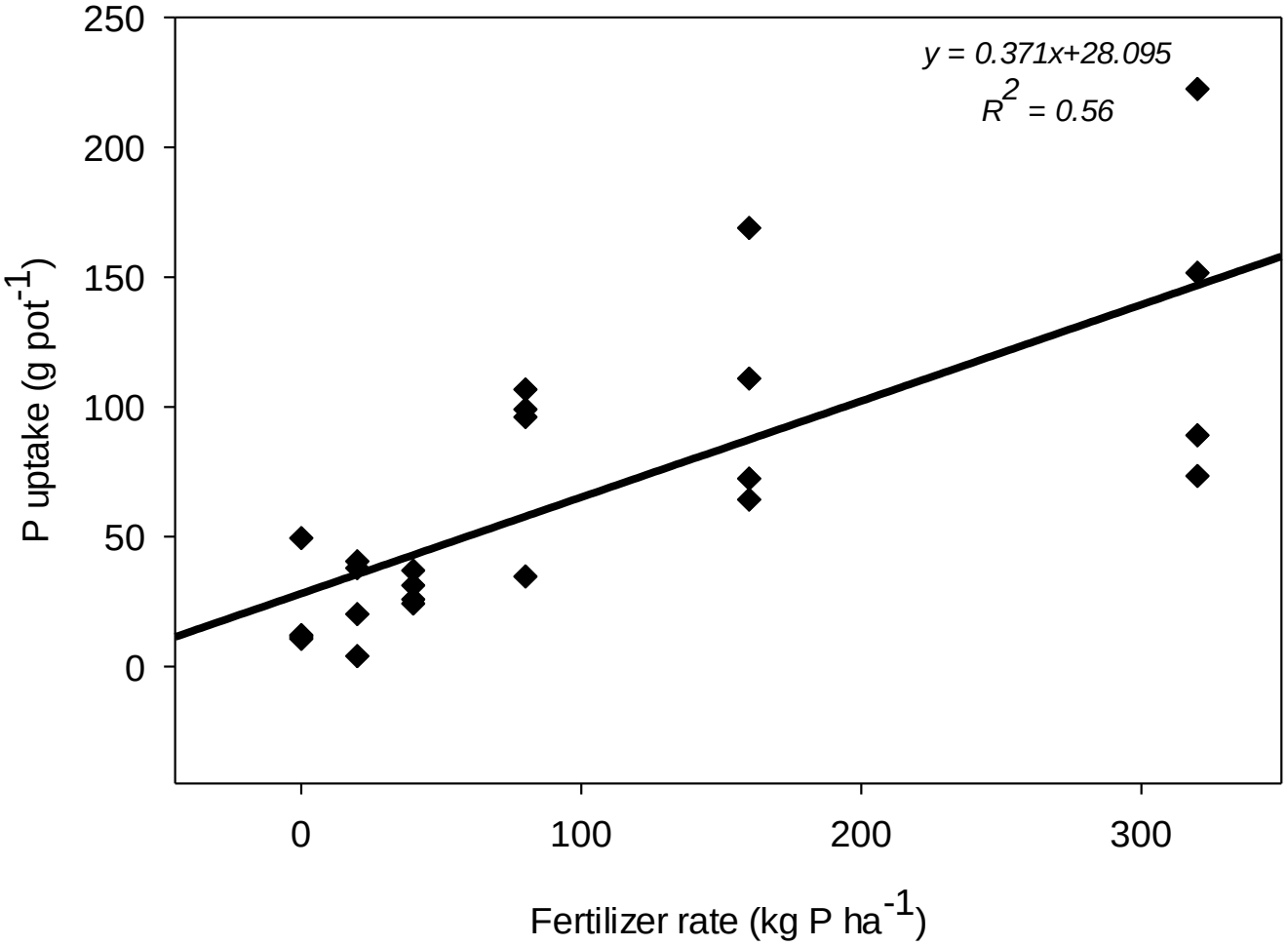

Fig. 3.20 Phosphorus fertilizer effect on phosphorus uptake in American skullcap in greenhouse 2

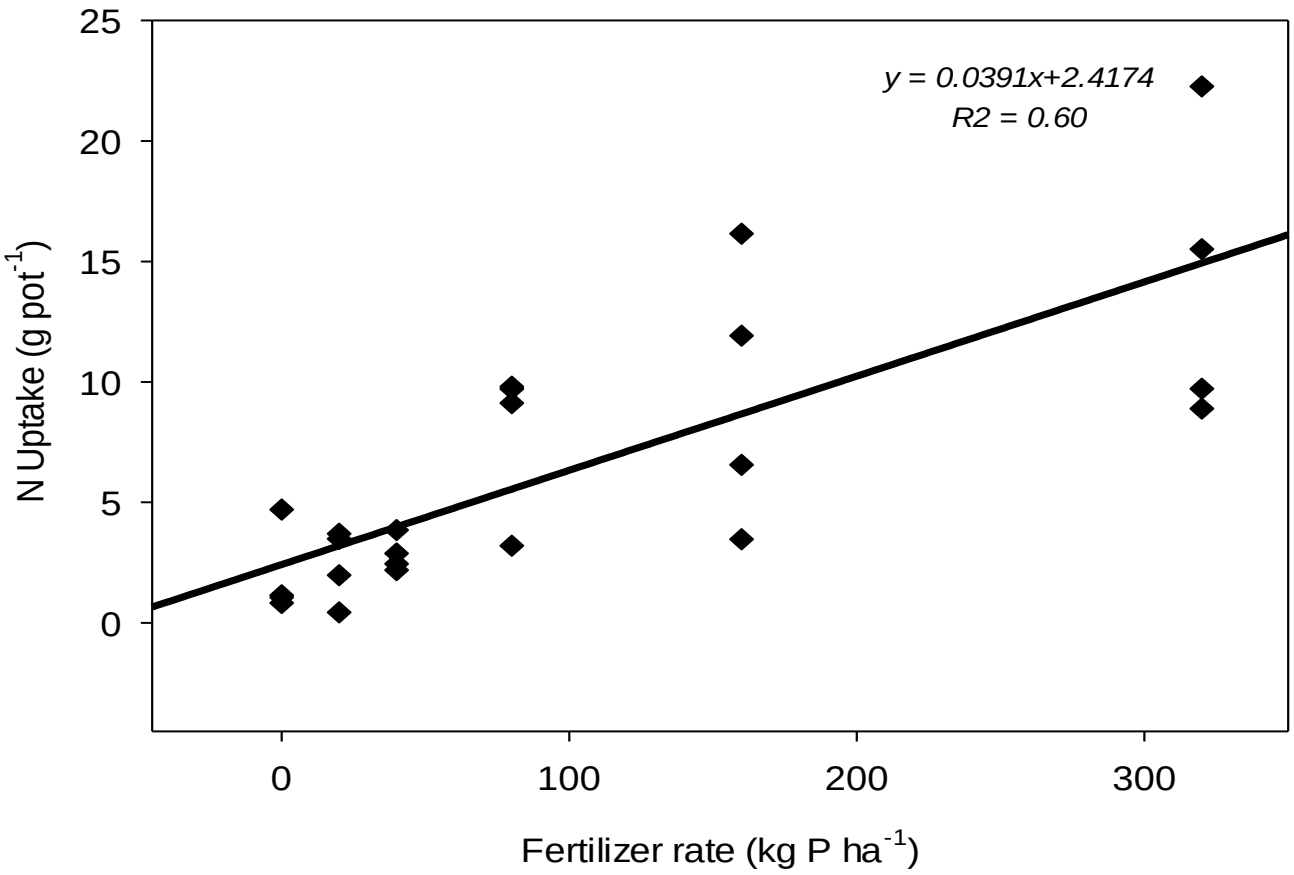

Fig. 3.21 Phosphorus fertilizer effect on nitrogen uptake in American skullcap in greenhouse 2

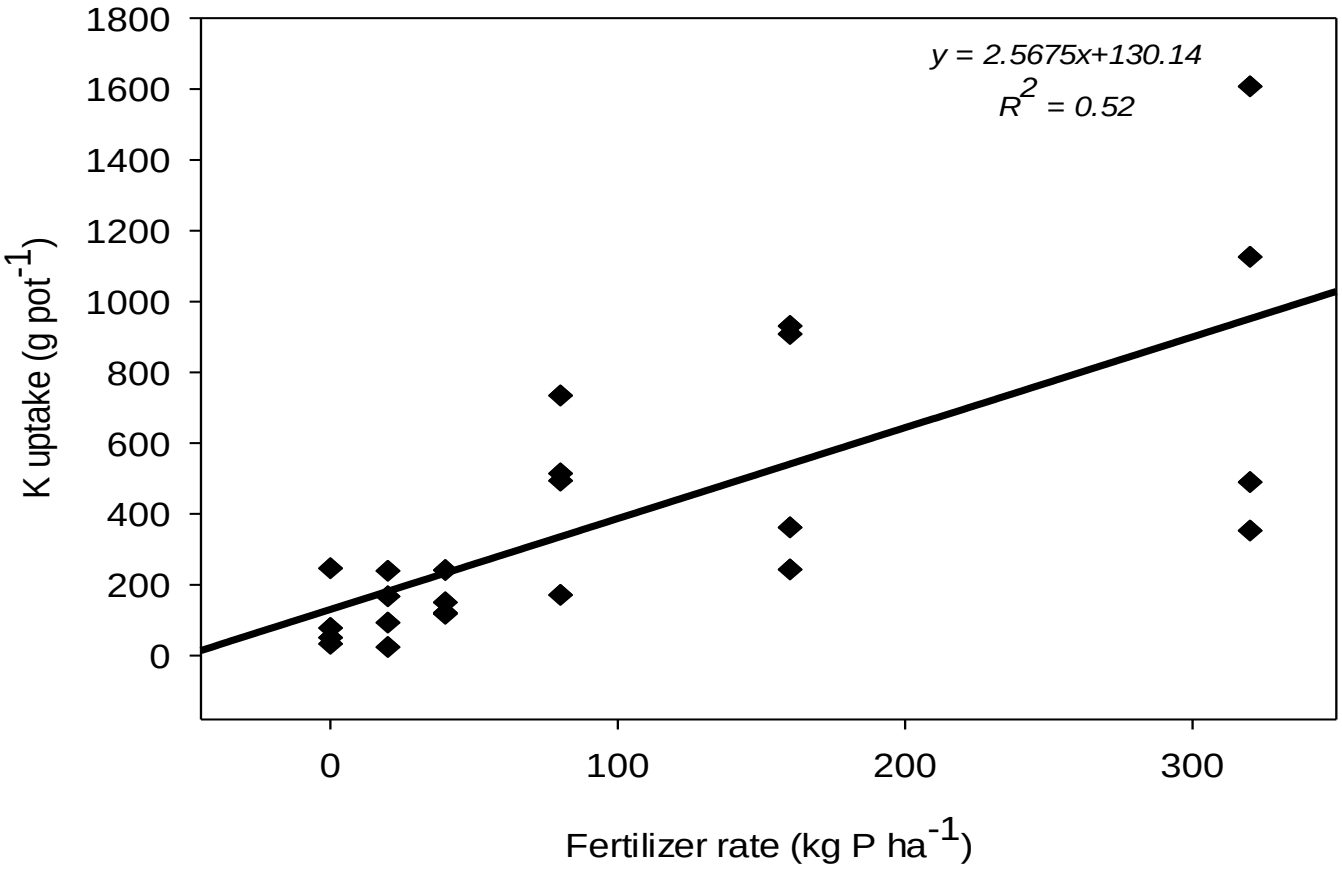

Fig. 3.22 Phosphorus fertilizer effect on potassium uptake in American skullcap in greenhouse 2

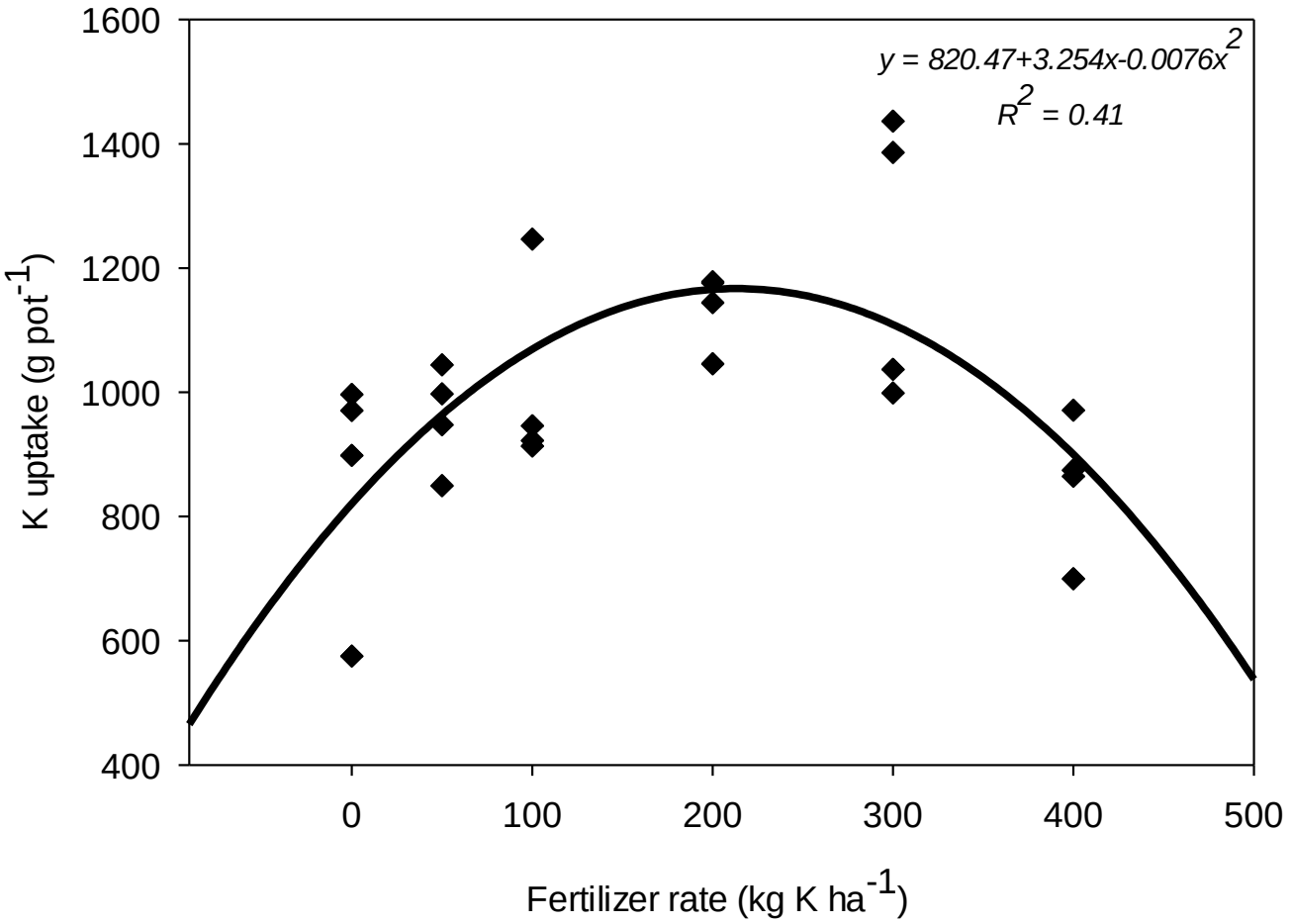

Fig. 3.23 Pottasium fertilizer effect on potassium uptake in American skullcap in greenhouse 2

Chapter 4. Summary and Conclusions

A field experiment was conducted in Shorter, Alabama to determine the effect of timing and frequency of harvest on shoot yield and flavonoid content in American skullcap (*Scutellaria lateriflora*). The experimental design was 2X2 split plot factorial in randomized complete block design with four replications of each treatment. The main factor was number of harvests in first season (2008): One harvest per season and two harvests per season and sub factors were timing of harvests in second season (2009): early harvest and late harvest. In the first year (2008) two harvests per season gave higher shoot dry matter yield (1708 Kg ha^{-1}) than one harvest per season (1256 Kg ha^{-1}). In second year (2009), there were no differences in yield between early and late harvest treatments.

The second experiment was conducted in a greenhouse to determine the effect of fertilization with N, P and K on dry matter yield and flavonoid content of American skullcap. The growing medium used was fritted clay (Xtrasorb Plus Absorbent, Moltan Company, Memphis, TN). The experimental design was completely randomized design with 4 replications. Dry matter yield, flavonoid yield, height and the nitrogen, phosphorus and potassium uptake increased for every increment of applied phosphorus. Fertilization with N, P and K significantly increased shoot biomass yield and flavonoid yield, as well as concentration of some flavonoids. Based upon the above results, American skullcap may be harvested twice in the first year and twice in the second year

of cultivation. A high rate of phosphorus may be applied, along with nitrogen, potassium and micro-nutrients. However, further research is needed to determine if three harvests may be carried out per year and if it is feasible to cultivate skullcap for more than two years in the same field. Further research is also needed to determine the optimum rates for NPK under field conditions.