CULTIVATION PRACTICES FOR *ASTRAGALUS MEMBRANACEUS* IN THE

SOUTHEASTERN UNITED STATES

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CULTIVATION PRACTICES FOR *ASTRAGALUS MEMBRANACEUS* IN THE SOUTHEASTERN UNITED STATES

Meili Wang

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CULTIVATION PRACTICES FOR *ASTRAGALUS MEMBRANACEUS* IN THE SOUTHEASTERN UNITED STATES

Meili Wang

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THESIS ABSTRACT

CULTIVATION PRACTICES FOR *ASTRAGALUS MEMBRANACEUS* IN THE SOUTHEASTERN UNITED STATES

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Astragalus membranaceus is a traditional Chinese medicinal herb and has been used for thousands of years. Information on its cultivation in Southeastern United States is lacking. In September 2006, three field experiments were planted in beds to test the effects of (1) deep tillage (subsoiling vs no subsoiling), (2) variety trial (seven variety from difference area), and (3) P (0, 29, and 58 kg ha^{-1}) and K (0, 56, and 112 kg ha^{-1}) fertilizer and lime on growth, root development, and astragaloside IV concentration in Auburn, Alabama on a Plinthic Kanhapludults soil. The fertility study was a 3 × 3 factorial augmented with a no-lime treatment at 29 kg P ha^{-1} + 56 kg K ha^{-1}.

Plant mortality was most severe with small seedlings in spring and summer, but was observed throughout the season, and was associated with insect damage to roots and underground stems, and with root and crown rot caused by *Pythium* and *Phytophthora* under wet conditions. Missing plants were reseeded in March 2007 and younger plants

harvested separately from older plants in October 2007. Subsoiling increased root weight, root diameter, and root length at 277 day (α=0.05) and distance from first branch to crown (α=0.1). Although deep tillage was somewhat beneficial for *A. membranaceus*, it did not sufficiently loosen soil to yield straight, unbranched roots. The application P with K fertilizer significantly (α=0.1) increased root to shoot ratio for 13-month plants.

Astragaloside IV and digoxin (internal standard) were clearly separated and resolved by HPLC-ELSD. Subsoiling significantly increased the concentration of astragaloside IV in all roots except big root of 7-month plants and subsoiling also significantly increased the total content of astragaloside IV in roots of 13-month plants. Small roots had higher concentrations of astragaloside IV than big roots for the same growing period and variety. The higher concentration of astraloside IV was found in 13-month plants than in 7-month plants. Potassium application had a significant (Pr=0.012) interaction effect with P in small roots of 7-month plants on astragaloside IV concentration and K suppressed concentration in absence of P. Phosphorus application significantly (Pr=0.037) affected concentration of astragaloside IV in big root of 7-month plants. The linear interaction effect of P × K was significant (Pr=0.009) in the root of 7-month plants. No P effect was found in small root of 7-month plants, big root of 13-month plants and small root of 13-month plants.

Lime had no effect on root growth and astragaloside IV. Varieties AM2, AM3, AM4 and AM5 had good adaptability, root weight (yield) and relatively high concentration of astragaloside IV in the roots and can be chosen to plant in southeastern U. S. as alternative crop.

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I. LITERATURE REVIEW ON *ASTRAGALUS MEMBRANACEUS*

Description of *Astragalus membranaceus*

Astragalus membranaceus is one of the important traditional Chinese medicinal herbs and has been used for thousands of years. It is also known as membranous milk-vetch or Astragalus root (English), huangqi (Chinese), Ogi (Japanese), and hwanggi (Korean) in different countries. The authentic botanical sources of Huangqi in Chinese Pharmacopoeia are *Astragalus membranaceus* (Fischer) Bunge and *Astragalus membranaceus* (Fischer) Bge. Var. *mongholicus* (Bge.) (Tu et al., 1988). Radix Astragali is the pharmaceutical name of the dried root of both varieties (Tu et al., 1988) and has medicinal function.

A. membranaceus is perennial herb. *A. membranaceus* is a member of the Fabaceae family which are legumes. *A. membranaceus* grows to about 40-100 cm height and sprawls as it matures. The fern-like fronds are approximately 20-35 cm long with angled stems covered by appressed white hairs. The oval or oblong-oval leaves are 3-6 cm long, with obsolete petioles. The light yellow flowers are 18-20 mm long and resemble that of peas. The two-valve seed pods have short, dark hairs. The seeds are kidney-shaped with dark brown color. The well grown *A. membranaceus* has a long, branched taproot. The taproot is cylindrical or nearly cylindrical with

lateral root disperse on the surface. The root has grayish yellow to yellowish brown epidermis and fibrous fracture (Tu et al., 1988; Upton et al., 1999).

The Adaptation of *Astragalus membrabnaceus*

The climatic condition affects the growth and quality of *A. membranaceus*. In China, the center of origin of wild *A. membranaceus* is the mountain areas in Sichuan province (Chen et al., 2006; Zhao, 2006), where the climate is moderate, the latitude N30-N33°, annual average temperature 4-12 ºC, annual precipitation 500-900 mm, and annual sunlight 1600-2600 hours. Although *A. membranaceus* still exists there, *A. membranaceus* is currently mainly widely distributed in the pine forest region and mountain areas in Northern China, Siberia, Northern Korea. *A. membranaceus* was also domesticated and is largely cultivated for medicinal use in Northern China, Northern Korea, South Korea and Japan (Chen et al., 2006; Zhao, 2006), where the latitude ranges from N37° to N52°. The climate in those areas is dry with snow and cold in winter. The latitude of Auburn, Alabama is 32° and annual average temperature 4-12 ºC, annual precipitation 500-900 mm, and annual sunlight 1600-2600 hours.

Plants have the ability to adapt and survive in different climates. The cultivation of *A. membranaceus* should be possible in Alabama. Successful cultivation should depend on growth performance, flowering, seeding, yield of roots and the content of medicinal component. For instance, the criteria successful cultivation of *A.*

membranaceus in China is that astragaloside IV concentration is greater than 4% (The State Pharmacopoeia Commission of P.R. China., 2000). *Astragalus membranaceus* has been successfully introduced and acclimated in moderate regions in some European countries, such as Germany, Poland and Turkey (Matkowski et al., 2003).

Seed Germination and Field Management

According to the standard operating procedure for *A. membranaceus* in China, before planting, the field should be prepared following this procedure: (1) Disk the field. (2) Apply enough fertilizer, (3) Kill and control soil-borne pests, for example, solarize the soil or spray low toxic and low residual insecticide (Yang et al., 2006; Zhang et al., 2005a).

The seed of *Astragalus membranaceus* is hard and tiny. Normally it is 1-2mm long. The seed has dormancy caused by an impermeability that developed with progressive dryness (Shibata and Hatakeyama, 1995; Shibata, Sakai and Shimomura, 1995). The seed coat is composed of thick compacted cell layers. This cell layer contains pectin and has poor permeability to water. This impermeability of the seed can be broken by scarification. Mechanical scarification is performed by putting the seeds in a bag and rubbing vigorously by hand or machine (Shibata and Hatakeyama, 1995; Yang et al., 2006; Zhang et al., 2005a). The stored seed and perhaps fresh seed need to be pre-soaked for 24 hours in warm water before sowing. One study shows that soaking the seed in 200 $mg \cdot kg^{-1}$ gibberellin for 24 hours can improve the rate of

germination (Wang, 2005). A period of cold stratification may also help stored seed to germinate. In one study (Shibata et al., 1995) on germination of *Astragalus membranaceus mongholicus (Bge.)*, Astragalus seeds were frozen with water at -22 ºC in a deep freezer for more than 30 days and rapidly thawed in warm water for a few minutes until the ice was melted. After freezing, the germination was nearly twice than those with no treatment (Shibata and Hatakeyama, 1995; Shibata, Sakai and Shimomura, 1995).

There are two methods to plant *A. membranaceus*. The first method is direct seeding. The second one is seedling transplantation. Normally, the in-row spacing is 20-45 cm for both methods in the field (Yang et al., 2006; Zhang et al., 2005a). The between-row spacing is 30-45 cm in the field (Zhang et al., 2005a). Different planting densities were tested and compared for appearance, grade and astragaloside IV concentration of Radix Astragali. The root length, diameter and yield of *A. membranaceus* did not show significant differences among the following density spacings, 10 × 20 cm, 15 × 20 cm, 20 × 20 cm, 25 × 20 cm (in-row spacing × between-row spacing). However, the concentration of astragaloside IV was highest in 20 × 20 cm density spacing and was significantly different than that in other density spacing treatment. Considering the cost, yield and astragaloside IV concentration, Ma et al. (2004b) concluded that the dense spacing of 20 × 20 cm was the best. Direct seeding can be performed in both spring and fall (Yang et al., 2006; Zhang et al., 2005a). In order to ensure that the weak cotyledons come out of the soil, the planting

depth needs to be adjusted according to the soil texture. For example, the recommended depth is 2-2.5 cm in sandy soil and 1-1.5 cm in sandy clay soil (Yang et al., 2006; Zhang et al., 2005a). For seedling transplantation, the seedlings need to be transplanted into their permanent positions as soon as the seedlings are large enough to handle. The seedlings are intolerant of root disturbance. The early transplanting of the seedlings at their early stage (the authors did not specify what size or how old the seedlings should be) will improve the rate of survival in the field.

Seedlings need to be irrigated regularly for the first couple of months (Yang et al., 2006; Janke and DeArmond, 2004). After the plant is big enough, about 10-15 cm high, the times of irrigation can be reduced. Thinning the plants is necessary at this time. The plant spacing is 15-20 cm within the row (Yang et al., 2006; Zhang et al., 2005a). From the second year, *A. membranaceus* grows fast and it is a fairly competitive herb once it gets established (Janke and DeArmond, 2004). Since enough manure, P and K fertilizers had been added in the field before planting, no more fertilizer is needed for the first couple of years of growth. In the third year, *A. membranaceus* grows fast and additional P and K fertilizer should be applied for the growth of root (Yang et al., 2006; Zhang et al., 2005a). Weed control needs to be done to ensure the normal growth of plant.

The Soil and Fertility Condition

A. membranaceus is a deep-rooted plant. According to previous studies, good

growing conditions for *A. membranaceus* consist of a dry and well-drained soil in a sunny position (Yang et al., 2006; Zhang et al., 2005a, c). It prefers a sandy soil. Taproot growth was significantly inhibited under water logging condition (Shibata et al., 1995). *A. membranaceus* grows well in mildly acid to alkaline soil with pH ranges from 6.5 to 8 (Yang et al., 2006).

Ma et al. (2000b) analyzed Astragali Radix from different regions of China and concluded Astragali Radix from Shanxi contained significantly higher amounts of isoflavonoids, saponins and polysaccharides. The main region for cultivation of Radix Astragali in Shanxi is Hengshan mountain areas (Zhang et al., 2005c). The soil texture in Hengshan mountain areas is loam and sandy loam. In Hengshan mountain areas the surface soil, which has relatively high pore space and high infiltration, can facilitate the root growth and the absorption of water and nutrients (Zhang et al., 2005c). In Japan, Shibata et al. (1996a) found the *A. membranaceus* plants grown in sandy and brown soil had best growth and high concentration of isoflavonoid in the taproots. Apparently, the physical property of sandy soil did not inhibit the growth and elongation of the taproot, whereas the development of lateral roots was limited (Shibata et al., 1996a). The suitable soil textures for *A. membranaceus* growth are loamy sand, loam, sandy loam and sandy clay (Liu and Wang, 1996; Yang et al., 2006; Zhang et al., 2005c). These soils are generally well drained. The taproot can easily grow deeply and produce less lateral roots in these soils.

Soil fertility greatly affects the growth rate, yield, the content of elements

including nutrient elements and trace elements, and of primary and secondary metabolites of *A. membranaceus* (Anetai et al., 1995; Zhang et al., 2005b, c). The studies by Zhang et al. (2005b) showed that *A. membranaceus* contains relatively high N and K, especially in the underground root. A high content of organic matter and available K in the soil is desirable for *A. membranaceus*'s growth. The concentration of N, P and K in root is higher than that in the aboveground parts and this content difference increases with the increase of growing time (Zhang et al., 2005b). This indicates that the products of photosynthesis are gradually transported to root.

Phosphorus was noted to enhance the growth and yield of *A. membranaceus*. *A. membranaceus* planted in different kinds of soil for one year grew best and had highest isoflavonoid concentration in sandy soil that had high P concentration in the soil (Shibata et al., 1996a). Anetai et al. (1995) planted *A. membranaceus* in the field and applied different amounts of phosphorus. They found the amounts of isoflavonoids and astragalosides I-IV in the Astragali Radix significantly increased with the increase of applied amounts of P. On the other hand, treatment of the soil with N or K fertilizer had little influence on the growth and yield of the plant and on the glycoside contents of the root (Anetai et al., 1995).

Although limited publications were found about the nodulation of *A. membranaceus*, the fact that N application did not increase growth (Anetai et al., 1995) suggested that the plants fixed N through nodules. Rhizobium-*Mesorhizobium* spp. was isolated from the root of *Astragalus sinicus* by Zhang (2000). Weir (2006)

inoculated *Mesorhizobium* spp to *A. membranaceus* and got effective nodules which were able to fix N_2. Dr. Zhang, who published the articles about *A. membranaceus* sent e-mail to me (Meili Wang) and wrote that he found *A. membranaceus* had nodules in deep soil level and could fix N_2 (Zhang, Q. Institute of Soil & Fertilizer, Shanxi Academy of Agricultural Sciences, Taiyuan, Shanxi Province, P.R. China, 2007, personal communications).

Some other studies (Anetai, et al. 1996; Zhang et al., 2005c; Zhao et al., 2002) found that the deficiencies of N, P and K in soil affected the growth of root and the ratio of root to shoot, especially under P deficient conditions. A deficiency of these nutrients also leads to less accumulation of dry material in root (Ma et al., 2004a; Tan et al., 2006b; Zhang et al., 2005c; Zhao et al., 2002). Tan et al. (2006b) used hydroponic methods to treat seedlings with different nutritional levels. The results indicated that N, P or K deficiency reduced the vigor of roots and increased the ratio of root to shoot in the order of –N> –P> –K> NPK. Nitrogen deficiency gave the highest root to shoot ratio. Anetai et al. (1995) applied different amount of manure in the field. After two years, the results showed that the rate of root to shoot decreased in the order of –P > –N > –K ≥ NPK. P deficiency gave the highest root to shoot ratio.

The root of *A. membranaceus* grows fast after July in North China and the content of N, P and K also greatly increases after July. After growing one year, one plant of *A. membranaceus* needs approximately 1.813 g N, 0.23 g P and 1.391 g K every year (Zhang et al 2005b). One study shows the diameter of *A. membranaceus*

root increases with increasing addition of N fertilizer at the same P and K level (120 kg P_2O_5 ha^{-1}, and 180 kg K_2O ha^{-1}) in the field under the condition of no inoculation with rhizobia (Zhao et al., 2002). Much more P fertilizer is normally taken up in summer (June and July) (Zhang et al., 2005b; Zhao et al., 2002). *A. membranaceus* has the property of luxury absorption to N, K, Fe and Zn, which results in high content of these elements in the underground parts (Zhang et al., 2005b). Accumulation of these elements is important for the medicinal function of traditional Chinese herbs.

The Relationship between Tillage and Root Morphology

Liu and Wang (1996) described four types of the root of *A. membranaceus* in the field (Figure 1), as follows: Type 1) the taproot is thick but shorter than 5 cm; the lateral roots are short and thick, too; Type 2) the taproot is shorter than 5 cm and has the two similar size lateral roots; Type 3) the taproot is longer than 30 cm and lateral roots are long and obvious; Type 4) the taproot is longer than 30 cm. Lateral roots are shorter, slender and fewer than above three types. The branches are 10 cm below the crown. *A. membranaceus* with Type 3 or Type 4 root has higher commercial quality than the other two types. Under the condition of dry weather, loamy sand, loam, or sandy loam soil, and the restricted soil layer deeper than 50 cm, the roots of *A. membranaceus* predominately belong to Types 3 and 4 (Liu and Wang, 1996).

Fig. 1-1. The types of *Astragalus membranaceus*'s root (Liu and Wang, 1996)

Many coastal plain soils of the southeastern US have a root restrictive layer, especially in the E horizon, that may impede plant growth (Busscher, Baue, and Frederick, 2000; Busscher, Frederick, and Baue, 2002, 2006; Hunt et al., 2004). If the taproot of *A. membranaceus* encounters a physical barrier in the soil, it is likely to bend or branch, rendering it poor quality for the medicinal plant market. High strengths in coastal plain soils can be reduced through deep tillage (Busscher, Frederick, and Baue, 2000, 2002, 2006). Deep tillage breaks the hardpan and should help the root of *A. membranaceus* grow through deeper subsoil.

In Japan, Shibata et al. (1996b) measured the soil hardness (penetration resistance) at different depths in the field, taproot length, and the number and length of 1st order lateral roots at different times. The result showed that the relationship between the taproot elongation and lateral root development with the soil hardness was close. A hard soil layer that had a penetration resistance of more than 588-1373

kPa might prevent taproot elongation and promote lateral root development (Shibata et al., 1996b). Except for the field experiment, the effect of soil compaction on root growth of *A. membranaceus* was performed by growing plants in PVC tubes. In a study of compaction using PVC tubes, soil penetration resistance above 980 kPa did not affect shoot growth, but it significantly shortened the taproot length and decreased the dry weight of taproot. The percentages of thick lateral roots, the number of lateral roots and dry weight of lateral roots significantly increased as the soil compaction levels increased. The concentration of astragaloside I, II, III, IV, total astragaloside and dilute ethanol-soluble extract in the taproot of *A. membranaceus* decreased as the soil compaction levels increased (Mia et al., 1999).

Different plowing conditions affect root growth and the amounts of glycoside in the roots. In a field experiment conducted in Japan, Mia et al. (1998) tested four tillage methods, tilling with trencher to 80 cm in depth, tilling with a trencher to 50 cm in depth, plowing with tractor to 25 cm in depth and no tillage. After 2-years of growth, the taproot dry weights of the plants grown in 80 cm deep trenches, and 50 cm deep trenches were significantly larger than those in no-plowing plots. Similarly, the taproots in 80 cm trenches, 50 cm trenches and tractor-plowed plots were significantly longer than those in no plowing plot. The plants grown in 80 cm trenches and 50 cm trenches had significantly fewer thick lateral roots and a large number of thin lateral roots than did those grown in no plowing plot. From these results, it can be concluded that *A. membranaceus* grown with deep tillage and loose soil condition

produced slender taproot with fewer thick-lateral roots (high quality), whereas the plants grown under no plowing condition produced short and thick taproot with large number of thick-lateral roots (low quality). *A. membranaceus* produces similar length taproots under both the shallow (25 cm) tillage and deep tillage condition. However, the shallow tillage results in lower dry weight and larger number of thick-lateral roots (Mia et al., 1998)

In another experiment, the taproots, and thick and thin lateral roots of *A. membranaceus* were assayed for their isoflavonoid (I-V) and astragaloside (I-IV) contents by HPLC. Isoflavonoids I-V were present in about the same concentration in those different root parts, regardless of the thickness and thinness of those roots. However, the concentration of astragalosides I-IV was greater in thinner roots; the thin lateral root contained much higher concentration of astragalosides than taproot and thick lateral roots (Anetai et al., 1996).

Astragalus membranaceus **Varieties**

Before *A. membranaceus* was domesticated, *A. membranaceus* grew wild in natural environments. However, the natural habitat areas of *A. membranaceus* are shrinking gradually because of the exhaustive exploitation (Zhao, 2006).To meet the increasing demands for natural medicinal plants, *A. membranaceus* is now largely cultivated. There are different varieties of *A. membranaceus* growing in different areas worldwide. Jiang et al. (2004) compared the active compounds of three different

varieties including wild *A. membranaceus*, cultivated *A. membranaceus* and cultivated *A. membranaceus mongholicus*, all of which grew in the same area. The result showed the concentration of astragaloside IV, total flavonoids and polysaccharides in wild *A. membranaceus* variety was higher than that in cultivated *A. membranaceus* and *A. membranaceus mongholicus* (Jiang et al., 2004). Zhang et al. (2002) compared the active compounds of wild *A. membranaceus* variety and cultivated *A. membranaceus* variety in the same area. Both varieties of wild *A. membranaceus* and cultivated *A. membranaceus* grew for three years and were harvested. They found total flavonoids and polysaccharides were higher in wild *A. membranaceus* than in cultivated *A. membranaceus* and astragaloside IV was similar in the two varieties (Zhang et al., 2002). Ma et al. (2000b) analyzed *Astragali Radix* from different regions and concluded the wild plants contained a slightly higher concentration of active constituents than the cultivated plants. This suggested the wild *A. membranaceus* has higher medicinal quality than cultivated *A. membranaceus* and we can use these wild varieties to produce some new varieties. It also suggested that cultivated plants grow under a more favorable environment for growth, but not for the concentration of active constituents.

Intra-species variation and interspecies hybridization occur naturally in *Astragalus* and produce new varieties. Some varieties of *A. membranaceus* have appressed white hairs on the leaves and some don't. The pollen, leaf vein, enzyme and root morphology are also different between the haired and unhaired *A. membranaceus*

(Xie et al., 2005). Intra-species hybidization might cause either high quality new varieties or the degeneration of cultivated varieties, in terms of the amount of medicinal components in them after 2-3 years growth. Some new varieties have been obtained by hybridization between different *A. membranaceus* varieties. Those new varieties possess an improved yield and higher concentration of active components (Xie et al., 2005).

Both environmental factors and varieties are vital to cultivation and able to affect bioactive component accumulation of medicinal plants. For example, under water deficient conditions, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), praline and soluble sugar of *A. membranaceus* were analyzed and compared in three varieties through outdoor potting cultivation method. The order of drought tolerance in three varieties is Mongolia > wild > Hebei (Tan et al., 2006a). Previous studies showed that the same medicinal plant from different regions have different therapeutic potency. A study by Cao et al. (2006) compared the root polysaccharide content in six varieties of *A. membranaceus*. Polysaccharide content differed in those varieties. A study by Li et al. (1992) of radix Astragali quality in China found that *A. membranaceus* from Shanxi Province contains more desirable trace elements (such as Fe, Zn Mn, Cr and Se), and fewer harmful trace elements (such as As and Pb) than those from Heilongjiang. *A. membranaceus* from Inner Mongolia contains more lead than those from Heilongjiang and Shanxi (Li et al., 1992). Ma et al. (2000b) analyzed Astragali Radix from different regions of China and concluded Astragali Radix from

Shanxi contained significantly higher amounts of isoflavonoids, saponins and polysaccharide than from other locations. This suggests that growth condition and varieties greatly affect the pharmacological contents.

For preserving and differentiating those varieties, internal transcribed spacer 1 of the nuclear ribosomal RNA gene of *A. membranaceus* was sequenced to confirm the different varieties of *A. membranaceus* (Yip and Kwan, 2006). Arbitrarily primed polymerase chain reaction was then used to obtain unique fingerprints for each sample after the species authentication. Therefore, *A. membranaceus* from different major cultivation places in China can be differentiated.

Pests and Diseases of *A. membranaceus*

A. membranaceus is susceptible to some plant diseases. Those diseases are powdery mildew caused by fungus *Erysiphic spp*, wilt and root or crown rot caused by fungus *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani* (Teng, Liang, and Chen, 2006; Luo et al. 2005; Yang et al., 2004). Mild and humid weather results in increased powdery mildew growth. We planted *A. membranaceus* in Alabama and found *Pythium* and *Phytophthora* root rot in the field. The root or crown rot caused by those fungi were where soils were kept continually wet, when the field was irrigated too much and poorly drained (Agrios, 2005).

Insect pests eat the leaves and roots of *A. membranaceus* and cause different degrees of damage. The main pests that can cause serious economic damage are

aphids, the underground larva of beetles, weevil and their larva (Yang et al., 2004). Grubs of white fringe beetle (*Naupactus* spp.) were found in our studied plots. They ate the root of seedling and caused serious damage. Three-cornered alfalfa hopper punctured and girdled the stems of *A. membranaceus* in the plots. The damaged stems were easily broken, but the new shoots grew from the old roots and replaced the damaged shoots.

For powdery mildew disease, 5% $NaHCO_3$ can be sprayed in the early stage. Fungicides, Chlorothalonil and Propiconazole, were often used to control other kinds of disease caused by fungi (Yang et al., 2004; Yang et al., 2006). For the insect pests, Chlorpyrifos and Carbaryl can be sprayed for some pests including soil-borne pests and aerial pest to prevent economic loss (Yang et al., 2004; Yang et al., 2006).

Pests can be control by cultural, biological and chemical methods. Cultural control includes weeding, good drainage and rotation with other non-leguminous crops. Biological control requires protecting natural enemies. Chemical control is the last method to be considered (Agrios, 2005; Everest et al., 2006).

Harvest Time and Drying

In China, *A. membranaceus* forms flowers and seeds mainly in fall. Therefore, *A. membranaceus* accumulates the bioactive constituents during this reproductive period. *A. membranaceus* mainly accumulates its root polysaccharides from August to the first frost day, in October or early November (Cao, et al. 2006; Ma, et al. 2002).

The optimal harvest time for polysaccharides is around the first frost day (Cao, et al. 2006). Astragaloside IV content was analyzed and compared in different months by Jia et al. (1998). Astragaloside IV content was higher in spring (end of April and early May) and fall (end of October and early November) than in other seasons (Jia et al. 1998). Spring and fall are the germination stage and dormancy stage of *A. membranaceus*. Therefore, the optimum harvesting time for astragaloside IV is spring or fall (Jia et al. 1998). Overall, the optimal harvesting time for highest yield of polysaccharide and astragaloside IV is fall, end of September to early November in north China (Cao, et al. 2006; Jia et al. 1998; Ma, et al. 2002).

A. membranaceus is a perennial herb. The levels of main constituents in *A. membranaceus* change according to the age of the plant. The contents of total astragaloside, total flavonids and astragaloside IV increase with growing time, whereas, the content of polysaccharides decrease with growth time (Zhang, Piao and Song, 2005). One-year-old plants and 2-year-old plants have similar concentration of total astragaloside, total flavonoids and astragaloside IV, whereas the concentration of these compounds in 3-year-old plants are 50% higher than those in 1 and 2-year-old plants. One-year-old plants have 50% higher concentration of polysaccharides than 2-year-old plants and the polysaccharides concentration of 3-year-old plants is 50% higher than in 2-year-old plants (Zhang, Piao and Song, 2005). Ma et al. (2002) studied the seasonal variations of active constituents in *A. membranaceus* and found total polysaccharides and γ-aminobutyric acid were significantly higher in 3-year-old

plants than in 2-year-old plants. The 1-year-old plants contained the highest concentration of total amino acid (Ma et al., 2002). Considering the planting costs and the desirable concentration of polysaccharides, astragalosides I-VII, triterpene glycosides, flavonoids and isoflavonoids, the optimal duration of cultivation is two or three years.

In China, traditionally, the harvested roots of *A. membranaceus* are dried in the sun. In Japan, outdoor drying resulted in higher concentration of dilute ethanol-soluble extracts and higher sucrose concentration than hot-air drying (above 50 ºC). The method of hot-air drying after harvesting reduced the concentration of dilute ethanol-soluble extract and sucrose (Anetai et al., 1998). According to the methods of tradition and this research, the outdoor or indoor drying method without high temperature is suitable for drying Astragali Radix.

Pharmacological Property and Analysis of Medicinal Compounds

Radix Aatragali has a long history of medicinal use in China. It is commonly used to treat pectoral, diuretic and childhood illnesses and acts as a tonic to supplement deficiencies in ancient China. Now in modern medicine, it is used as an immune modulator, an adjunctive therapy to chemo- and radiation therapy in cancer patients (Upton et al., 1999; Han, 2003).

Only in the last two decades have the pharmacological properties of individual compounds in *A. membranaceus* been examined. The medicinal compounds are found

mainly in roots. Major medicinal compounds in *A. membranaceus* are polysaccharides, triterpene glycosides including astragalosides I-VII, flavonoids and isoflavonoids (He and Findlay, 1991; Jiang, Ge, and Xue, 2004; Shirataki, et al., 1997; Yu and Liu, 1993; Zheng et al., 1998, 2002). The extensive studies have been conducted to investigate the medicinal functions of bioactive compounds. Four compounds were extracted from Radix Astragalus by Kim et al. (2003) and the extraction induced the release of growth hormone in pituitary cell culture. Flavonoids and saponins in *A. membranaceus* have been found to have free-radical-scavenging ability and antioxidant activity (Wang et al., 2003). Those bioactive compounds in the root are able to prevent lipid peroxidation (Choi et al., 2002; Toda and Shirataki, 1998). The extract of *A. membranaceus* has been demonstrated to have broad anti-inflammatory effect (Zhang et al., 2003). Further clinical studies also indicated that *Radix Astragali* has cardioprotective effects (Miller, 1998). Based on above stated medicinal functions, *A. membranaceus* may be used as an alternative remedy for prostate, carcinoma, lung and liver cancer (Chu, Wong, and Mavligit, 1988; Sun et al., 1983). The extract of *A. membranaceus* can effectively inhibit the growth of bacteria including gram negative and gram positive bacteria (Yao et al., 2006). The crude saponin, especially astragalosides I and IV, has the immunity function (Bedir et al., 2000; Ganzera et al., 2001; Han, 2003; Mao et al., 2005).

Cyclolanostane-type saponins are the major compounds responsible for the pharmacological effects in *A. membranaceus* (Anonymous, 2003; Block and Mead,

2003; McKenna, Hughes and Jones, 2002). Saponins in *Radix Astragali* are mainly comprised of astragalosides I, II, III and IV. Astragaloside IV has been regarded as one of *A. membranaceus* characteristic active constituents whose presence forms part of the quality assurance of *A. membranaceus* and products containing it (Ma et al., 2000b; State Pharmacopoeia Commission of P.R. China., 2000; Tu et al., 1988). He and Findlay (1991) extracted the roots of *A. membranaceus* with n-Butanol and separated the astragaloside with Si gel chromatograph. After those procedures, Astragaloside I (40 mg), Astragaloside II (20 mg) , Astragaloside IV (70mg), unknown compound-3 (25 mg), unknown compound-1 (10 mg), unknown compound-5 (40mg) were obtained (He and Findlay, 1991). Obviously, the amount of Astragaloside IV was higher than other compounds. On the other hand, Astragalosides I and II are chemically unstable and easily converted to astragaloside IV through loss of acetoxyl groups (Han et al., 2007). Therefore they are often commercially unavailable even though they are important bioactive saponins. So astragaloside IV is normally used as a marker compound for quality control (Ma et al., 2002; State Pharmacopoeia Commission of P.R. China., 2000.).

The morphological appearance of *A. membranaceus* and its adulterants, such as *Astragalus hoantchy*, *Astragalus lehmannianus* and *Astragalus aksuensis*, show a great resemblance, but astragaloside IV can't be detectable in adulterants by using same TLC and HPLC methods (Ma et al., 2002). Therefore, in other words, we analyze for astragaloside IV to be sure the roots are from *A. membranaceus*.

The marker compound, astragaloside IV, can be extracted from the root of *A. membranaceus -Radix Astragali*. Traditionally, the soxhlet apparatus is used to extract the ground root powder with 80% methanol (Feng, Feng and Guan, 2005; Gong, Yang and Zeng, 2005). Now ultrasonicator is often used to improve the extraction rate and this method normally has better extraction result than the conventional soxhlet extraction (Feng, Feng and Guan, 2005; Gong, Yang and Zeng, 2005; Li, Tan, and Ma, 2007; Valachovic, Pechova and Mason, 2001).

Astragaloside IV can be detected by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Li et al., 2003, Yang et al., 2007). The structure of astragaloside IV does not contain a UV-chromophore, which makes the application of direct HPLC with UV detection rather limited (Oleszek, 2002; Gu, Wang and Fawcett, 2004). A pre-column derivatization method is used to improve the sensitivity and specificity of HPLC (Yao, 2000). However the derivatization method also has some weakness, such as time consuming sample preparation and potential health risk to the operator. Mass spectrometry (MS) and evaporative light-scattering detection (ELSD) are best methods to detect astragaloside IV (Ganzera, et al., 2001; Gu, Wang and Fawcett, 2004; Li and Fitzloff, 2001; Shen et al., 2006).

Perspective of *A. membranaseus*: Marketing and Challenge to Cultivate

The Dietary Supplement Health and Education Act of 1994 has made it possible to market herbal medicines as dietary supplements and this has led to an increasing

demand for medicinal plants (FDA, http://www.cfsan.fda.gov/~dms/dietsupp.html). The price of Astragalus root (Oregon grown) was $19 per pound for dried and ground (www.eco-herbs.com, 2007). The price of Astragalus Root slices is $44 per pound and Astragalus Root powder is $18.4 per pound (Frontier Natural Products Co-op, http://www.frontiercoop.com**).** There are Astragalus supplements such as astragalus capsules and products on the market. Because of its market value, *A. membranaseus* could be an alternative crop for production in Alabama. Production practices need to be developed.

There are problems growing Astragalus in Alabama. Fifty to sixty percent of the seedlings that emerged in the fall of 2006 were dead by spring and summer 2007 in Auburn, Alabama. Plant mortality was most severe with small seedlings, but was observed throughout the season. Pests ate roots and caused serious damage. Chlorpyrifos (Duraguard ME, 0.4 g (AI) L^{-1}) was sprayed three times to control the fringe beetle in May, 2007. The insecticide spray was not effective to control the underground beetle, because it was not a good time to kill them and it was too late for saving the seedling plants. Root rot, caused by *Pythium* and *Phytophthora* fungi, was serious too in late spring and summer. .

In China, the same problem also existed. Before planting, the underground pests were controlled and killed by solarizing the soil or spraying low toxic and low residual insecticide (Yang et al., 2006; Zhang et al., 2005a). For soil-borne pests, such as white fringe beetle, can be controlled with insecticide (Dr. Xingping Hu, personal

communication, 2008). In spring, the soil can be treated to kill eggs and larvae; in fall, an experimental gas product is being tested that can be used to induce adults to go to other places to lay eggs (Dr. Xingping Hu, personal communication, 2008). Under high soil moisture and wet conditions, *Astragalus* is susceptible to root rot caused by fungi, which is the main constraint to cultivation in the southeastern U.S.. Land must be well drained for *Astragalus.* Therefore, after *A. membranaseus* grows for three to five months, we should reduce irrigation times. Soil moisture must be monitored to prevent over watering. Loose soil and raise beds can be used to control soil moisture. Resistant varieties can be selected to plant in the southeastern U.S.

Deep tillage is beneficial for the growth of *A. membranaceus* and can improve the content of astragaloside. In the processes of transplanting, partial damage of small fragile roots is unavoidable. Therefore, direct seeding is preferable to transplanting. Soil fertility is not major concern as long as P and K soil tests are in medium to high level, but more research is needed on nutrient effects on root yield and astragaloside content.

A. membranaseus belongs to the legume family and can fix N through nodules. However, *A. membranaceus* failed to nodulate with indigenous rhizobia. We did not find nodules on the root in all the trials. The inoculation study was performed in green house in Sep. 2007. The seeds were inoculated with (Rhizobium spp.) and the functional nodules were found on the roots after 45 days growing. Inoculation with rhizobia specific to Astragalus is highly recommended.

II. THE EFFECTS OF TILLAGE, VARIETY AND FERTILIZER ON THE GROWTH AND ROOT DEVELOPMENT OF *ASTRAGALUS MEMBRANACEUS* IN FIELD

Abstract

Astragalus membranaceus is a traditional Chinese medicinal herb that has been used for thousands of years. Information on its cultivation in the southeastern United States is lacking. In September 2006, three field experiments were established to test the effects of (1) deep tillage (subsoiling vs no subsoiling), (2) varieties (7) trial, and (3) P (0, 29, and 58 kg ha^{-1}) and K (0, 56, and 112 kg ha^{-1}) fertilization and lime on growth and root development in Auburn, Alabama on a Marvyn loamy sand (Plinthic Kanhapludults). Plant mortality was most severe with small seedlings, but was observed throughout the season. Mortality was was associated with insect damage to roots and underground stems and with root and crown rot caused by *Pythium* and *Phytophthora* under wet conditions. Subsoiling increased root weight and crown diameter, increased root length at 277 day (α=0.1) and distance from first branch to crown (α=0.1). Although deep tillage was somewhat beneficial for *A. membranaceus*, it did not sufficiently loosen soil to yield straight, unbranched roots. AM2, AM3, AM4 and AM5 cultivars had good adaptability, root weight (yield) and root quality and can be chosen to plant in southeastern U. S.. Shoot height, root weight, and root diameter of 7-month plants increased with phosphorus application. The lime effect was not significant at this site where the soil pH was below 5.9.

Introduction

Astragalus membranaceus is one of the important traditional Chinese medicinal herbs and has been used for thousands of years. It was used to treat pectoral, diuretic and childhood illnesses and acts as a tonic to supplement deficiencies in ancient (Upton et al., 1999; Han, 2003). Its common name is membranous milk-vetch or Astragalus root. This genus contains *A. mollissimus* (woolly locoweed) – a noxious weed in western U. S.; while cicer milkvech (*A. cicer*) is a forage legume in Northern U. S. and Canada. *A. membranaceus* is widely distributed in the pine forest region and mountain areas in Northern China, Siberia, and Northern Korea. Now, it is domesticated and is largely cultivated for medicinal use in Northern China, Northern Korea and Japan (Chen et al., 2006; Zhao, 2006). It has been successfully cultivated in North America, Canada and Europe (Matkowski et al., 2003).

Research has shown its roots can increase the production of interferon and macrophages and thus help restore normal immune function in cancer patients (Block and Mead, 2003; McKenna et al., 2002). The Dietary Supplement Health and Education Act of 1994 has made it possible to market herbal medicines as dietary supplements and this has led to an increasing demand for medicinal plants (FDA, http://www.cfsan.fda.gov/~dms/dietsupp.html). The price of Astragalus root (Oregon grown) was $19/lb for dried and ground (www.eco-herbs.com, 2007). It's worthwhile to evaluate the potential of *A. membranaceus* as an alternative crop for production in Alabama and to develop practices for production of *Astragalus*. However, the information on its cultivation in Southeastern United States is lacking

Root yield can be affected by varieties, soil strength, and by P and K fertilizer (Anetai et al., 1995; Ma, et al., 2000b; Shibata et al., 1996a). According to previous

studies, good growing conditions for *A. membranaceus* consist of a dry and well-drained soil in a sunny position (Yang et al., 2006; Zhang et al., 2005a, c). It prefers a sandy soil. *A. membranaceus* grows well in mildly acid to alkaline soil with pH ranges from 6.5 to 8 (Yang et al., 2006). Many coastal plain soils of the southeastern U. S. have a root restrictive layer that may impede plant growth (Busscher et al., 2000, 2002, 2006; Hunt et al., 2004). Deep tillage breaks the hardpan (Busscher et al., 2000, 2002, 2006) and should help the root of *A. membranaceus* grow through deeper subsoil.

Before *A. membranaceus* was domesticated, it grew wild in natural environments. However, the natural habitat areas of *A. membranaceus* are shrinking gradually because of the exhaustive exploitation (Zhao, 2006). To meet the increasing demands for natural medicinal plants, *A. membranaceus* is largely cultivated in the field nowadays. There are different varieties of *A. membranaceus* growing in different areas worldwide.

Soil fertility greatly affects the growth rate, yield, the content of elements including nutrient elements and trace elements, and of primary and secondary metabolites of *A. membranaceus* (Anetai et al., 1995; Zhang et al., 2005b, c). Phosphoric acid was noted to enhance the growth and yield of *A. membranaceus* (Anetai et al., 1995). On the other hand, treatment of the soil with nitrogen or potassium fertilizer had little influence on the growth and yield of the plant and on the glycoside contents of the root (Anetai et al., 1995). Some other studies (Anetai et al. 1996; Zhang et al., 2005c; Zhao et al., 2002) found that the deficiencies of N, P and K in soil affected the growth of root and the ratio of root to shoot, especially when P deficiency happened. The deficiency of these nutrients also leads to the less

accumulation of dry material in root (Ma et al., 2004a; Tan et al., 2006b; Zhang et al., 2005c; Zhao et al., 2002).

Plants have the ability to adapt and survive in different climate and area. Based upon the range in climatic conditions under which *A. membranaceus* is cultivated, including South Korea, we hypothesized that it would be possible to cultivate *A. membranaceus* in Alabama. The goal was to assess the suitability of *A. membranaceus* for cultivation in Alabama and to develop preliminary recommendations on its cultivation. Specific objectives of this research were to (1) determine the appropriate tillage practices for Astragalus root on a sandy, well drained Coastal Plain soil with hardpan, (2) select varieties of *A. membranaceus* to grow in Alabama, and (3) determine *A. membranaceus* P, K, and lime response on root yield.

Materials and Methods

Study Site

The study site was a Marvyn loamy sand (Fine-loamy, kaolinitic, thermic, Plinthic Kanhapludults) at Auburn, Alabama (32°35'N, 85°29'W). The mean annual temperate is 18ºC and annual precipitation is 1400 mm. The area we used had been uncultivated for the last five years and had a cover of grass and weeds. The soil was classified as Marvyn loamy sand (Fine-loamy, kaolinitic, thermic, Plinthic Kanhapludults). Soil profile description is shown in Table 2-1. In September, 2006, before fertilizer and lime were applied, a composite soil sample collected from the top 15 cm was analyzed by Auburn University Soil Testing Laboratory (Table 2-2). We conducted three experiments at the same time, a tillage trial, a variety trial and a fertility trial.

Site Preparation and Experimental Designs, Fertilization, Beds Creation and Subsoiling

Randomized complete block designs with four replications were used in each experiment. Soil testing was performed before fertilizer application and after harvesting. No nitrogen fertilizer was applied.

Tillage trial

The field size was 15.2 m × 15.2 m. The field was disked in order to incorporate surface vegetation and to create a plow pan in August, 2006. To adjust soil pH to 6.5, ground limestone (2469 kg ha^{-1}) was broadcast on the field on September 19, 2006. Triple superphosphate (39 kg P ha^{-1}) and muriate of potash (37 kg K ha^{-1}) were broadcast and incorporated to assure that these nutrients were not limiting factors for the growth of *A. membranaceus*.

Two disk plows were used to create ridges and a bedder was driven over the ridge to form the soil into raised beds 10 cm high and 91 cm wide. There were two treatments, (1) in-row subsoiling (SS) and (2) no subsoil control (NoSS). Treatment SS was established by using an in-row subsoiler to a depth of 35 cm. The subsoiler was attached to the tractor at the time the beds were formed and the shanks were adjusted according to the row spacing. The plot size was 0.91 m × 9.1 m. On September 22, 2006, all the beds were covered with landscape fabric and forty-four holes per plot with approximate diameter of about 5 cm were cut in fabric. The hole spacing in the row was 41cm. In SS treatment, the holes were located above the subsoiler path.

Variety trial

The field size measured 18 m × 25 m and was prepared, limed and fertilized the

same as the tillage trail. An in-row subsoiler was used to break up any plow pan that may exist on the site and the beds were constructed the same as in the tillage trial. The beds were constructed so that each bed straddles path of two subsoilers. That allowed us to plant so that roots penetrate soil loosened by subsoiler shanks. The plot size was 0.91 m × 5.3 m. On September 22, 2006, all the plots were covered with landscape fabric and twenty-four holes per plot with a diameter of about 5 cm were cut in fabric in each plot. The hole spacing was 41cm and all the holes were located above the subsoiler path.

Fertility trial

The field size was 19 m × 22 m and was prepared in August, 2006, in the same manner as the other two trials including subsoiling under the beds. The design of experiment (Table 2-3) was a 3 × 3 factorial of P (0, 29, 58 kg P ha^{-1}) and K (0, 56, 112 kg K ha^{-1}) augmented with a no-lime treatment at mid levels of P and K (29 kg P + 56 kg K ha^{-1}). The ten treatments are listed in Table 2-3. Fertilizer and limestone was applied by hand to each plot on September 19, 2006. The application of limestone (2469 kg ha^{-1}) was based on soil test in order to bring pH to 6.5. Plot size was 0.91 m × 4.5 m.

Seeds, Planting and Thinning

Seven varieties of *A. membranaceus* were selected in variety trial (Table 2-4). AM1 and AM2 were donated by USDA. AM3, AM4 and AM6 were purchased from companies in U. S.. AM5 was purchased South Korea. AM7 was purchased from Anguo, Hebei (38°25'N, 115°18'E), China. AM3 was used in tillage trial and fertility trial.

The seeds of *A. membranaceus* were soaked in warm water for 24 hours before

sowing and directly planted in the field. Assuming full stands, the planting density was 14,546 plants ha^{-1}. On September 25, 2006, 5-6 seeds were planted per hill and covered with less than 0.7 cm soil. The in-row spacing was 45.7 cm and between-row spacing was 70 cm. Seven days after sowing, first stand counts were taken in all three experiments. Ten days after sowing, second stand counts were taken in the variety trial. The hills where there were no plants present were reseeded between October 20 and October 31, 2006. Irrigation was applied frequently until emergence because of drought conditions. At same time, Astragalus seeds were planted in the greenhouse and after ten days the seedlings were transplanted to field to replace the missing or dead plants. The plants were thinned to one plant per hill at about three weeks after planting.

On November 21, pine bark mulch was spread on landscape fabric for weed control. The weeds next to plants were pulled by hand. The weeds between beds were eliminated by spraying glyphosate herbicide (at 1.9kg ai ha^{-1} RoundUp®, Monsanto, Missouri, USA).

In winter, the above-ground portion of plants wilted and dried due to frost. With the rising temperature in spring 2007, the plants recovered and shoots regrew. Regrowth stand counts were taken on March 30, 2007. Percent recovery was calculated by dividing the result of stand counts after winter by stand counts before winter. To replace the dead plants, 10 day-old seedlings were transplanted to field in April, 2007.

Inoculation Study

Seeds of *A. membranaceus,* (AM3 and AM6) were inoculated with bacterial *Rhizobium spp.* developed for *Astragalus* species (Nitragin, Inc, WI, USA) and

planted in greenhouse on July 27, 2007. The roots were gently washed off with water to observe the nodules on Sep. 10, 2007.

Observations and Data Collection

During the growing period, the percent emergence and qualitative observations were made on growth, diseases and insect pests..Date of flowering, fruit development and fruit maturation were recorded. When the two-valve seed pods turned dark color, which means seeds were mature, seeds were collected and air dried and stored for future experiments. The effective nodules on the roots were observed in the inoculation study.

Harvest in Fall

In all three experiments, plants were dug with shovel and harvested in November 2007. Plants that were planted in October 2006 and harvested in November 2007 were labeled as 13-month plants. The plants that were planted in spring 2007 and harvested in November 2007 were labeled as 7-month plants.

Drying and Sample Grinding

Shoots were dried in oven at 65 °C for two days. Roots were dried in a forced air dryer at 40 °C for four days. After drying, dry weight of the shoots and roots were measured. All the plant materials (shoot and root) were ground to fine powder in Wiley mill.

Tillage trial

On December 5, 2007, 64 days after planting, we dug a trench at the front of each plot and harvested two plants. Two plants from each plot were harvested on May 31, June 30, and August 1, 2008, 214 day, 242 days and 277 days respectively after *A. membranaceus* was planted. When harvesting, the presence of hardpan was observed

and its depth was measured. The length of root was measured. Whether the taproot penetrated hardpan or not was determined. Observations were recorded on number of branches on the taproot and whether the taproot was straight or curved. The plants were air dried under room temperature and dry weights of roots and shoots determined.

On February 21, 2008, the soil was thoroughly moistened and soil strength was measured using a CP40II recording cone penetrometer (ICT International Pty Ltd, Armidale, New South Wales, 2350, Australia). Each datum is the average of 15 insertions made within individual plots, with readings taken in 1.5 cm increments. In other words, 15/plot × 4 plots = 60 measurements per treatment.

Plant height was measured from soil surface to the tip of main stem before harvesting on October 20, 2007. Fresh weight of the shoots and roots were measured at harvest in fall. Root branches were counted. The diameter of taproot at crown and root length was also measured.

Variety trial

On October 2, 2006, the first stand counts were taken one week after sowing. On October 5, second stand counts were taken.

Plant height was measured from soil surface to the tip of main stem before harvesting on October 20, 2007. Fresh weight of the shoots and roots were measured at harvest in fall and dry matter yield was determined. Root branches were counted. The diameter of taproot at crown and root length was measured respectively.

The aboveground parts of 13-month plants were analyzed for N, P, K, Ca, Mg, Cu, Fe, Zn, Mn and B. Total N was determined by the combustion method using a LECO CHN-600 (LECO Corporation, St. Joseph, MI). ICAP (Thermo Jarrell Ash,

Franklin, MA) was used to determine P, K, Ca, Mg, Cu, Fe, Zn, Mn and B.

Fertility trial

Plant height was measured from soil surface to the tip of main stem before harvesting on October 20, 2007. Fresh weight of the shoots and roots were measured at harvest in fall. Root branches were counted. The diameter of taproot at crown and root length was measured respectively. After harvesting in fall, soil in each plot was analyzed for pH, N, P and K by Auburn University Soil Testing Laboratory using Procedures used for Soil and Plant (Donohue, 1992) (Table 2-5, 2-6, 2-7).

The aboveground parts of both 7-month and 13-month plants in T1, T3, T7 and T9 were analyzed for N, P, K, Ca, Mg, Cu, Fe, Zn, Mn and B. The aboveground parts of 13-month plants in T5 and T10 were also analyzed. Total N was determined by the combustion method using a LECO CHN-600 (LECO Corporation, St. Joseph, MI). ICAP (Thermo Jarrell Ash, Franklin, MA) was used to determine P, K, Ca, Mg, Cu, Fe, Zn, Mn and B.

Because of poor stands and presence of plants of different ages within same plots, yield was calculated on a per plant basis for both 7-month and 13-month old plants.

Statistical Analysis

Analysis of variance was performed on raw data using version 9.1 of SAS to test main effects and interactions (SAS Institute Inc., 2003). Parameters were analyzed using PROC MIXED as randomized complete block factors. The treatment means were compared using Duncan's multiple range tests. All statistical tests were made at $\alpha = 0.10$ significance level.

Results

Seed Emergence, Seedling Survival and Disease Control

In the tillage trial, germination seven days after sowing was 69%. About 85% of the hills had seedlings. In winter (December 2006, January and February 2007), the aboveground parts were killed due to frost. In April, 2007, plants grew new stems and leaves and the percent recovery and stands were 83% after winter.

In the variety trial, seven varieties of *A. membranaceus* were assessed for plant growth and root development. After sowing, first stand counts and second stand counts were taken. The percent germination after sowing seven days and ten days are shown in Table 2-8. The percent germination of AM1 was 5% greater after sowing ten days than after sowing seven days. For the other varieties, germination did not change much after seven days. AM3 had highest germination rate (89%) and AM5 had lowest germination rate (4%).

After reseeding and transplanting to replace the missing or dead plants, populations were close to 100% in early December. The percent recovery was above 80% for all of varieties after winter.

Mortality was high in all trials and many plants died in spring and summer 2007. The height of above ground parts were only had about 10 to 20 cm and the roots were still small and slender at that time. In the variety trial, the mortality was about 70% for AM7 and about 50% for the remaining accessions. The mortality was about 50% in fertility trial and about 60% in tillage trial. Soil-borne insects fed on the root and crown of the seedlings which caused the roots to break. However, some plants that were cut above crown by insects still had the ability to produce new shoots.

Chlorpyrifos (Duraguard ME, 0.4 g (AI) L^{-1}) was sprayed three times to control those soil-borne insects in May, 2007.

Plant mortality was most severe with small seedlings, but was observed throughout the season. Root rot, caused by *Pythium* and *Phytophthora* fungi, which was identified by The Auburn University Plant Diagnostic Lab on June, 2007, caused aboveground parts to wilt (photo 1, 2). A plant having good growth with normal root is showed in photo 3.

Because of high mortality rate and poor stands, all the plants were harvested in late October and November.

General Growing Information

All plants had flowers with white-yellow color in June, 2007, except AM7 (Photo 4, 5). AM7 did not produce flowers or pods. One month after flowering, the flowers turned into seeds. At end of August, 2007, the pods became dry with dark brown color. The two-valve seed pods had short, dark hairs on them. The seeds in the pods are very tiny and kidney-shaped with dark brown color. We did not find nodules on the root in the field, when we dug the roots.

Plant Growth and Root Development of *A. membranaceus* (Tillage Trial)

Soil strength

After *A. membranaceus* was harvested, we measured soil strength from soil depth 15 mm to 465 mm in the field (Fig. 2-1). Between 15 mm and 60 mm soil depth, no significant differences were found between SS treatment and NoSS treatment. Soil strength was significantly greater in NoSS treatment than in SS treatment at soil depth from 75 mm to 300 mm. Between 315 mm and 465 mm soil depth, no significant

differences was found between SS treatment and NoSS treatment. At depth of 180 mm, soil strength was 3840 kPa in NoSS treatment, almost 5 times greater than in SS treatment (Table 2-9).

Monthly intervals harvest

At harvest, the presence of hardpan was observed and its depth was estimated by inserting a steel wire until there was strong resistance to penetration. The depth to hardpan averaged 25±3.2 cm in SS treatment and 19±1.1 cm in NoSS treatment. At 64 days after planting, the roots were still very small and slender. The taproot penetrated straight into hardpan and no branch was found on root at 64 days. At 214, 242 and 277 days after planting, although taproot penetrated hardpan, the taproot was bent at the point where branches (lateral roots) were present in both SS and NoSS treatments.

At 64, 214, 242 and 277 days after planting, root weight, shoot weight, shoot height and root diameter did not differ statistically between SS and NoSS treatment (Table 2-10).

At 277 days after planting, root length (Pr=0.04) and the distance from crown to first branch (Pr=0.093) differed statistically between the two treatments. Root length was longer in SS treatment (57.0 cm) than NoSS treatment (49.7 cm) (Table 2-10). Subsoiling increased root length by 13%. The distance was longer in SS treatment (2.8 cm) than NoSS treatment (1.7 cm) (Table 2-10). The distance from first branch to crown increased 39% in SS treatment compared to the NoSS treatment. At 64, 214 and 242 days after planting, root length and the distance from first branch to crown did not differ statistically between SS and NoSS treatment (Table 2-10).

Harvest in the fall

Subsoiling had no significant effect on shoot or root weight for either 7-month plants or 13-month plants (Table 2-11). Root weight was close to each other in SS and NoSS treatment for both age plants. Subsoiling increased average number of root branches by 36 % in 7-month plants ($p \leq 0.066$), but had no effect on root branch numbers in 13-month plant (Table 2-11). Average root length did not differ significantly between SS and NoSS treatments in 7-month plants, but subsoiling increased root length by 13.3% (Pr=0.046) in 13-month plants (Table 2-11). Subsoiling had no significant effect on root diameter for 7-month and 13-month plants (Table 2-11).

Plant Growth and Root Development of *A. membranaceus* (Variety Trial)

With 7-month plants, shoot height among varieties differed statistically (Pr=0.010) and AM5 had highest shoot height (Table 2-12). With 13-month plants, shoot height did not differ statistically among varieties, however significant differences were observed with the Duncans Multiple Range Test and AM5 was tallest. Shoot weight tested significant among varieties for both 7-month (Pr=0.004) and 13-month plants (Pr=0.020). AM5, AM2, AM1 and AM4 had highest shoot weight in 7-month plants and AM7 had lowest shoot weight (Table 2-12). With 13-month plants, AM4, AM5 and AM2 had highest shoot weights and AM3 had lowest shoot weight.

Number of root branches per plant and root length did not differ statistically with 7-month and 13-month plants (Table 2-12).

Root diameter differed statistically among varieties for both 7-month (Pr=0.014) and 13-month plants (Pr=0.026). AM5 had the biggest root diameter in 7-month

plants, and AM5 and AM2 had the biggest root diameter in 13-month plants (Table 2-12).

Root weight differed statistically among varieties for both 7-month (Pr=0.029) and 13-month (Pr=0.086) plants. In 7-month plants, root weight was greatest in AM5. With 13-month plants, root weight was greatest in AM7, AM5 and AM4 (Table 2-12).

Concentration of N, P, K, Ca, Mg, Zn, Cu, Fe, and B

No significant differences among varieties were observed in concentration of N, P, K, Ca, Mg, Cu, Fe, and B in above-ground plant parts (Table 2-13). Concentration of Mn and Zn differed significantly among varieties, at Pr=0.001 and Pr=0.062, respectively (Table 2-13). AM7 had highest concentration of Mn and Zn. AM2 had lowest concentration of Mn and AM6 had lowest concentration of Zn.

Plant Growth and Root Development of *A.membranaceus* (Fertility Trial)

Soil fertility in the field

Before fertilizer application, Mehlich-1 extractable P rated medium level (14 mg P kg^{-1}) and extractable K rated high high level (60 mg K kg^{-1}) (Table 2-2) according to the procedures used by Auburn University Soil Testing Laboratory (Adams, J.F., C.C. Mitchell, and H.H. Bryant.1994. Soil test fertilizer recommendations for Alabama crops. Ala. Agric. Exp. Sta. Dep. Ser. No. 178. Auburn University, AL). After harvest, K rated medium for all treatments and K application didn't significantly increase soil available K (Table 2-5). After harvesting, P rated for zero and 29 kg P ha^{-1} application treatments and rated high for 58 kg P ha^{-1} application treatments. Phosphorus application significantly increase soil available P

(Table 2-5). Ground limestone was broadcast to adjust soil pH to 6.5 except treatment 10 (T10). After harvesting, soil pH ranged from 5.8 to 6.0 in T1 to T9 and soil pH was 5.3 in T10, which was lower than other treatments (Table 2-6). Compared with T10 (0-29-58, without lime), treatment 5 (T5, 0-29-58, with lime) significantly increased soil pH. Lime application did not significantly increase soil available Ca and Mg (Table 2-6).

Plant Growth, P effect, K effect, P×K interaction effect and lime effect

After P fertilizer application, differences in root weight of 7-month plants were significant at Pr=0.083 (Table 2-14). A breakdown of linear and quadratic effects revealed that only the linear effect was significant (Pr=0.068) and root weight significantly increased with P application (Table 2-15, Fig. 2-8). There was no significant difference in root weight for 13-month plant after P fertilizer application.

The main effect for K fertilizer application on root weight was not significant (Table 2-14) for either 7-month or 13-month plant. However, the linear interaction of P and K did test significant (Pr=0.056) for root weight of 13-month plants (Table 2-15). Without K application, root weight decreased with the level of P application (Fig. 2-9). Similarly, without P, root weight root weight appeared to decrease with K application. At the high rate of P, K application appeared to increase yield, but not to the point of the no fertilizer treatment (Fig. 2-9). There was no P effect and K effect on root to shoot ratio for either 7-month or 13-month plants (Table 2-14). However, the linear interaction P and K significantly (Pr=0.077) affected root to shoot ratio for 13-month plants (Table 2-15). Without K application, root to shoot ratio decreased with the level of P application for 13-month plants. With K application, root to shoot

ratio increased with P application at 112 kg K ha^{-1} , such that at the high levels of P and K, the root to shoot ration was at or slightly higher than the root:shoot ratio without fertilizer. (Fig. 2-10).

Fertilization with P significantly increased shoot height (Pr=0.052) for 7-month plants (Table 2-14). Shoot height was higher at 29 kg P ha^{-1} than without P application (Fig. 2-11).

Fertilization with P significantly affected root diameter (Pr=0.081) (Table 2-14) for 7-month plant. The quadratic effect for P was significant (Pr=0.069) (Table 2-15) and root diameter was significantly higher at 29 kg P ha^{-1} than at 0 or 58kg P ha^{-1} (Fig. 2-12). Fertilization with P did not significantly affect shoot height and root diameter for 13-month plants. The effect of P fertilizer on root length and number of root branches did not test significant for either 7-month or 13-month plants (Table 2-14). Application of K fertilizer had no significant effect on shoot height, root diameter, root length and root branch for either 7-month or 13-month plants (Table 2-13). The interaction of P and K did not significantly affect shoot height, root diameter, root length and root branch for either 7-month or 13-month plants (Table 2-14).

There was no significant effect of lime on root weight, root to shoot ratio, shoot height, root diameter, root length and root branch at 29kg P + 56kg K ha^{-1} for either 7-month or 13-month plants (Table 2-16).

Concentration of N, P, K, Ca, Mg, Zn, Cu, Fe, Mn and B

Concentrations of N, P, K, Ca, Mg, Zn, Cu, Fe, Mn and B in aboveground parts of 13-month and 7-month plants in T1(0-0-0, with lime) (control), T3 (0-58-0, with lime), T5 (0-29-56, with lime), T7 (0-0-112, with lime), T9 (0-58-112, with lime) and

T10 (0-29-56, without lime) are shown in Table 2-17. One of the objectives was to determine if application of fertilizer at the highest rates (58 kg P ha^{-1}, 112 kg K ha^{-1}) altered nutrient concentrations in the shoots under the condition of applied lime. For all of these elements, no significant difference was found for all selected treatments. The other aim was to compare no lime application with application of lime at middle amount application of fertilizer (29 kg P ha^{-1}, 56 kg K ha^{-1}). No significant difference was found between lime and no lime treatment (Table 2-17).

Discussion

Seed Emergence, Seedling Survival, General Growing, and Disease Control

In the variety trial, the percent germination between seven days and ten days after sowing varied a little from 0.1 to 1.0% for variety AM6 and AM7 (Table 2-8). The percent germination between seven days and ten days after sowing didn't change for variety AM2, AM3, AM4 and AM5 (Table 2-8). Therefore, seeds of Astragalus need about seven days to germinate after sowing. AM5 in variety trial had very low percent germination (4%) which was probably because the seeds were stored for a long time. We collected some seeds of AM5 in November and December, 2006 and planted them in a greenhouse. Percent germination of these fresh AM5 seeds was 92%, which was much higher than the original seeds. In spring 2007, the seedlings from these fresh seeds were transplanted in the field. Therefore, when plants were harvested, many of AM5 were 7-month old and only few plants of AM5 were 13-month old, which made the data less representative for 13-month old AM5.

The percent germination of AM3 in the variety trial was 90%. However, the

percent germination of AM3 in tillage and fertility trial was 54% and 69%, respectively. The main reason for the low emergence in the tillage and fertility trials was that too much soil covered the seeds when we sowed by hand. The seeds of *A. membranaceus* are very tiny and about 2-5 mm long. The germinated seeds had difficulty emerging if more than 0.7 cm soil covered them. In the field, frequent irrigation was necessary under drought condition until emergence.

All of the varieties had flowers and produced seeds except AM7. AM7 failed to produce flowers and pods. Based upon the ability to reproduce via flowers and seeds, accessions AM1 - AM6, are adapted to southeastern U. S., whereas AM7 is not.

A. membranaceus belongs to legume family and can fix nitrogen through nodules. However, we did not find nodules on the root in the field, which might be caused by no appropriate rhizobia present. Limited publications were found about the nodulation of *A. membranaceus*. Weir (2006) inoculated *A. membranaceus* with *Mesorhizobium* spp for other Astragalus species that had never been tested on A. membranaceus and obtained effective nodules. In our inoculation study, seed was inoculated with (Rhizobium spp.) resulted in seedlings with functional nodules with pink centers on the roots after 45 days growing (Photo 7). This suggests that Rhizobium bacteria appropriate to *Astragalus* was not present in the field in Auburn, but that N fixation may be achieved with inoculation with appropriate Rhizobia.

In winter (December 2006, January and February 2007), the above-ground part of the plant was wilted and dried due to frost. In the middle of March, 2007 new leaves came out from the stems and the plants began to grow again. The percent recovery after winter ranged from 81% to 95% for different varieties (Table 2-8). Percent recovery for each variety was above 80%, which means *A. membranaceus* can

tolerate low temperature of winter in the southeastern U. S. (USDA hardiness zone 8a/-9.5 to -12.2 C) (http://www.usna.usda.gov/Hardzone/hzm-se1.html) and has the ability to recover and live very well in spring.

Mortality was high and about 50-60% plants died in spring and summer 2007. Chlorpyrifos (Duraguard ME, 0.4 g (AI) L^{-1}) was sprayed three times to control soil-borne insects in May, 2007. Later, in Feb. 2008, this soil-borne insect was identified as white fringe beetle (*Naupactus* spp.) by the Auburn University plant Diagnostic Lab (Dr. Charles Ray, Insect Diagnostician) (Photo 6). Because we didn't know what kind of insect fed on the root during the plants growing period, the effective control method was not found in time to use in the experiments. Soil-borne pests, such as white fringe beetle, can be controlled with insecticide (Dr. Xingping Hu, personal communication). In spring, the soil can be treated by pesticide to kill eggs and larvae; in fall, a special gas can be used to induce adults to go to other places to lay eggs (Dr. Xingping Hu, personal communication). Root rot, caused by *Pythium* and *Phytophthora* fungi and these fungal organisms 7were often found where soil is kept continually wet. Therefore, after *A. membranaceus* grows for three to five months, we should reduce irrigation. Soil moisture should be checked often and prevent to prevent over watering.

Plant Growth and Root Development of *A. membranaceus* (Tillage Trial)

Soil strength was significantly greater in NoSS treatment than in SS treatment at depth from 70 mm to 300 mm. No significantly difference was found between SS treatment and NoSS treatment at other soil depths (Table 2-9). According to Yang et al. (2006), and Zhang et al. (2005a, c), *A. membranaceus* prefers a sandy soil with low

soil strength so that Astragalus' roots can easily grow deep. The physical property of sandy soil did not inhibit the growth and elongation of the taproot, whereas the development of lateral roots was limited (Shibata et al., 1996a). Liu and Wang (1996), Yang et al. (2006) and Zhang et al. (2005c) reported that loamy sand, loamy, sandy loam and sandy clay are suitable soil types for growing *A. membranaceus.* The soil texture in our experimental field was loamy sand in Ap and E horizon, which should have been suitable for Astragalus' growth. However, below E horizon, the soil texture was sandy clay and sandy clay loam which should not have been suitable for Astragalus' growth (Table 2-1).

Many Coastal Plain soils of the southeastern U. S. have a root restrictive layer that may impede plant growth (Busscher et al., 2000, 2002, 2006; Hunt et al., 2004). High strengths in coastal plain soils can be reduced through deep tillage (Busscher et al., 2000, 2002, 2006). Deep tillage breaks the hardpan and should help the root of *A. membranaceus* grow into the deeper subsoil. If the hardpan was still there, when taproot of *A. membranaceus* encountered this physical barrier (hardpan) in the soil, it was likely to bend or branch, rendering it poor quality for the medicinal plant market.

A hard soil layer that had a penetration resistance of more than 588-1373 kPa might prevent taproot elongation and promote lateral root development (Shibata et al., 1996b). The soil strength was 596 kPa at 135 mm depth in SS treatment, which means that even with in-row subsoiling the soil strength within the rooting zone was sufficient to promote branching. In the NoSS treatment, soil strength was 585 kPa at 75 mm depth, which means that SS treatment was only 60 mm deeper than in NoSS treatment to reach similar soil strength. This result indicated that hardpan was not completely broken in SS treatment, because it is normal that the subsoiler can disrupt

hardpan ground down to at least 30 cm depth. The hardpan was still there and causes high soil strength in both treatments. All of the activity, such as disking, making bed and deep tillage, might destroy the soil structure and contribute to high soil strength, which made roots branch and prevented root elongation.

Subsoiling increased root length at 277 days after planting by 7.3 cm and distance between crown and first root by 1.1 cm. Although statistically significant, these differences (1.1 cm) are not meaningful to the trade in Astragalus root. Subsoiling did not improve shoot weight, root weight, shoot height, root diameter and root branches (Table 2-10).

At different harvest times (after 64, 214, 242 and 277 days), the trend of shoot weight, root weight, shoot height, root diameter, root length and the distance from first branch to crown were greater in SS treatment than in NoSS treatment (Fig. 2-2, 2-3, 2-4, 2-5, 2-6, 2-7). Those trends indicated in-row subsoiling was beneficial for the growth of *A. membranaceus* According to the study of Liu and Wang (1996), high market value Astragalus root had long taproots. Therefore in-row subsoiling has the potential to increase the market value of Astragalus root.

Subsoiling significantly increased the number of root branches of 7-month plants and increased root length of 13-month plants (Table 2-11). The root with longer root length and less branch has high market value. Subsoiling improved market value on root length, and at the same time decreased root quality on root branches, when *A. membranaceus* was young (7 month). After growing 13 months, there was no difference between SS and NoSS treatment, which indicated subsoiling didn't improve root quality based upon branching with the growing time increase. The deep tillage was not beneficial in terms improving root growth with high quality

characteristics. All the other measured parameters didn't show the significant difference between SS treatment and NoSS treatment for both age plants. Following are some reasons that may have contributed to this caused this not significant difference:

(1) As a result of the serious damage caused by insect pests feeding on roots in spring 2007 and root rot was caused by fungi *Pythium* and *Phytophthora*,.mortality was high and about 60% plants died in spring and summer 2007. To make up both kinds of damage, the 10-day old seedlings, which were planted in greenhouse, were transplanted to the field. In the processes of transplanting, some damage to these small fragile roots was likely. Their damage in young age will affect the root growth and root development in the future. Damaged roots would have affected both treatments equally, since a damaged root will have branching regardless of soil conditions.

(2) In the SS treatment, in-row subsoiler created two narrow slits near the outside of each bed. We attempted to place the seed of *A. membranaceus* over these slits. At harvest, we used a steel wire to locate the slit in relation to the harvested plants. It was found that many plants were 5 – 9 cm away from subsoiler slits, which means that many plants in the SS treatment may not have benefited from the loosened soil from the subsoiling operation. This may explain the lack of significant differences between SS treatment and NoSS treatments for many parameters.

Plant Growth and Root Development of *A. membranaceus* in Variety Trial

High quality roots should have long root length, big root diameter and less branches (Liu and Wang, 1996). No variety in this experiment had all three root

characteristics. Among 7-month plants, AM5 had greatest and AM7 had lowest shoot weight, root weight and root diameter. Among 13-month plants, AM7 had greatest root weight and root length and AM5 had biggest root diameter (Table 2-12). Within 13-month plants, AM4 had lowest root length and AM1 had lowest root diameter (Table 2-12).

Although AM7 had greatest root weight and root length, its mortality was higher than other varieties in the field (70% vs 50%). AM7 was susceptible to the disease caused by *Pythium* and *Phytophthora* and only 4 or 5 plants of AM7 were left alive at harvest in some plots, which suggests that AM7 had lower adaptability than other varieties to southeast US. AM7 originated from Anguo, Hebei (38°25'N, 115°18'E), China, where the annual precipitation is 300-800mm, average January temperature is -7 ºC and July temperature is 18-27 ºC. The annual precipitation in Auburn is 1340 mm, average January temperate 8.5 ºC and July temperature 26 ºC. Especially the summer in Auburn is humid with high temperature. Those differences of temperature and humidity probably made AM7 less able to adapt to the new environment.

AM5 was purchased South Korea and grew very well in Auburn, Alabama. South Korea is in monsoonal region, summer is hot and humid and rainfall is over 1000 mm, which is similar to the summer in Auburn. Therefore, AM5 achieved high yield and good root characteristics, such as big root diameter.

Considering all of the study results, AM2, AM3, AM4 and AM5 had good adaptability, root weight (yield) and root quality and can be chosen to plant in southeast U. S. as an alternative crop.

Plant Growth and Root Development of *A. membranaceus* in Fertility Trial

In fertility trial, only phosphorus and potassium fertilizer were applied in field and no nitrogen was applied. Nutrients in soil were unbalanced and nitrogen was deficiency for the growth of *A. membranaceus*. Some studies (Anetai et al. 1996; Zhang et al., 2005c; Zhao et al., 2002) found that the deficiencies of nitrogen in soil affected the growth of root. The deficiency of nitrogen also leads to the less accumulation of dry material in root (Ma et al., 2004a; Tan et al., 2006b; Zhang, et al., 2005c; Zhao et al., 2002). In our study, main P effect was only found on root weight, shoot height, and root diameter with 7-month plants. The increase in shoot height, root weight, and root diameter of 7-month plants with phosphorus application level (Table 2-14, Fig. 2-8) was consistent with Shibata et al. (1996a), who reported highest yield on soil with high P. No P effect was found on root to shoot ratio, root length, root diameter and root branch for 13-month plants. No main K effect was found either. Nitrogen deficiency counteracted the P and K effect and weakened the growth of *A. membranaceus*.

In this study, the linear interaction of P and K on root weight and root to shoot ratio of 13-month plants was significant. No interaction effect of P and K was found on shoot height, root diameter, root length and root branch for either 7-month or 13-month plants. Therefore, potassium did work effectively on *A. membranaceus*. These results contradicted the conclusion by Anetai et al. (1995), who stated that soil treatment with potassium fertilizer had little influence on the growth and yield of the plant. Without K application, root weight and root to shoot ratio decreased with the level of P application for 13-month plants. With K application, root weight and root to shoot ratio increased with P application for 13-month plants (Fig. 2-9, 2-10).

A. membranaceus is a perennial herb and is often harvested after planting for

two or three years. We only grew Astragalus for 7 months and 13 months, respectively. The growing period was too short and *A. membranaceus* didn't reach its maximum nutrient absorbing time, but it is long enough to observe differences. The main reason for weak nutrient effect was probably is the high mortality rate affected quality of data and results. If the stands were better, there would be good enough plants to produce high quality of data and results. Then the P effect, K effect, P×K interaction effect and lime effect might be clearly displayed.

There was no lime effect on root weight, root to shoot ration, shoot height, root diameter, root length and root branch at 29kg P + 56kg K ha^{-1} for either 7-month or 13-month plants (Table 2-16). In fertility trial, soil pH ranged from 5.8 to 6.0 after lime application, which means that we failed to achieve the desired soil pH of 6.5. For the no lime application treatment, soil pH was 5.3 (Table 2-5), which was much lower than the treatments receiving lime. According to Yang et al. (2006) *A. membranaceus* grows well in mildly acid to alkaline soil with pH ranges from 6.5 to 8. In Zhang's (2005c) study soil pH ranged from 7.8 to 8.0. Therefore, low soil pH in all the treatments may have negatively affected the growth of *A. membranaceus* and reduced the lime effect.

Concentration of N, P, K, Ca, Mg, Zn, Cu, Fe, Mn and B in Variety and Fertility Trial

For leguminous plants, the critical value of nitrogen is 3 to 4.25 percent, phosphorus is 0.20 to 0.25 percent, potassium is 1.75 to 2.00 percent (AESL Plant Analysis Handbook. http://aesl.ces.uga.edu/publications/plant/Nutrient.htm). The concentration of N, P and K in variety (all the seven varieties) and fertility trial was

lower than those critical values and the concentration of Ca, Mg, Cu, Fe, Mn, B and Zn was in the normal range.

In the variety and fertility trial, only phosphorus and potassium fertilizer were applied in field and no nitrogen was applied, because we assumed that *A. membranaceus* would be self-sufficient in N through N fixation. Zhao (2002) reported that the best ratio of nitrogen, phosphorus and potassium (N: P_2O_5: K_2O) in applied fertilizer was 1:0.8-1.2:1.2-1.8, which indicated that nitrogen application was recommended for the cultivation of *A. membranaceus*. The nutrients in the field were unbalanced for the growth of *A. membranaceus*. We did not find nodules on the root in the field, when we dug the root out from soil. So *A. membranaceus* couldn't fix nitrogen from air in this experiment and the only way to get nitrogen was from soil. According to the Liebig's Law of the Minimum (Foth and Ellis, 1997), yield is proportional to the amount of the most limiting nutrient. Nitrogen was probably deficient nutrient element for the growth of *A. membranaceus*, so its deficiency limited root growth and shoot growth. Nitrogen deficiency affected other elements' absorbance and led to no significant difference was found on the concentration of N, P, K, Ca, and Mg in all treatment.

Conclusions

Plant mortality was most severe with small seedlings, but happened throughout the season. Under high soil moisture and wet conditions, *Astragalus* is susceptible to root rot caused by fungi, which is the main constraint to cultivation in southeastern U. S.. Land must be well drained for *Astragalus.* Loose soil and raise beds can be used to control soil moisture.

Although, in-row subsoiling management (in SS treatment) reduced the soil strength and was somewhat beneficial for *A. membranaceus,* subsoiling did not adequately improve desired characteristics of increased shoot weight, root weight, shoot height, root diameter and decreased root branches.

The varieties, AM2, AM3, AM4 and AM5 had the best adaptability in terms of root weight (yield) and root quality and may be planted in southeastern U. S. as alternative crop.

No nodules were found on the roots. It is necessary to inoculate with rhizobia specific to *Astragalus* spp and to produce functional nodules. There was no lime effect on root, but low soil pH in all the treatments may have negatively affected the growth of *A. membranaceus* and reduced the lime effect. For the good growth of *A. membranaceus*, soil pH should be adjusted to above 6.0 with lime.

Fertility is not a major concern as long as P and K soil tests are in medium to high level in our study. The other reason for weak nutrient effect was probably due to the high mortality rate, which affected quality of data and results.

Further research on disease and pest control, variety selection and fertility for *A. membranaceus* are needed in order to develop recommendations for its cultivation in the southeastern U. S..

Table 2-1. Soil profile description in field.†

Horizon	Depth	Color	Texture	Structure
Ap	0 to 10 cm	brown	loamy sand	weak medium subangular blocky
E	10 to 23 cm	yellowish brown	loamy sand	weak medium subangular blocky
Bt1	23 to 61 cm	yellowish red	sandy clay	moderate medium subangular blocky
Bt2	61 to 76 cm	strong brown	sandy clay loamy	moderate medium subangular blocky
Btv	76 to 89 cm	strong brown	sandy clay loamy	moderate medium subangular blocky
BC	89 to 109 cm	strong brown	sandy clay loamy	weak medium subangular blocky
C	109+ cm	yellowish red	sandy clay	Structureless single grained

†On site evaluation and soil profile description performed by Rick Smith.

Table 2-2. Soil pH and Mechlich I extractable nutrients (mg kg^{-1}) in study field before planting.†

pH	P	K	Mg	Ca
5.65	14	60	64	259

† Adapted from Auburn University Soil Testing Laboratory report
pH was in 1:1 soil:water (v/v); P, K, Mg and Ca are NH_4OAc extractable bases.

Table 2-3. Experiment design and fertilizer application in fertility trial.

	Phosphorous (kg P ha^{-1})	Potassium (kg K ha^{-1})	Lime (kg ha^{-1})
T1	0	0	2469
T2	29	0	2469
T3	58	0	2469
T4	0	56	2469
T5	29	56	2469
T6	58	56	2469
T7	0	112	2469
T8	29	112	2469
T9	58	112	2469
T10	29	56	0

Table 2-4. The origin of *A. membranaceus.*

Accession number	Latin name	Origin of the seeds
AM1	*Astragalus membranaceus*	USDA, PI 515968, 89i (South Korea).
AM2	*Astragalus membranaceus*	USDA, W6 22350, 2002i (United States)
AM3	*Astragalus membranaceus*	Horizon Herbs, LLC. William, OR 97544
AM4	*Astragalus membranaceus*	Elixir Farm Botanical LLC. Brixey, MO 65618
AM5	*Astragalus membranaceus*	South Korea
AM6	*Astragalus membranaceus*	Johnny's selected seeds, Winslow, Maine 04901
AM7	*Astragalus membranaceus*	Anguo, Hebei , China

Note: Ultimately all seeds originated from China or Korea.

Table 2-5. Soil pH and Mehlich I extractable nutrients (mg kg^{-1}) in fertility trial after harvesting.†

	pH	P	K	Mg	Ca
0-0-0 (Lime)	6.00	18	41	72	452
0-29-0 (Lime)	5.95	23	32	66	432
0-58-0 (Lime)	5.90	29	38	71	481
0-0-56 (Lime)	5.95	17	38	61	406
0-29-56 (Lime)	5.93	20	41	71	460
0-58-56 (Lime)	5.93	28	39	65	423
0-0-112 (Lime)	5.88	17	43	69	446
0-29-112 (Lime)	5.83	21	42	62	391
0-58-112 (Lime)	5.80	24	45	61	382
Pr>F	NS‡	0.004	NS	NS	NS

† Adapted from Auburn University Soil Testing Laboratory report
‡ Not significant at $\alpha = 0.10$ significance level.

Table 2-6. Soil pH and Mehlich I extractable nutrients (mg kg^{-1}) after harvesting.†

	pH	P	K	Mg	Ca
0-29-56 (Lime)	5.93	20	41	71	460
0-29-56(no lime)	5.33	18	37	44	225
Pr>F		NS‡	NS	NS	NS

†: Adapted from Auburn University Soil Testing Laboratory report
‡: Not significant at $\alpha = 0.10$ significance level.

Table 2-7. Soil pH and Mehlich I extractable nutrients (mg kg^{-1}) tillage and variety trial after harvesting.†

	pH	P	K	Mg	Ca
Tillage trial	6.00	18	48	75	472
Variety trial	5.80	22	53	91	550

† Adapted from Auburn University Soil Testing Laboratory report

Table 2-8. Percent emergence, percent stands and recovery after winter for each variety of *Astragalus membranaceus* in variety trial.

	Percent emergence 7 days after sowing (%)†	Percent emergence 10 days after sowing (%)‡	hills having seedlings (%)§	Percent recovery (%)¶
AM1	25.4	30.2	60.4	81
AM2	48.0	48.0	81.0	91
AM3	89.5	89.5	94.7	91
AM4	40.3	40.3	81.0	92
AM5	3.7	3.7	27.1	95
AM6	71.1	71.2	98.9	92
AM7	19.1	20.1	60.4	85

†Planted on 9/25/2006 and counted on 10/2/2006 (7 days)
‡ Planted on 9/25/2006 and counted on 10/5/2006 (10 days)
§ Planted on 9/25/2006 and counted on 10/5/2006 (10 days)
¶ counted on 3/30/2007

Table 2-9. The effect of subsoiling on soil strength (kPa) at 15 cm depth intervals in tillage trial on Feb. 21, 2008.

Treatment†	15 mm	30 mm	45 Mm	60 mm	75 mm	90 mm	105 mm	120 mm	135 mm	150 mm	165 mm	180 mm	195 mm	210 mm	225 mm
SS	257	304	359	512	452	520	502	554	596	650	731	820	939	1019	1122
NoSS	303	469	421	524	585	757	952	1252	1714	2343	2565	2840	2779	2495	2576
Pr>F	0.121	0.262	0.158	0.919	0.013	0.014	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

†SS = subsoiling; NoSS = no subsoiling.

(Table 2-9 continued.)

Treatment	240 mm	255 mm	270 mm	285 mm	300 mm	315 mm	330 mm	345 mm	360 mm	375 mm	390 mm	405 mm	420 mm	435 mm
SS	1297	1383	1451	1463	1434	1431	1407	1364	1368	1383	1390	1419	1453	1476
NoSS	2581	2408	2138	1877	1799	1435	1264	1234	1189	1174	1121	1142	1150	1141
Pr>F	<0.01	<0.01	0.091	0.098	0.092	0.485	0.463	0.486	0.303	0.221	0.162	0.204	0.165	0.166

Table 2-10. The effect of subsoiling on shoot weight, root weight, shoot height, root length, root diameter and distance from first branch to crown in *Astragalus membranaceus* harvested on different dates in SS and NoSS treatments.

Treatment	Shoot weight (g plant^{-1})	Root weight (g plant^{-1})	Shoot height (cm)	Root length (cm)	Root diameter (mm)	Root branch	Distance from first branch to crown (cm)
Harvest at 64 days							
SS	0.02	0.04	1.80	12.2	1.50	-†	-
NoSS	0.03	0.05	2.25	13.8	1.98	-	-
Pr>F	NS‡	NS	NS	NS	NS	-	-
Harvest at 214 days							
SS	7.5	2.6	38.5	30.0	8.9	8	10.0
NoSS	5.9	2.1	31.7	27.3	7.67	6	6.2
Pr>F	NS	NS	NS	NS	NS	NS	NS
Harvest at 242 days							
SS	37.4	10.3	57.4	39.1	12.8	7	2.9
NoSS	34.8	10.4	55.5	36.6	11.5	8	2.2
Pr>F	NS	NS	NS	NS	NS	NS	NS
Harvest at 277 days							
SS	87.2	18.6	66.2	57.0	18.5	10	2.8
NoSS	65.9	21.6	65.0	49.7	17.8	9	1.7
Pr>F	NS	NS	NS	**0.040**	NS	NS	**0.093**

† - means no branch was found on roots.

‡: Not significant at $\alpha = 0.10$ significance level.

Table 2-11. The effect of subsoiling on shoot weight, root weight, shoot height, root length, root diameter and root branch of *Astragalus membranaceus* harvested in Nov. 2007, in SS and NoSS treatment.

	Shoot weight (g/plant)		Root weight (g/plant)		Shoot height (cm)		Root length (cm)		Root diameter (mm)		Root branch (number/plant)	
Treatment	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month
SS	23.1	63.6	19.1	26.3	55.4	67.2	44.9	57.8	11.1	19.1	14.5	7.4
NoSS	21.9	58.2	20.7	26.2	54.3	68.6	41.3	50.1	118	20.2	9.2	10.3
Pr>F	NS†	NS	NS	NS	NS	NS	NS	**0.045**	0.697	0.290	**0.066**	0.145

†: Not significant at α = 0.10 significance level.

Table 2-12. Shoot weight, root weight, shoot height, root length, root diameter and root branch of seven *A. membranaceus* varieties in variety trial.

	Shoot height (g/plant)		Shoot weight (g/plant)		Root weight (g/plant)		Root length (cm)		Root diameter (mm)		Root branch (number/plant)	
Variety	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month
AM1	49.3 b†	67.5 bc	29.4 abc	57.0 bc	14.7 bc	23.4 b	38.1 a	54.0 ab	11.2 b	18.2 c	10.5 a	10.0 a
AM2	56.6 ab	70.7 abc	34.3 ab	71.0 abc	18.4 ab	26.6 b	39.9 a	60.2 a	11.7 b	21.9 ab	10.2 a	9.4 a
AM3	51.2 b	67.0 c	20.9 c	55.6 c	14.2 bc	24.6 b	40.4 a	53.5 ab	11.1 b	18.7 c	9.3 a	9.8 a
AM4	51.1 b	70.7 abc	24.9 abc	89.1 a	15.9 bc	28.3 ab	41.2 a	51.0 b	12.1 b	19.4 bc	11.1a	9.0 a
AM5	59.3 a	75.2 a	36.0 a	76.5 ab	24.2 a	29.6 ab	41.4 a	57.2 ab	15.5 a	23.6 a	12.5 a	10.1 a
AM6	49.5 b	67.3 bc	22.9 bc	59.2 bc	16.3 bc	20.6 b	43.7 a	58.6 ab	11.2 b	20.0 bc	11.7 a	8.9 a
AM7	40.7 c	74.2 ab	7.8 d	60.6 bc	9.6 c	37.9 a	37.6 a	60.8 a	7.7 c	20.4 bc	8.6 a	8.9 a

† Columns followed by same letter are not significantly different by Duncan multiple range test ($\alpha = 0.10$)

Table 2-13. Concentration of elements in above-ground portion of 13-month plants of *Astragalus membranaceus* in variety trial.

Treatment	N	P	K	Ca	Mg	Cu	Fe	Mn	Zn	B
	%	%	%	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
AM1	1.98 a†	0.15 a	1.37 a	1.00 a	0.28 a	10.13 a	343.20 a	34.08 b	18.19 ab	7.90 a
AM2	1.49 a	0.12 a	1.15 a	0.51 a	0.14 a	6.99 a	63.13 a	17.24 c	14.76 bcd	1.93 a
AM3	1.54 a	0.12 a	1.18 a	0.55 a	0.14 a	12.39 a	70.67 a	27.10 bc	16.06 bc	8.01 a
AM4	1.65 a	0.13 a	1.20 a	0.59 a	0.16 a	5.32 a	69.04 a	21.40 c	13.73 cd	1.77 a
AM5	1.60 a	0.14 a	1.10 a	0.72 a	0.21 a	7.92 a	88.23 a	35.29 b	19.04 ab	7.72 a
AM6	1.46 a	0.10 a	1.15 a	0.57 a	0.17 a	3.62 a	68.81 a	19.23 c	11.62 d	2.85 a
AM7	2.10 a	0.16 a	1.57 a	0.56 a	0.17 a	8.56 a	195.35 a	51.13 a	21.05 a	6.63 a

† Columns followed by same letter are not significantly different by Duncan multiple range test ($\alpha = 0.10$)

Table 2-14. Root weight, root to shoot ratio, shoot height, root length, root diameter and root branch of *A. membranaceus* in fertility trial.

	Root weight (g/plant)		Root/shoot ratio		Shoot height (cm)		Root length (cm)		Root diameter (mm)		Root branch (number/plant)	
Treatment	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month
0-0-0-L†	16.2	32.1	0.8	0.4	47.5	67.7	38.7	48.9	12.1	19.1	9.3	6.1
0-29-0-L	17.0	24.3	1.1	0.4	49.9	67.0	43.0	47.8	10.7	18.8	9.5	6.8
0-58-0-L	15.4	18.1	0.8	0.3	48.0	63.1	43.1	43.7	10.2	16.9	9.8	5.3
0-0-56-L	10.9	19.8	1.1	0.3	45.9	64.1	38.8	47.1	8.9	17.3	12.3	5.6
0-29-56-L	17.9	25.1	0.9	0.4	49.5	66.6	45.3	52.5	12.5	18.6	10.5	6.3
0-58-56-L	18.8	22	1.4	0.3	51.6	67.5	42.4	49.5	9.8	18.7	9.1	7.0
0-0-112-L	15.3	23.5	0.6	0.3	49.8	67.8	46.3	47.1	11.7	19.6	10.5	6.3
0-29-112-L	19.5	27.1	0.8	0.3	54.3	69.1	44.9	50.6	13.1	19.6	9.6	5.6
0-58-112-L	22.6	23.4	1.1	0.4	48.6	62.5	43.1	48.6	11.1	18.5	10.9	4.9
P effect	**0.083**	NS‡	NS	NS	**0.052**	NS	NS	NS	**0.081**	NS	NS	NS
K effect	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P×K effect	NS	**0.053**	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

†: L is lime application

‡: Not significant at α = 0.10 significance level.

Table 2-15. Significance levels for linear and quadratic effects of P and K fertility on root weight, root to shoot ratio, shoot height, root length, root diameter and root branch of *A. membranaceus* in fertility trial as determined by orthogonal contrasts.

	Root weight (g/plant)		Root/shoot ratio		Shoot height (cm)		Root length (cm)		Root diameter (mm)		Root branch (number/plant)	
	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month
P linear effect	**0.068**	NS†	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P quadratic effect	NS	NS	NS	NS	NS	NS	NS	NS	**0.069**	NS	NS	NS
K linear effect	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
K quadratic effect	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P × K linear effect	NS	**0.056**	NS	**0.077**	NS	NS	NS	NS	NS	NS	NS	NS
P × K quadratic effect	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

†: Not significant at α = 0.10 significance level.

Table 2-16. The effect of lime application on root weight, root to shoot ratio, shoot height, root length, root diameter and number of root branch of *Astragalus membranaceus* in fertility trial.

	Root weight (g/plant)		Root/shoot ratio		Shoot height (cm)		Root length (cm)		Root diameter (mm)		Root branch (number/plant)	
Treatment	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month	7-month	13-month
0-29-56 (lime)	17.9	25.1	0.9	0.4	49.5	66.6	45.3	52.5	12.5	18.6	10.5	6.3
0-29-56 (no lime)	13.8	26.8	0.6	0.4	50.3	63.5	42.8	49.0	10.8	19.0	9.4	5.7
Pr>F	NS†	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

†: Not significant at α = 0.10 significance level.

Table 2-17. Concentration of elements in above ground of 7-month and 13-month of *A. membranaceus* plants in fertility trial.

Treatment	N	P	K	Ca	Mg	Cu	Fe	Mn	Zn	B
	%	%	%	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
7-month plants										
0-0-0(lime)	1.84	0.15	1.28	0.99	0.26	22.04	163.78	33.39	21.83	2.28
0-58-0(lime)	2.07	0.17	1.39	1.39	0.34	8.83	92.43	42.91	18.74	4.45
0-0-112(lime)	1.78	0.14	1.42	0.80	0.21	6.96	86.67	42.88	20.35	5.55
0-58-112(lime)	1.94	0.15	1.44	0.91	0.24	9.46	80.50	40.69	21.43	3.60
Pr>F	NS†	NS	NS	NS	NS	NS	NS	NS	NS	NS
13-month plants										
0-0-0(lime)	1.43	0.15	1.14	0.59	0.18	15.75	45.18	20.99	19.57	2.59
0-58-0(lime)	1.78	0.18	1.30	0.68	0.22	9.83	61.90	26.35	19.81	3.51
0-0-112(lime)	1.56	0.14	1.30	0.59	0.17	10.52	50.62	24.15	16.75	1.71
0-58-112(lime)	1.45	0.14	1.24	0.59	0.18	5.56	66.05	22.15	17.20	1.02
Pr>F	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
13-month plants										
0-29-56(lime)	1.34	0.14	1.12	0.52	0.16	6.82	44.50	18.46	15.50	1.03
0-29-56(no lime)	1.69	0.15	1.33	0.60	0.19	7.61	54.15	32.37	19.53	6.21
Pr>F	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

†: Not significant at $\alpha = 0.10$ significance level.

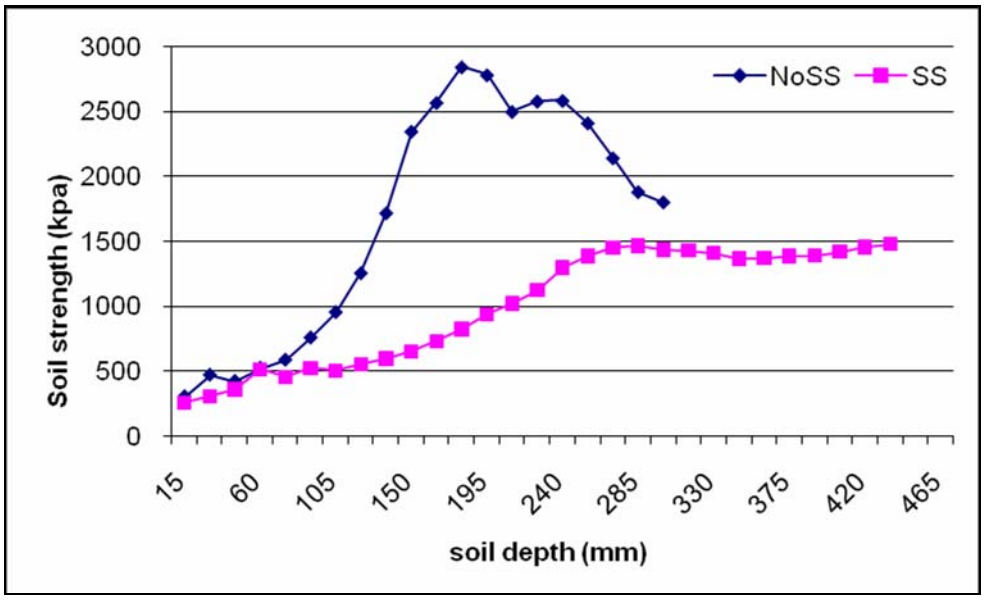

Fig. 2-1. The effect of subsoiling on soil strength in tillage trial on Feb. 21, 2008.

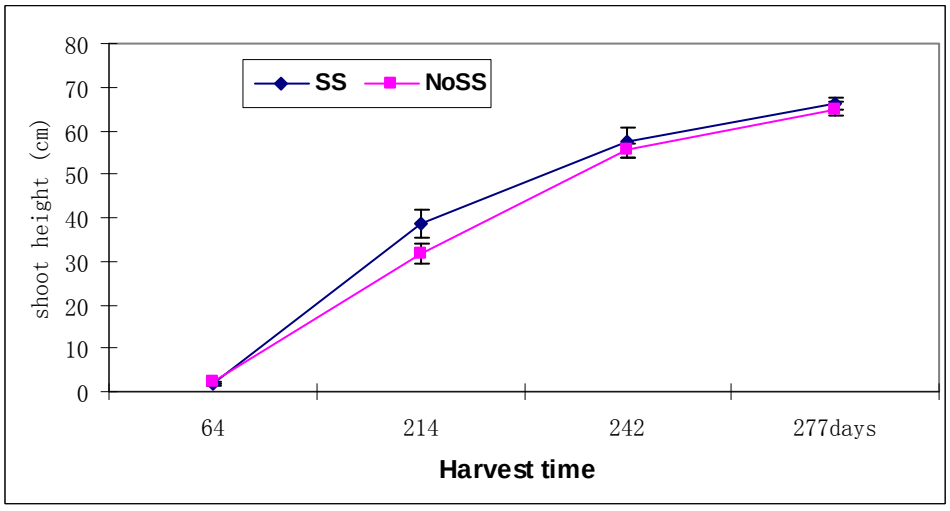

Fig. 2-2. The effect of subsoiling on shoot height in tillage trial (The bars indicate standard error of the mean).

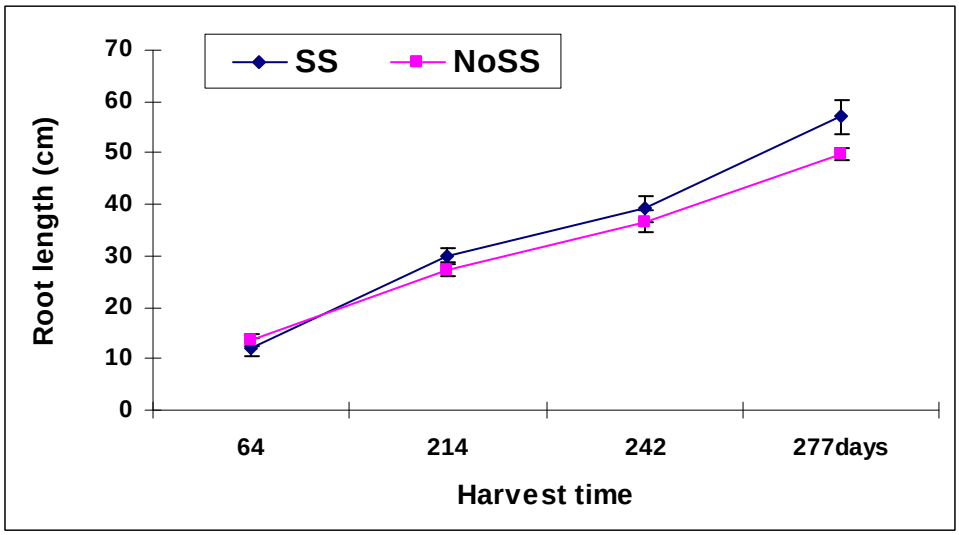

Fig. 2-3. The effect of subsoiling on root length in tillage trial (The bars indicate standard error of the mean).

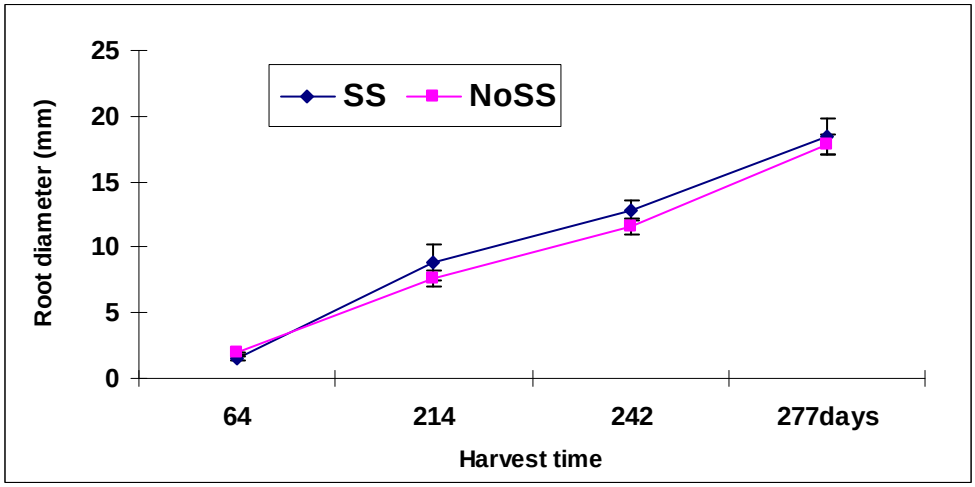

Fig. 2-4. The effect of subsoiling on root diameter in tillage trial (The bars indicate standard error of the mean).

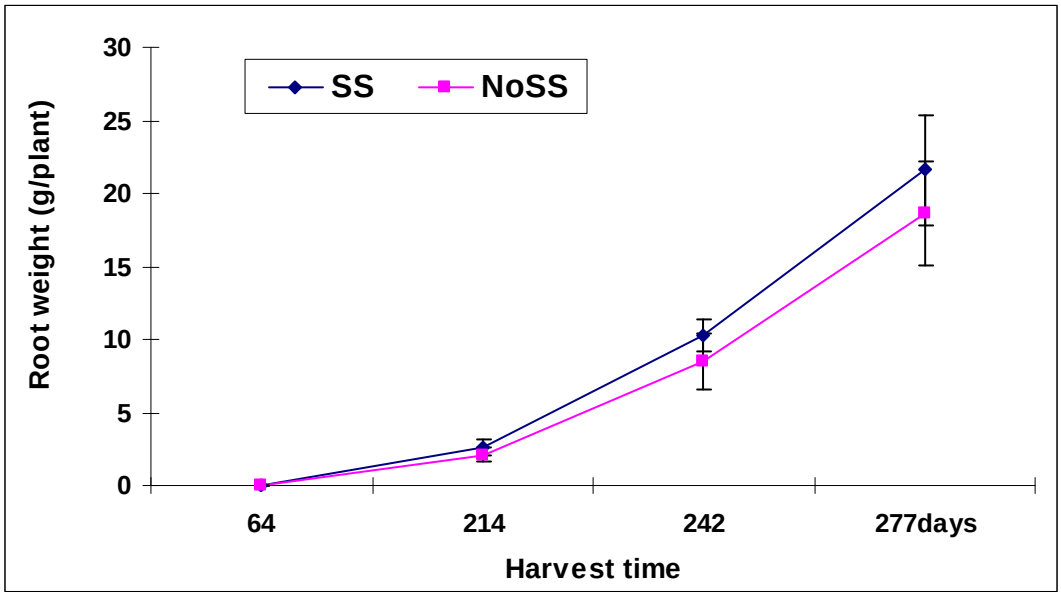

Fig. 2-5. The effect of subsoiling on root weight in tillage trial (The bars indicate standard error of the mean).

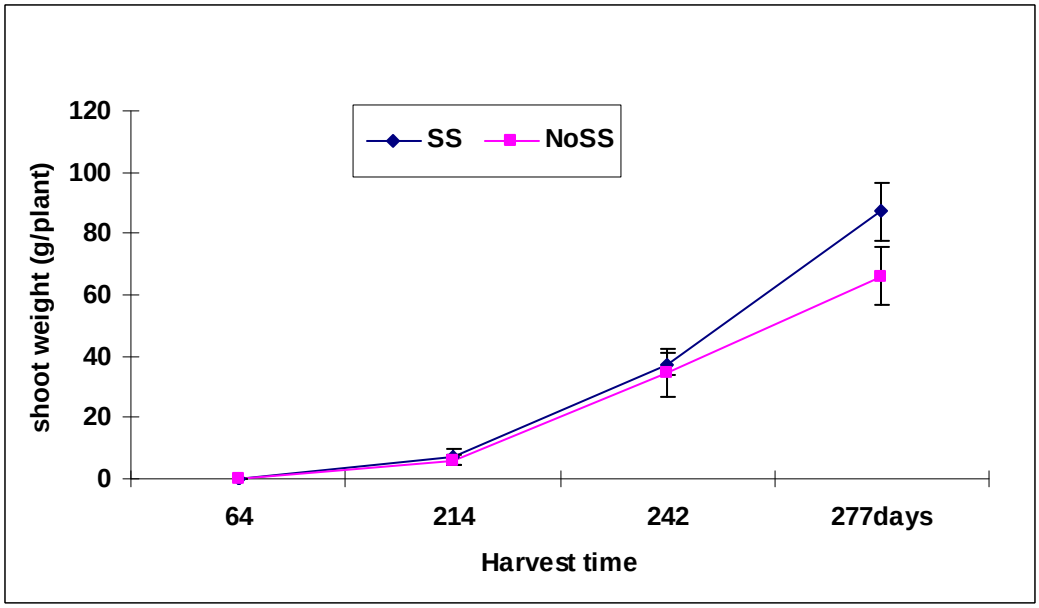

Fig. 2-6. The effect of subsoiling on shoot weight in tillage trial (The bars indicate standard error of the mean).

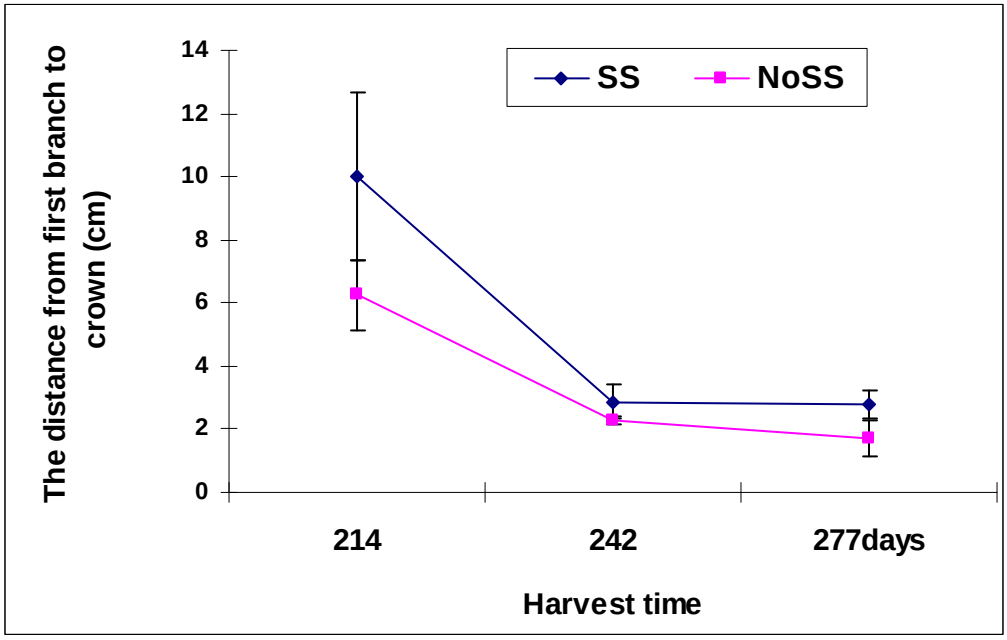

Fig. 2-7. The effect of subsoiling on the distance from crown to first branch in tillage trial (The bars indicate standard error of the mean).

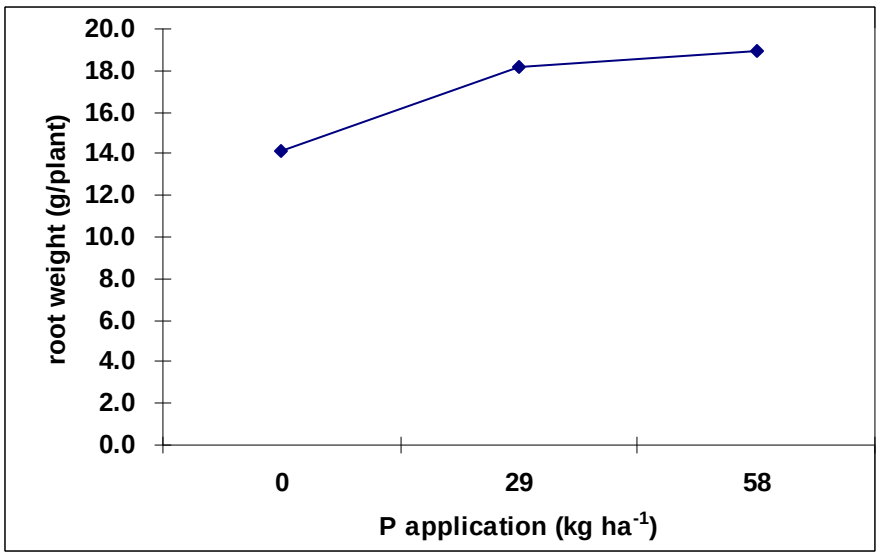

Fig. 2-8. The effect of fertilization with phosphorus on root weight of 7-month plants.

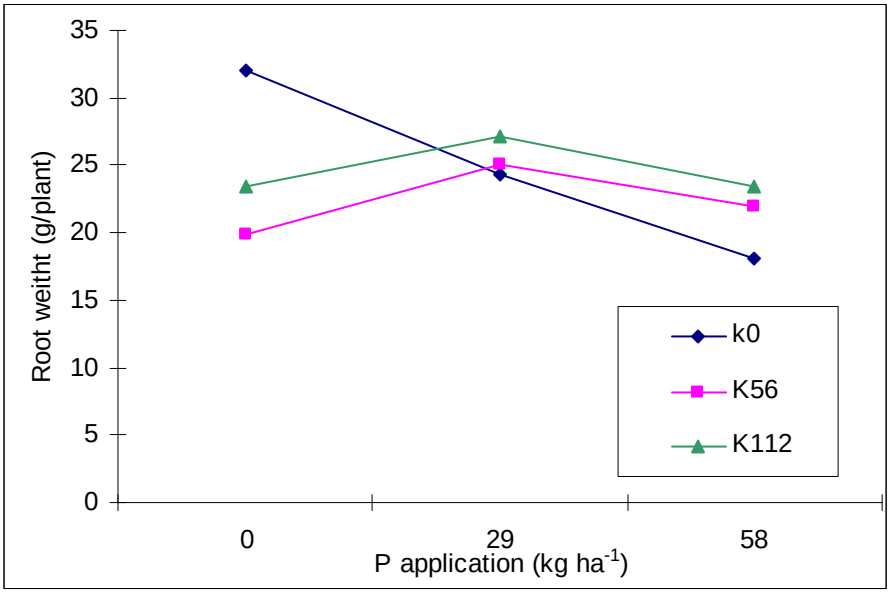

Fig. 2-9. The effect of phosphorus and potassium application on root weight of 13-month plants.

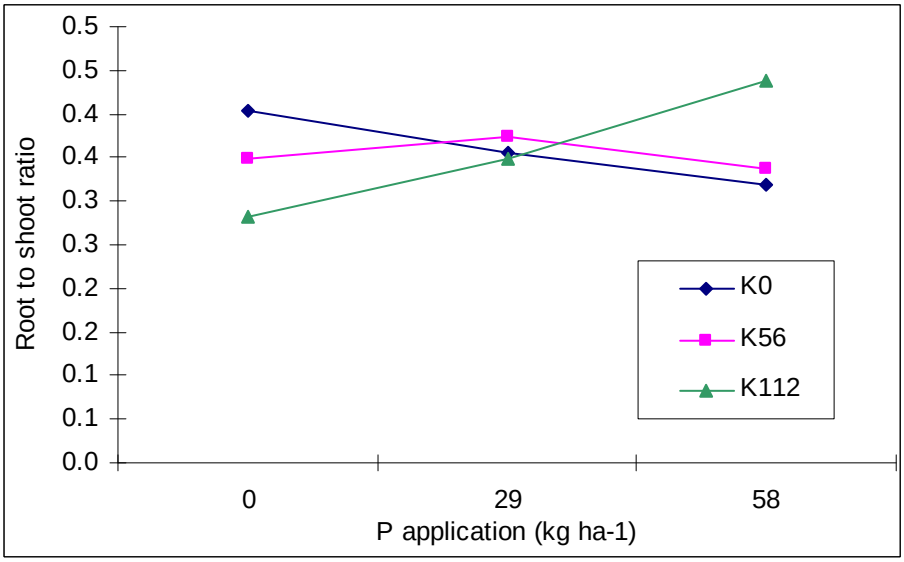

Fig. 2-10. The effect of phosphorus and potassium application on root to shoot ratio of 13-month plants.

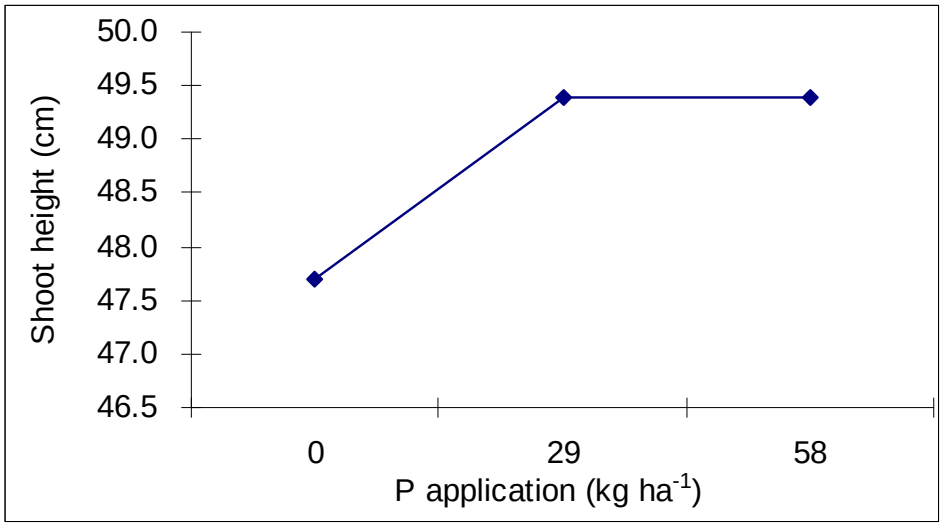

Fig. 2-11. The effect of phosphorus application on shoot height of 7-month plants.

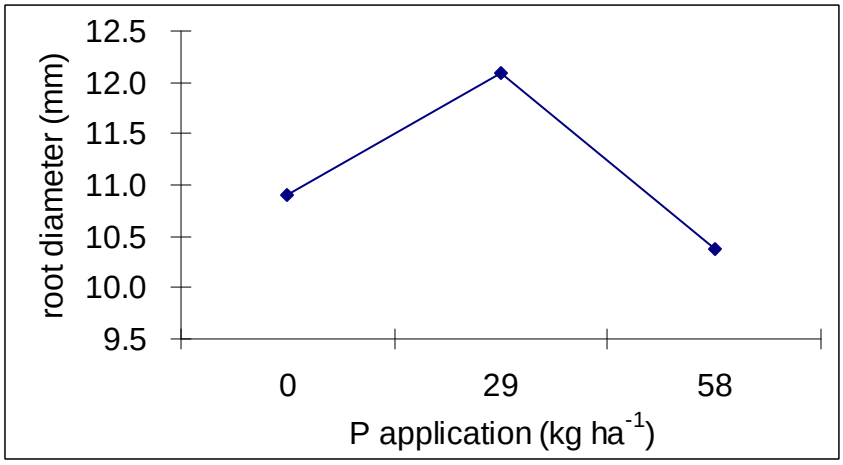

Fig. 2-12. The effect of fertilization with phosphorus on root diameter of 7-month plants.

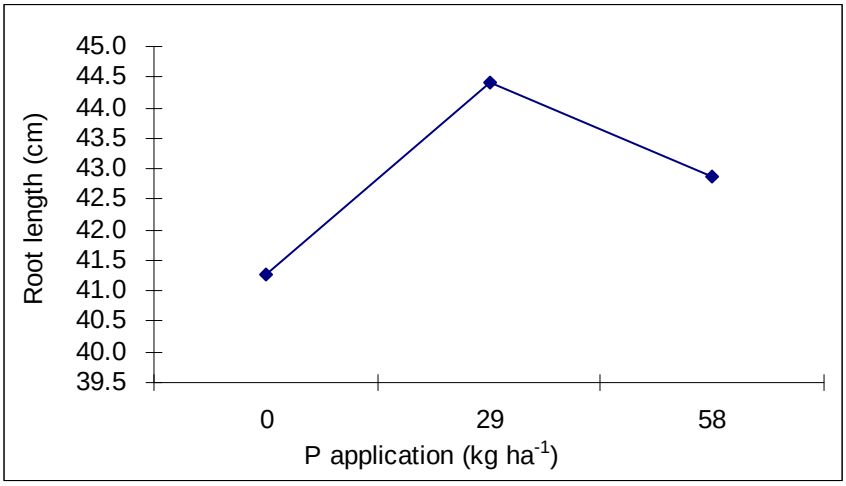

Fig. 2-13. The effect of fertilization with phosphorus on root length of 7-month plants.

Photo 1. Wilted shoot of *A. membranaceus.*

Photo 2. Rot root on *A.membranceus.*

Photo 3. Healthy plant of *A. membranaceus* with normal root.

Photo 4. An erect variety of *A. membranaceus* (AM3).

Photo 5. The decumbent variety of *A. membranaceus* (AM7).

Photo 6. Larva found with damaged seedling of *A. membranaceus.*

Photo 7. After 45 days, nodules on the roots of *A. membranceus* from seeds inoculated with rhizobium inoculants specific to Astragalus species.

III. THE EFFECT OF TILLAGE, VARIETY AND FERTILIZER ON ASTRAGALOSIDE IV IN *ASTRAGALUS MEMBRANACEUS*

Abstract

Astragalus membranaceus is a traditional Chinese medicinal herb and has been used for thousands of years. In September 2006, three field experiments were planted in beds to test (1) the effects of deep tillage (subsoiling vs no subsoiling), (2) seven varieties from different areas, and (3) P (0, 29, and 58 kg ha^{-1}) and K (0, 56, and 112 kg ha^{-1}) fertilizer and lime (0 and 2469 kg ha^{-1}) on astragaloside IV concentration in Auburn, Alabama on a Plinthic Kanhapludults soil. Each experiment consisted of a randomized complete block design with four replications. The fertility study was a 3×3 factorial augmented with a no-lime treatment at 29 kg P ha^{-1} + 56 kg K ha^{-1}. Astragaloside IV and the internal standard (digoxin) were clearly separated and resolved by high-performance liquid chromatography coupled with evaporative light scattering detection (HPLC-ELSD). This is the first study to test and validate that digoxin as an internal standard can be used to accurately and precisely measure Astragaloside IV with HPLC. Subsoiling significantly increased the concentration of astragaloside IV in all roots except big root of 7-month plants and significantly increased the total content of astragaloside IV in roots of 13-month plants. Small roots had higher concentrations of astragaloside IV than big roots for the same growing period and variety. The higher concentration of astraloside IV was found in 13-month plants than in 7-month plants. Varieties AM3, AM4, AM2 and AM5

had relatively high concentrations of astragaloside IV in the roots. Potassium application had an interaction effect with P (Pr=0.012) in small roots of 7-month plants on astragaloside IV concentration and K suppressed concentration in absence of P. Phosphorus application increased (Pr=0.037) concentration of astragaloside IV in big root of 7-month plants. There was a linear interaction effect (Pr=0.009) of P × K in the roots of 7-month plants. No P effect was found in small root of 7-month plants, big root of 13-month plants and small root of 13-month plants. Lime had no effect on astragaloside IV.

Introduction

Astragalus membranaceus is one of the important traditional Chinese medicinal herbs and has been used for thousands of years. Radix Astragali is the pharmaceutical name of the dried root of both varieties (Tu et al., 1988) and has medicinal function. It was used to treat pectoral, diuretic and childhood illnesses and acts as a tonic to supplement deficiencies in ancient (Upton et al., 1999; Han, 2003).

Both environmental factors and varieties are vital to cultivation and able to affect bioactive component accumulation of medicinal plants. In Japan, Shibata et al. (1996b) found that *A.membranaceus* plants grown in sandy and brown soil had best growth and high concentration of isoflavonoid in the taproots. Shibata et al. (1996b) measured the soil hardness (penetration resistance) at different depths in the field and soil strength affected the taproot elongation and lateral root development. The

concentration of astragaloside I, II, III, IV, total astragaloside and dilute ethanol-soluble extract in the taproot of *A.membranaceus* decreased as the soil compaction levels increased (Mia et al., 1999).

In another experiment, the taproots, and thick and thin lateral roots of *A.membranaceus* were assayed for their isoflavonoid (I-V) and astragaloside (I-IV) contents by HPLC. Isoflavonoids I-V were present in about the same concentration in those different root parts, regardless of the thickness and thinness of those roots (Anetai et al., 1996). However, the concentration of astragalosides I-IV was greater in thinner roots; the thin lateral root contained much higher concentration of astragalosides than taproot and thick lateral roots.

There are different varieties of *A.membranaceus* growing in different areas worldwide. Ma et al. (2000b) analyzed Astragali Radix from different regions of China and concluded Astragali Radix from Shanxi contained significantly higher amounts of isoflavonoids, saponins and polysaccharides (Ma et al., 2000b). Jiang et al. (2004) compared the active compounds of three different varieties including wild *A.membranaceus* , cultivated *A.membranaceus* and cultivated *A.membranaceus mongholicus*, all of which grew in the same area. The result showed the concentration of astragaloside IV, total flavonoids and polysaccharides in wild *A.membranaceus* variety was higher than that in cultivated *A.membranaceus* and *A.membranaceus mongholicus* (Jiang, Ge, and Xue, 2004).

Soil fertility greatly affects primary and secondary metabolites of

A.membranaceus (Anetai et al., 1995; Zhang et al., 2005b, c). Anetai et al. (1995) planted *A.membranaceus* in the field and applied different amounts of phosphorus. They found the amounts of isoflavonoids and astragalosides I-IV in the Astragali Radix significantly increased with the increase of applied amounts of phosphoric acid. On the other hand, treatment of the soil with nitrogen or potassium fertilizer had little influence on the growth and yield of the plant and on the glycoside contents of the root (Anetai et al., 1995).

The marker compound, astragaloside IV, can be extracted from the root of *Radix Astragali*. Traditionally, the soxhlet is used to extract the ground root powder with 80% methanol (Feng, Feng and Guan, 2005; Gong, Yang and Zeng, 2005). Now ultrasonicator is often used to improve the extraction rate and this method normally has better extraction result than the conventional soxhlet extraction (Feng, Feng and Guan, 2005; Gong, Yang and Zeng, 2005; Li, Tan, and Ma, 2007; Valachovic, Pechova and Mason, 2001). A good method to detect astragaloside IV is to use high-performance liquid chromatography (HPLC) with evaporative light-scattering detection (ELSD) (Ganzera, et al., 2001; Gu, Wang and Fawcett, 2004; Li and Fitzloff, 2001; Shen et al., 2006).

The objectives of this research are to assess the effect of deep in-row tillage with a subsoiler on astragaloside content on coastal plain soil with hardpan, the best variety of *A.membranaceus* to grow in Alabama, and to test the optimal P and K fertility and lime response on *A. membranaceus* lime response on *A. membranaceus*

root yield and astagaloside content. This chapter addresses these effects on astragaloside content.

Materials and Methods

Three field experiments were carried out in Auburn, Alabama to determine the effects of tillage, varieties and P and K fertilizer and lime on astragaloside IV in *Astragalus membranaceus*. A randomized complete block design with four replications was used in each experiment. Details of the experiments are provided in Chapter II. Roots were obtained from these three experiments carried out in Auburn, Alabama. Roots were dried in a forced air dryer at 40 °C for four days, after which roots were stored in dry and cool storage room.

Following harvest of the experiments and determination of fresh and dry weight, as described in Chapter II, roots were cut and separated according to root diameter. The roots whose diameter was less than 2 mm were labeled as small roots. The roots whose diameter was greater than 2 mm were labeled as big roots. All the plant materials were ground in a Wiley mill. The ground roots were analyzed were analyzed for their concentration of astragaloside IV using high-performance liquid chromatography coupled with evaporative light scattering detection (HPLC-ELSD).

A commercial sliced sample of *A. membranaceus* was obtained from Spring Wind Herbs, Inc. (Berkeley, CA). The sample was imported from China. The sample was ground to analyze the concentration of astragaloside IV using the same method

as our samples.

An astragaloside IV reference standard with purity of 95% was purchased from ChromaDex (Irvine, CA). The internal standard, digoxin with purity of 95%, was obtained from Sigma-Aldrich (St. Louis, MO, US). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was obtained from a Nanopure water system (Barnstead, Newton, MA).

Apparatus

High-performance liquid chromatography (HPLC) analyses were performed on a Waters HPLC system (equipped with two 510 pumps, Automated Gradient Controller, 717 Auto sampler, and 746 Data Module Integrator; Waters, Milford, MA, US) and a Sedex 75 ELS detector (Sedex, Alfortville, France).

Preparation of standard solution

The stock solution of astragaloside IV was prepared by weighing (5mg) with a CHAN electrobalance (Cerritos, CA) and dissolving it in 10-mL volumetric flask with methanol. The internal standard stock solution was prepared by dissolving 10 mg digoxin in 10 mL methanol. Astragaloside IV calibration working standard curve solutions (15-80μg mL^{-1}) were prepared with a constant concentration of 30 μg mL^{-1} digoxin. All stock solutions were stored at -20 ºC.

Sample preparation

Finely powdered root (0.5 g) was placed in a 20 mL sample glass vial. Digoxin stock solution (60 μg) and methanol (3 mL) were added. The mixture was shaken

and mixed on vortex (Fisher) for 20 second. The mixtures were sonicated at 25-30 ºC for 15 minutes. After the mixtures were centrifuged at 1,500 rpm for 10 minutes, the supernatant was collected. The residue of root sample was extracted again and both supernatants were combined. The combined extracts were evaporated at 30 ºC to dryness under stream of nitrogen and the residue was dissolved in 2 mL of methanol. Prior to HPLC-ELSD analysis, all samples were filtered through a 0.2 μm nylon filter (Millipore).

Chromatographic conditions

For all separations, a Luna C-18(2) analytic column (150 × 4.6 mm, 5μm particle size) from Phenomenex (Torrance, CA, US) was used and protected by a SecurityGuard C-18 guard column (Phenomenex, Inc., Torrance, CA, US). The mobile phase consisted of acetonitrile (A) and water (B), which were applied in the following gradient elution: 0-13min, from 27A/73B to 30A/70B; 13-25min, from 30A/70B to 38A/62B; 25-35min, from 38A/62B to 70A/30B; 35-40min, from 70A/30B to 80A/20B; 40-40.5min, from 80A/20B to 27A/73B; 40.5-45min, from 27A/73B to 27A/73B. Those chromatographic conditions were adapted and modified from Ganzera (Ganzera, et al., 2001) and Li (Li and Fitzloff, 2001). All gradient steps were linear. The mobile phase was purged with helium for 30 minutes before running. The flow rate was set at 1 mL/min and the injection volume was 10μL. The ELS detector was adjusted to 40 ºC, at gain 8 and with a nitrogen pressure of 3.4 bar.

Calibration

Calibration curves were plotted as the peak area ratio (peak area ratio equaled peak area of astragaloside IV divided by peak area of digoxin) versus the concentration of astragaloside IV. The linearity was evaluated by linear regression analysis calculated by the least squares regression method using the formula Y = a + bx, where Y = peak area ratio and x = concentration of astragaloside IV (μg mL^{-1}). The limit of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 or 10, respectively. The LOD of the described method was 6 μg mL^{-1} (60ng) and LOQ was 12 μg mL^{-1} (120ng).

Validation

The validation of this HPLC-ELSD quantification method was determined. The precision (intraday variability) involved comparing, on three different days) and accuracy (inter-day variability by comparing). One experimental dried, powdered root sample was aliquoted into 12 each of 0.5 g samples. Three aliquots (or 3 replicates) were analyzed as described above (spiked with 60 ug digoxin and extracted twice with 3 ml methanol) in order to determine the amount of astragaloside in the original sample. The remaining aliquots were spiked with astragaloside IV (20, 40 or 60 μg, n=3) and digoxin (60 μg). Therefore, the three concentrations resulting from spiking with astragaloside IV were 10, 20, and 30 μg mL^{-1}. The variation was evaluated by making one injection from each of the three

replicates. Variations were expressed by the relative standard deviations (R.S.D.) percent, which equals standard deviation divided by mean, multiplied by 100.

The accuracy of this method was determined by measuring recovery of astragaloside IV. Triplicate analysis of astragaloside IV (10, 20, and 30 μg mL^{-1}) was used to calculate recovery. The average recoveries were determined by the following formula: recovery (%) = (observed concentration − original concentration)/spiked concentration×100%, and R.S.D. values were calculated.

Concentration and Total Content of Astragaloside IV in the Plants

Concentration of astragaloside IV was calculated based on the linear calibration curve for astragaloside IV, $X = \frac{Y + 0.0745}{0.0278}$ (X = concentration of astragaloside IV in the final solution (μg mL^{-1}); Y = peak area of astragaloside IV divided by peak area of digoxin). Concentration of astragaloside IV in the plant (μg g^{-1} plant) was calculated according to this following formula:

$$\frac{\text{the concentration of astragaloside IV in the solvent } (\mu g/mL) \times 2(mL)}{\text{weight of sample (g)}}$$

Total content astragaloside IV (mg $plant^{-1}$) was calculated according to this following formula:

[(small root weight × concentration of astragaloside IV in small root weight) + (big root weight × concentration of astragaloside IV in big root weight)] × 0.001.

Statistical Analysis

Analysis of variance was performed on raw data using version 9.1 of SAS to test main effects and interactions (SAS Institute Inc., 2003). Parameters were analyzed using PROC MIXED as randomized complete block factors. The treatment means were compared using Duncan's multiple range tests. All statistical tests were made at $\alpha = 0.10$ significance level.

Results

Validation of HPLC Method

Astragaloside IV and the internal standard were clearly separated and resolved using HPLC-ELSD. Digoxin (30 μg mL^{-1}) was eluted at 9.94 minutes and astragaloside IV (40 μg mL^{-1}) was eluted at 23.08 minutes in standard solution used for extraction (methanol) (Fig. 3-1). Digoxin (30 μg mL^{-1}) was eluted at 9.86 minutes and astragaloside IV was eluted at 23.02 minutes in sample extraction solution (Fig. 3-2). Over the range of 10 to 80 μg mL^{-1}, linearity of calibration curve for astragaloside IV is $y=0.0278x - 0.0745$, where y = peak area ratio; x = concentration of astragaloside IV (μg mL^{-1}) ($R^2=0.9932$) (Fig. 3-3). The LOD of 6 μg mL^{-1} (60ng) and LOQ of 12 μg mL^{-1} (120ng) indicate the sensitivity of this method.

Astragaloside IV spiked root samples were performed to evaluate the intra-

and inter-day reproducibility (Table 3-1). R.S.D. (%) were found to be less than 11.21%, 4.69%, and 2.97% for 10, 20, and 30 μg mL^{-1} spiked sample, respectively. The average recovery of astragaloside IV was 93.23% (n=3) (Table 3-2).

Concentration and Total Content of Astragaloside IV in Tillage Trial

Tillage did not significantly affect concentration of astragaloside IV in big roots (diameter ≥ 2 mm) of 7-month plants (Table 3-3). However, subsoiling significantly increased concentration of astragaloside IV in small roots (diameter ≤ 2 mm) of 7-month plant (Pr=0.06) by 23 %. Subsoiling significantly increased concentration of astragaloside IV in big and small roots in 13 month plants (Table 3-3).

Subsoiling significantly increased total content of astragaloside IV in 13-month plants (Pr=0.025), but had no effect on total content of astragaloside IV in 7-month plants (Table 3-4).

Concentration and Total Content of Astragaloside IV in Variety Trial

Significant differences among varieties were observed in concentration of astragaloside IV in big and small roots of both 7-month and 13-month plants (Table 3- 5). In big roots of 7-month plants, AM4 had significantly higher concentration of astragaloside IV than other varieties with 64.5 μg g^{-1}, and AM7 had lowest concentration of astragaloside IV with 22.1 μg g^{-1}. In big roots of 13-month old

plants, AM3 had significantly higher concentration of astragaloside IV than other varieties with 172.9 μg g^{-1}, while AM7 had the lowest concentration of astragaloside IV with 21.3 μg g^{-1}. In small roots of 7-month plants, AM4 had highest concentration of astragaloside IV with 204.3 μg g^{-1} and AM7 had lowest concentration of astragaloside IV with 34.0 μg g^{-1}. AM4 did not differ significantly from AM1, AM3 and AM5. In small roots of 13-month plants, AM3 and AM2, with 320.8 μg g^{-1} and 297.5 μg g^{-1} had significantly higher concentration of astragaloside IV than did AM6 and AM7. AM7 had lowest concentration of astragaloside IV with 100.1 μg g^{-1} (Table 3- 5).

The concentration of astragaloside IV in the commercial sample was 134 μg g^{-1} (not shown).

The mean total content of astragaloside IV in 7-month plants differed statistically. AM4 had highest total content of astragaloside IV, followed by AM2 and AM3, and AM7 had lowest total content of astragaloside IV (Table 3-6). The average of total content of astragaloside IV in 13-month plant did not differ statistically as determined by the F test. However, the Duncan test revealed that total content of astragaloside IV in AM3 was significantly higher than total content of astragaloside IV in AM7. AM7 had lowest total content of astragaloside IV (Table 3-6).

Concentration and total content of astragaloside IV in fertility trial

Concentration of astragaloside IV

Concentration of astragaloside IV in big roots of 7-month plants ranged from 53 μg g^{-1} to 134 μg g^{-1} and main P effect was significant (Pr=0.037) (Table 3-7). The P quadratic effect was significant (Pr=0.031), but the linear effect did not test significant by orthogonal contrasts (Table 3-8). The P × K linear interaction effect was significant (Pr=0.028) (Table 3-8). At 58 kg P ha^{-1}, concentration of astragaloside IV increased with increasing level of K (Table 3-8, Fig. 3-4). There was little difference in concentration between the highest fertilizer rates and the unfertilized treatment (Fig. 3-4). K linear and quadratic effects were not significant (Table 3-8).

In small root of 7-month plants, concentration of astragaloside IV ranged from 91 μg g^{-1} to 201 μg g^{-1}. Main effects for P and K did not test significant. The interaction of P X K was significant (Pr=0.012) (Table 3-7 and 3-8). When P was applied without K, concentration of astragaloside IV decreased with increasing level of P (Fig. 3-5). Similarly, without P application, application of K also decreased astragaloside IV concentration. At 56 kg K ha^{-1}, concentration of astragaloside IV increased with increasing level of P and K application in presence of P appeared to counteract detrimental effect of P (Fig. 3-5). Highest concentration of astragaloside IV was obtained without fertilizer application.

In big root of 13-month plants, concentration of astragaloside IV ranged from

75 μg g^{-1} to 157 μg g^{-1}. In small root of 13-month plants, concentration of astragaloside IV ranged from 214 μg g^{-1} to 343 μg g^{-1} (Table 3- 7). No significant effects of fertilization with P or K were found in big and small roots of 13-month plants (Table 3- 7). However, the P × K linear interaction effect was significant (Pr=0.040) in small roots of 13-month plants, as determined by orthogonal contrasts (Table 3-8). Without K application, concentration of astragaloside IV decreased with increasing level of P and without P application, K application also decreased concentration of astragaloside IV (Fig. 3-6).

Lime application significantly (Pr=0.078) increased concentration of astragaloside IV by 49 % in small root of 13-month plants (Table 3- 9). No lime effect was found in big roots of 7-month and 13-month plants and in small root of 7-month plants.

Total content of astragaloside IV

Total content of astragaloside IV ranged from 0.96 to 2.94 mg per plant in the root of 7-month plants (Table 3- 10). Highest content of astragaloside IV was obtained with 0-58-112 (lime) treatment. P effect was significant (Pr=0.052) and total content of astragaloside IV averaged over K rates increased with increasing level of P (Fig. 3-7). However, the P × K interaction effect was significant (Pr=0.073). Analysis of orthogonal contrasts showed that the P × K interaction effect was linear (Pr=0.009) (Table 3- 11). Without K application, total content of

astragaloside IV decreased with increasing level of P and without P application, total content of astragaloside IV decreased with increasing level of K (Fig. 3-8). With the high P application of 58 kg P ha^{-1}, total content of astragaloside IV increased with increasing level of K (Fig. 3-8). The high P and K rates gave only slightly higher yield of astragaloside IV than did no fertilizer.

Total content of astragaloside IV in the roots of 13-month plants ranged from 2.50 to 5.31 mg per plant. Highest astragaloside IV content was obtained with 0-0-0 (lime) treatment. The main effects for P and K did not test significant, but P × K interaction effect was significant (Pr=0.060) (Table 3-10). The P × K interaction effect was linear as determined by orthogonal contrasts (Pr=0.012) (Table 3-11). Without K application, total content of astragaloside IV decreased with increasing level of P. Similarly, without P application, total content of astragaloside IV decreased with increasing level of K. With P application, total content of astragaloside IV increased with increasing level of K (Fig. 3-9). However, highest astragaloside IV content was obtained with no fertilizer.

Despite a trend towards higher content of astragaloside IV with lime than without lime in 7-month plants, no significant lime effect on astragaloside IV content was found in the roots of either 7-month or 13-month plants (Table 3- 12).

Discussion

Astragaloside I has been reported to alter immune function (Bedir et al. 2000), while astragaloside IV has been used as marker compounds in *A. membranaceus* for quality control (Ma et al., 2002; The State Pharmacopoeia Commission of P.R. China., 2000.). Roots were dried in a forced air dryer at 40 °C for four days, then the roots were stored in dry and cool storage room until they were ground and analyzed. Anetai (1998) found that hot-air drying (above 50 ºC) after harvesting reduced the concentration of dilute ethanol-soluble extract. Therefore, forty degree was chosen for stability of astragaloside IV to approximate temperature when roots are dried in sun which is the traditional method used (Yang et al., 2006; Zhang, et al., 2005a).

Validation of HPLC Method

The reverse-phase high performance liquid chromatography (HPLC) with UV detector had been used for the analysis of astragaloside IV (Ma et al., 2002). However, the sensitivity of this method was low, because astragaloside IV has a weak chromophoric group in the UV region. Ganzera et al. (2001) and Li and Fitzloff (2001) used evaporative light scattering diction to analyze astragaloside IV and improved the sensitivity. We adopted parts of their methodology and modified it to use digoxin as internal standard. Acetonitrile (A) and water (B) were still used as the mobile phase, but different gradient elution was adjusted to separate astragaloside IV and digoxin.

This is the first study to test and validate that digoxin as an internal standard can be used to measure Astragaloside IV with HPLC-ELSD. The selection of digoxin to be tested as an internal standard in this study was based on previous study on ginsenosides in ginseng (Kim et al. 2007).

Digoxin was added to the standard and experimental samples. Internal standards are used when the quantity of sample analyzed or the instrument response varies from run to run and is difficult to control. It is also used when sample loss is unavoidable during sample preparation steps prior to analysis (Li, 2001, 2002; Skelly et al., 1990 ; Srinivas, 1998). The use of an internal standard can improve method accuracy and robustness. The average recovery of astragaloside IV was 93.23% (n=3) (Table 3- 2), which means the accuracy of this method was good.

Astragaloside IV and the internal standard were clearly separated and resolved in standard and experimental samples under the conditions described above (Fig.1, 2). The fact that astragaloside IV and the internal standard were clearly resolved in the standard and experimental samples indicated that this method can be used for the quantitative determination of astragaloside IV in *A. membranaceus* samples. Because the periodic problem with slight shift in retention time of astragaloside IV and multiple peaks due to other endogenous root compounds, it was sometimes necessary to spike root extract with astragaloside IV standard, from which the peak of astragaloside IV can be determined depend on the original peak area before it was spiked.

Good linearity in the range of 10 to 80 μg mL^{-1} (R^2=0.9932) (Fig. 3-3) and the LOD of 6 μg mL^{-1} (60ng) and LOQ of 12 μg mL^{-1} (120ng) indicate that this method was sensitive for the analysis of astragaloside IV.

The average recovery of astragaloside IV was 93.23% (n=3) (Table 3-2). The validation study showed good reproducibility for the quantification of astragaloside IV. These results demonstrated that this method was precise, accurate and sensitive for the quantitative determination of astragaloside IV in *A. membranaceus*.

Concentration and Content of Astragaloside IV in Tillage Trial

When the roots of *A. membranaceus* encounter a physical barrier in the soil, it is likely not only that the roots will bend or branch, but also that the accumulation of active compounds in the root will be affected (Mia et al., 1999). In a study of compaction using PVC tubes, the concentration of astragaloside IV and total content of astragaloside IV in the taproot of *A. membranaceus* decreased as the soil compaction levels increased (Mia et al., 1999). High strengths in coastal plain soils can be reduced through deep tillage (Busscher et al., 2000, 2002, 2006). Deep tillage breaks the hardpan and should help the root of *A. membranaceus* grow through deeper subsoil. Subsoiling significantly increased the concentration of astragaloside IV in small roots of 7-month plants, and in small and big roots of 13-month plants, (Table 3-3) and also increased the total content of astragaloside IV in roots of 13-month plants (Table 3-4). These results confirm the findings of Mia et al. (1999) that

reducing soil compaction increases astragaloside IV concentration. Deep tillage enhanced the accumulation of astragaloside IV, and thereby enhanced root medicinal quality.

Concentration and Content of Astragaloside IV in Variety Trial

A higher concentration of astraloside IV was found in 13-month plants than in 7-month plants, such as the big roots of AM1, AM3, AM4, AM5 and AM6 varieties. The concentration of astragaloside IV was higher in roots of 13-month plants than in roots of 7-month plant, is consistent with Zhang, Piao and Song (2005), who reported that concentration of astragaloside IV increased with age.

The result of small roots had higher concentrations of astragaloside IV than big root for the same growing period and variety is consistent with Anetai et al (1996), who assayed the taproots, and thick and thin lateral roots of *A. membranaceus* for astragaloside IV concentration by HPLC. They reported that the concentration of astragalosides IV was much higher in thin (small) roots and the thin (small) lateral root than taproot (big) and thick (big) lateral roots (Anetai et al., 1996). However, the small root only accounted small part (about 12.9% for 7-month plant and 10.7% for 13-month plant) of total root weight and the content of astragaloside IV in small roots did not markedly affect total content of astragaloside IV in Astragalus roots.

Significant differences among varieties were observed in concentration and total content of astragaloside IV in big and small roots of 7- and 13-month plants

(Table 3-5). AM3, AM4, AM2 and AM5 had relatively high concentration of astragaloside IV in the roots, which suggests that these varieties may be more suitable to plant in southeast US.

The concentration of astragaloside IV in the commercial sample was 134 μg g^{-1}. The commercial sample was sliced from big roots (diameter > 2 mm), therefore it should be compared with the large roots from our sample. Although the concentration of astragaloside IV in the commercial sample was higher than in the big roots of 7-month plants of all the varieties and most varieties at 13 months, it was lower than the concentration of astragaloside IV in AM3 at 13 months (Table 3-5). However, AM4 (85.6 μg g^{-1}) and AM5 (113.1 μg g^{-1}) still had acceptable concentration of astragaloside IV, based on and compared with the commercial sample. It is noteworthy that the concentration of astragaloside in small roots of most varieties was higher than the concentration in the commercial sample (Table 3-5). These results imply AM3, AM4 and AM5 have the potential to produce appropriate amount astragaloside IV in the roots.

The total content of astragaloside IV was highest in AM3 in 13-month plants and AM4 was highest in 7-month plants. AM7 had the lowest total content of astragaloside IV in both 7- and 13-month plants. After one year growth, AM3 had higher concentration and content of astragaloside IV than other varieties. Therefore, AM3 had the best root quality in terms of astragaloside content.

The lack of correlation between astragaloside IV concentration and

concentration of nutrient elements (N, P, K, Ca, Mg, Zn, Cu, Fe, and B) in above-ground parts suggests that these elements did not influence the differences among varieties in concentration of astragaloside IV in root in our experiment (results not shown).

Concentration and Content of Astragaloside IV in Fertility Trial

Soil fertility greatly affects the accumulation of primary and secondary metabolites in *A. membranaceus* (Anetai et al., 1995; Zhang et al., 2005b, c). In our experiment, a P quadratic effect in big roots of 7-month and linear P × K interaction in big roots of 7-month plants and small roots of 13 month plants was not significant (Table 3-8), and the overall effect of fertilization with P and K was negligible (Fig. 3-5, 3-6). No conclusive advantage was shown for fertilizer application small roots of 7 month plants or big and small roots of 13-month plants.

Phosphorus application increased total content of astragaloside IV in roots of 7-month plants with increasing level of P, when averaged over K treatments (Fig. 3-7). This result is consistent with Anetai et al. (1995), who found that the total content of astragaloside IV significantly increased with the increasing amounts of phosphoric acid in a field experiment where different amounts of P was applied.

The linear interaction effect of P × K was also significant (Pr=0.009). Without K application, total content of astragaloside IV decreased with increasing level of P. With K application, total content of astragaloside IV increased with increasing level

of P (Fig. 3-8). The fact that fertilization with K affected astragaloside IV content contradicts Anetai et al. (1995), who found potassium fertilizer had little influence on the glycoside (that includes astragaloside IV) contents in the root.

One of the reasons was that as long as P and K soil tests are in medium to high level (Table 2-2 and Table 2-4 in Chapter II) and fertility might be for lack of significant effect a major limitation for accumulation of astragaloside IV. The second reason was no nodules were found on the roots after harvesting, which indicated that the roots couldn't fix N_2 from environment. Therefore nitrogen may have been deficiency for the growth of *A. membranaceus* in this study. The third reason for lack of significant effects from nutrient applications on astragaloside IV was probably the high mortality rate, which affected small sample size. If the plant stands were better, there would be enough plants to increase the sample size and possibly strengthen treatment effect of P, K, P × K interaction effect and lime effect.

Lime application significantly (Pr=0.078) increased concentration of astragaloside IV in small roots of 13-month plants (Table 3-9), but the lime effect was not significant on concentration of astragaloside IV in roots of 7-month and in big roots of 13-month plants. Lime application did not affect the total content of astragaloside IV in roots of 7-month and 13-month plants (Table 3-12). According to Yang et al. (2006), *A. membranaceus* grows well in mildly acid to alkaline soil with pH ranges from 6.5 to 8. In our fertility trial, soil pH ranged from 5.8 to 6.0 after lime application and was lower than data reported by Yang et al. (2006). Therefore,

lime application was not sufficient to achieve the desired pH and therefore the lime effect on astragaloside IV was very weak. Even so, the increase in pH from 5.8 to pH 6.0 had little effect on astragaloside IV concentration and an alternative interpretation might be that Astragalus does not require pH 6.5 to accumulate astragaloside IV, which suggest that *A. membranaceus* might tolerate soils that are more acid than is suggested by the literatures.

Zhao et al. (2002) reported that the best ratio of nitrogen, phosphorus and potassium (N: P_2O_5: K_2O) for optimum astragaloside IV concentration is when the applied fertilizer was 1:0.8-1.2:1.2-1.8. In our fertility trial, only phosphorus and potassium fertilizer were applied in field and no nitrogen was applied, based upon the assumption that *A. membranaceus* would fix N and not require N fertilizer. Some studies (Anetai, et al. 1996; Zhang et al., 2005c; Zhao et al., 2002) found that the deficiencies of nitrogen in soil reduced the growth of root. The deficiency of nitrogen also leads to the less accumulation of dry material in root (Ma et al., 2004a; Tan et al., 2006b; Zhang, et al., 2005c; Zhao et al., 2002). In our study, nitrogen deficiency may have limited the P, K and lime effect on the accumulation of astragaloside IV in the roots.

Conclusions

Aastragaloside IV and digoxin (internal standard) were clearly separated and resolved by HPLC-ELSD. This method was used to measure astragaloside IV concentration and the validation procedure indicated it was precise, accurate and sensitive for the quantitative determination of astragaloside IV.

Subsoiling increased the concentration of astragaloside IV in roots and also significantly increased the total content of astragaloside IV in roots of 13-month plants. Small roots had higher concentrations of astragaloside IV than big roots of the same age and variety. The higher concentration of astraloside IV was found in 13-month plants than in 7-month plants. AM3, AM4, AM2 and AM5 had relatively high concentration of astragaloside IV in the roots. Compared with commercial sample, AM3, AM4 and AM5 had acceptable concentration of astragaloside IV, which indicated these varieties have the most promising potential for cultivation in southeast US.

Phosphorus and potassium, applied separately had detrimental effects on the concentration and total content of astagaloside IV, and when applied in combination resulted in similar or only slightly higher concentration and yield than the unfertilized treatment. This suggests that P and K fertility are not major constraints provided that both P and K are within the sufficiency range for the accumulation of astragaloside IV in the roots. Lime had no effect on astragaloside IV.

Table 3-1. The precision of the HPLC-ELSD method for the determination of astragaloside IV (n=3)

	original concentration ($\mu g\ mL^{-1}$)	Spiked concentration ($\mu g\ mL^{-1}$)	Observed concentration − original concentration ($\mu g\ mL^{-1}$)	R.S.D. (%)‡
Day1	19.24	10	9.30 (0.84) †	9.01
	19.24	20	18.89 (0.89)	4.69
	19.24	30	28.58 (0.42)	1.48
Day2	19.24	10	9.24 (1.03)	11.11
	19.24	20	19.28 (0.84)	4.34
	19.24	30	29.23 (0.76)	2.59
Day3	19.24	10	8.13 (0.91)	11.21
	19.24	20	18.47 (0.44)	2.39
	19.24	30	28.91 (0.86)	2.97

† The data in parentheses are standard deviation of the mean

‡ R.S.D. (relative standard deviations) (%) = standard deviation divided by mean, multiplied by 100.

Table 3-2. Accuracy of HPLC-ELSD method for the determination of astragaloside IV (n=3)

Spiked concentration ($\mu g\ mL^{-1}$)	Recovery (%)†	R.S.D. (%)‡
10	88.9 (6.58) §	7.41
20	94.4 (2.03)	2.14
30	96.4 (1.08)	1.12
Average of recovery	93.23	

† Recovery (%) = (observed concentration − original concentration)/spiked concentration×100%.

‡ R.S.D. (relative standard deviations) (%) = standard deviation divided by mean, multiplied by 100.

§ The data in parentheses are standard deviation of the mean

Table 3-3. The effect of subsoiling on concertration of astragaloside IV in tillage trial

Treatment	Big roots 7-month μg g^{-1}	Small roots 7-month μg g^{-1}	Big roots 13-month μg g^{-1}	Small roots 13-month μg g^{-1}
SS†	178	319	646	200
NoSS‡	131	259	454	165
Pr>F	NS§	**0.062**	**0.005**	**0.022**

† SS was subsoiling treatment

‡ NoSS was No subsoiling treatment

§ Not significant at α = 0.10 significance level.

Table 3-4. The effect of subsoiling on total content of astragaloside IV per plant in tillage trial

Treatment	7-month (mg $plant^{-1}$)	13-month (mg $plant^{-1}$)
SS†	3.44	15.6
NoSS‡	3.87	10.8
Pr>F	NS§	**0.025**

† SS was subsoiling treatment

‡ NoSS was No subsoiling treatment

§ Not significant at α = 0.10 significance level.

Table 3-5. The effect of variety on concentration of astragaloside IV in variety trial

Variety	Big 7-month $\mu g\ g^{-1}$	Big 13-month $\mu g\ g^{-1}$	Small 7-month $\mu g\ g^{-1}$	Small 13-month $\mu g\ g^{-1}$
AM1	37.2 bc†	40.6 cd	139.8 ab	250.0 ab
AM2	47.4 b	44.2 cd	119.0 b	297.5 a
AM3	45.1 b	172.9 a	148.1 ab	320.8 a
AM4	64.5 a	85.6 bc	204.3 a	237.2 ab
AM5	28.8 cd	113.1 b	161.0 ab	179.8 ab
AM6	41.7 b	48.8 cd	103.5 bc	269.3 bc
AM7	22.1 d	21.3 d	34.0 c	100.1 c

†Means in same column followed by the same letter do not differ at $\alpha = 0.10$ level of probability as determined by the Duncans Multiple Range Test.

Table 3-6. The effect of variety on total content of astragaloside IV per plant in variety trial

Variety	7-month (mg $plant^{-1}$)	13-month (mg $plant^{-1}$)
AM1	0.482 bc†	1.58 ab
AM2	0.931 ab	1.91 ab
AM3	0.824 abc	3.17a
AM4	1.24 a	2.76 ab
AM5	0.637 bc	1.11 ab
AM6	0.875 abc	1.79 ab
AM7	0.417 c	0.84 b

†Means in same column followed by the same letter do not differ at $\alpha = 0.10$ level of probability as determined by the Duncans Multiple Range Test.

Table 3-7. The effect of P and K application on concentration of astragaloside IV in fertility trial

Treatment Rate of N-P-K (kg ha^{-1})	Big 7-month μg g^{-1}	Small 7-month μg g^{-1}	Big 13-month μg g^{-1}	Small 13-month μg g^{-1}
0-0-0 (Lime)	123	201	135	343
0-29-0 (Lime)	84	129	140	313
0-58-0 (Lime)	97	117	117	214
0-0-56 (Lime)	83	115	122	275
0-29-56 (Lime)	75	152	75	312
0-58-56 (Lime)	126	161	126	291
0-0-112 (Lime)	63	125	122	238
0-29-112 (Lime)	53	165	151	230
0-58-112 (Lime)	134	91	157	277
P effect	**0.037**	NS†	NS	NS
K effect	NS	NS	NS	NS
P×K interaction effect	NS	**0.012**	NS	NS

Note: means are adjusted for missing values by Mixed model.

† Not significant at α = 0.10 significance level.

Table 3-8. Significance levels for orthogonal contrasts for linear and quadratic effects of P and K on concentration of astragaloside IV in fertility trial

	Big 7-month	Small 7-month	Big 13-month	Small 13-month
P linear effect	NS†	NS	NS	NS
P quadratic effect	**0.031**	NS	NS	NS
K linear effect	NS	NS	NS	NS
K quadratic effect	NS	NS	**0.085**	NS
P × K linear interaction effect	**0.028**	NS	NS	**0.040**
P × K quadratic interaction effect	NS	**0.086**	NS	NS

† Not significant at α = 0.10 significance level.

Table 3-9. The effect of lime application at medium P and K level on concentration of astragaloside IV in fertility trial .

Rate of N-P-K (kg ha^{-1})	Big 7-month µg g^{-1}	Big 13-month µg g^{-1}	Small 7-month µg g^{-1}	Small 13-month µg g^{-1}
0-29-56 (Lime)	75	75	152	312
0-29-56 (no lime)	56	90	124	210
Pr>F	NS†	NS	NS	**0.078**

† Not significant at α = 0.10 significance level.

Table 3-10. The effect of P and K application on total content of astragaloside IV per plant in fertility trial

Treatment Rate of N-P-K (kg ha^{-1})	7-month (mg $plant^{-1}$)	13-month (mg $plant^{-1}$)
0-0-0 (Lime)	2.24	5.31
0-29-0 (Lime)	1.60	3.54
0-58-0 (Lime)	1.56	2.63
0-0-56 (Lime)	0.96	3.11
0-29-56 (Lime)	1.65	2.50
0-58-56 (Lime)	2.36	3.26
0-0-112 (Lime)	0.97	3.15
0-29-112 (Lime)	1.17	4.35
0-58-112 (Lime)	2.94	5.00
P effect	**0.052**	NS
K effect	NS†	NS
P×K interaction effect	**0.073**	**0.060**

† Not significant at α = 0.10 significance level.

Table 3-11. Significance levels for orthogonal contrasts for linear and quadratic effects of P and K on total content of astragaloside IV in fertility trial

	7-month	13-month
P linear effect	NS†	NS
P quadratic effect	NS	NS
K linear effect	NS	NS
K quadratic effect	NS	NS
P × K linear interaction effect	**0.009**	**0.012**
P × K quadratic interaction effect	NS	NS

† Not significant at α = 0.10 significance level.

Table 3-12. The effect of lime application at medium P and K level on total content of astragaloside IV per plant in in fertility trial.

Treatment Rate of N-P-K (kg ha^{-1})	7-month (mg $plant^{-1}$)	13-month (mg $plant^{-1}$)
0-29-56 (Lime)	1.65	2.50
0-29-56 (no lime)	0.86	2.84
Pr>F	NS†	NS

† Not significant at α = 0.10 significance level.

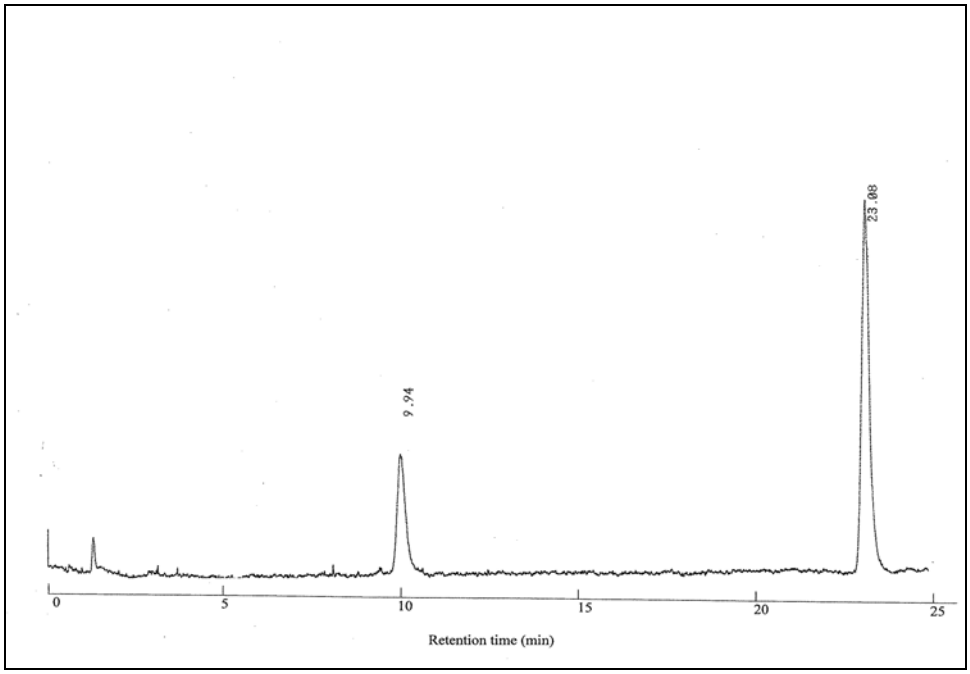

Fig. 3-1. Astragaloside IV and internal standard (digoxin) in methanol (retention time for digoxin was at 9.94 min and for astragaloside IV was at 23.08 min).

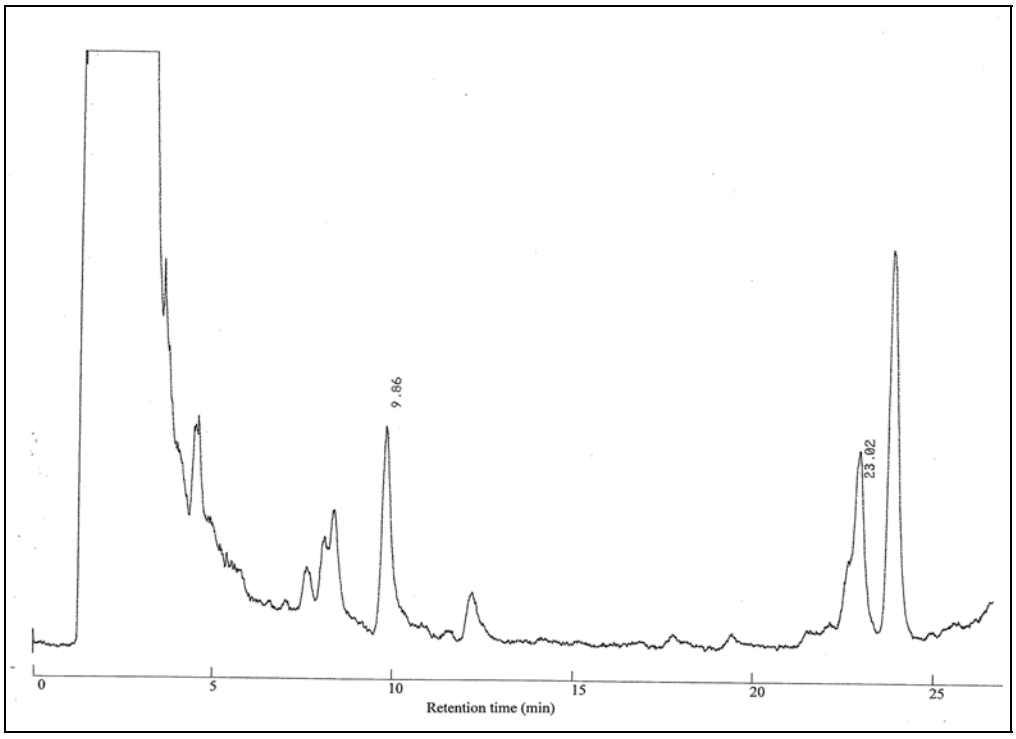

Fig. 3-2. Astragaloside IV and internal standard (digoxin) in methanol extract of root of *A. membranaceus* (retention time for digoxin was at 9.86 min and for astragaloside IV was at 23.02 min).

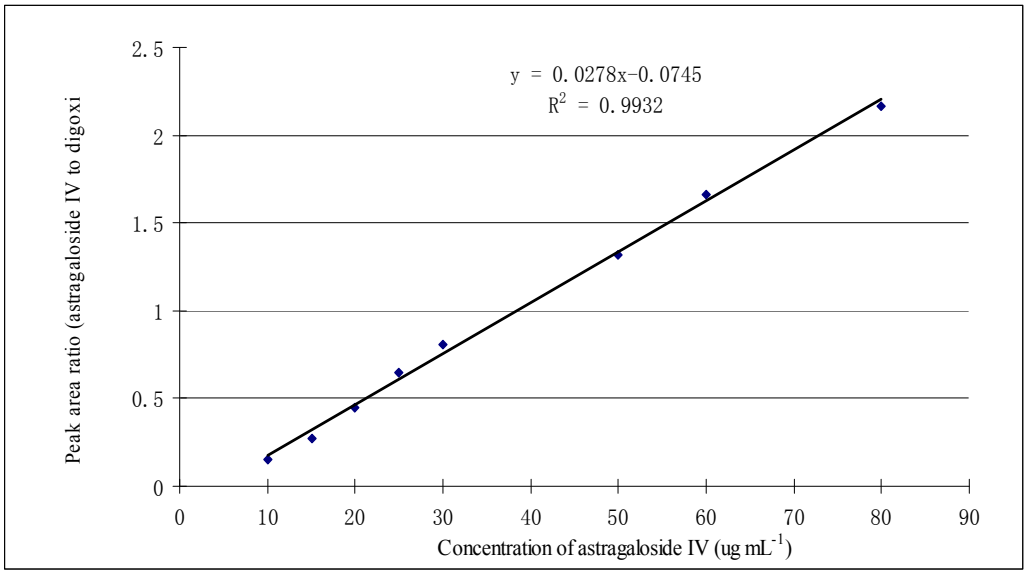

Fig. 3-3. Standard curve of astragaloside IV

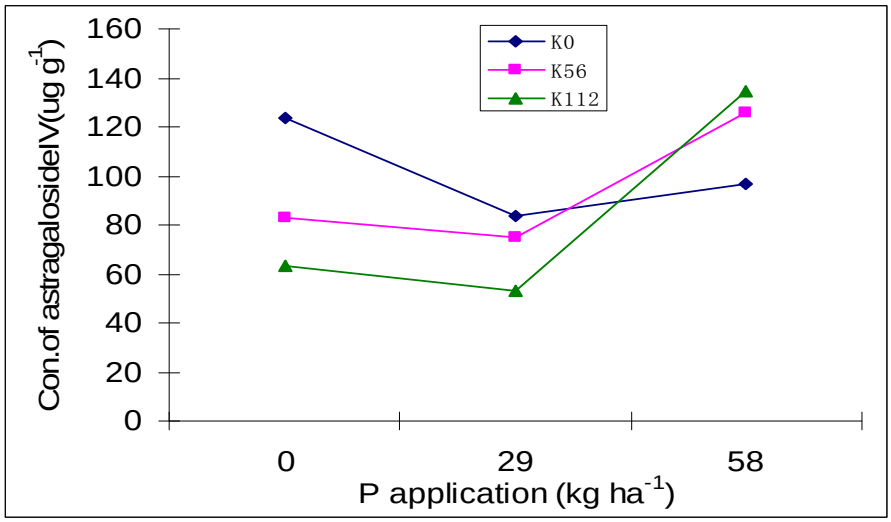

Fig. 3-4. Effects of P and K application on concentration of astragaloside IV in big root of 7-month plant

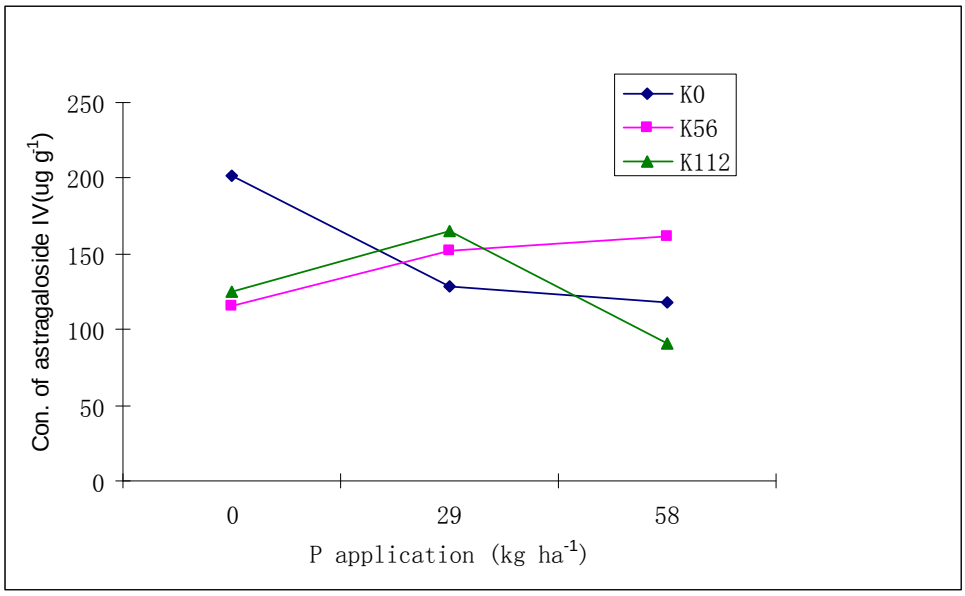

Fig. 3-5. Effects of P and K application on concentration of astragaloside IV in small root of 7-month plant

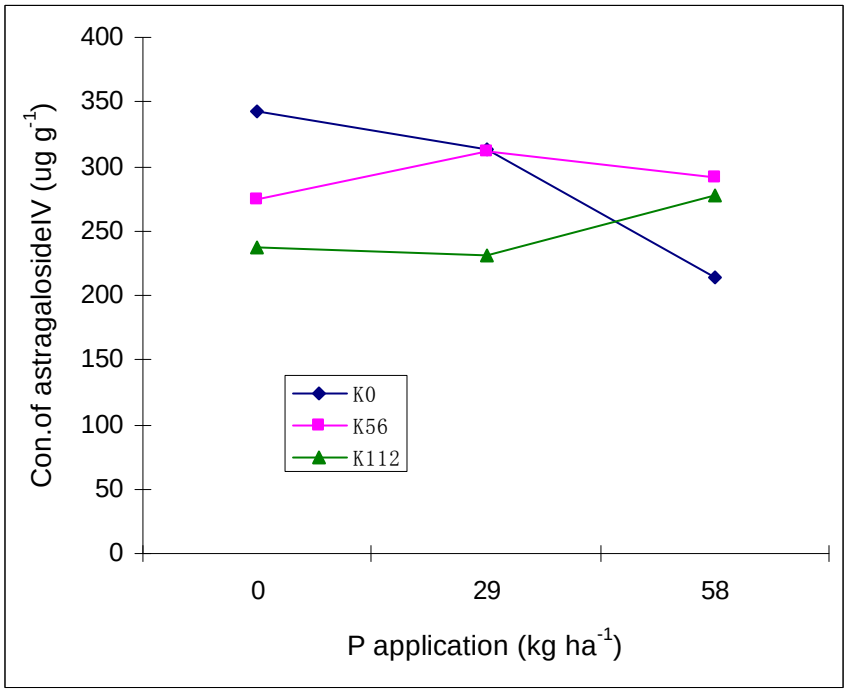

Fig. 3-6. Effects of P and K application on concentration of astragaloside IV in small root of 13-month plant

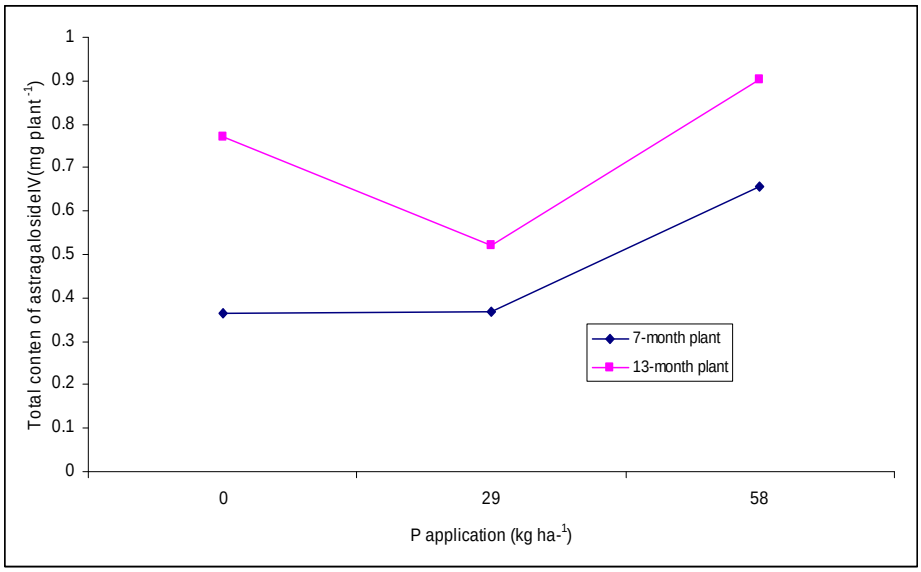

Fig. 3-7. Main effects of P application on total content of astragaloside IV in root of 7- and 13-month plants

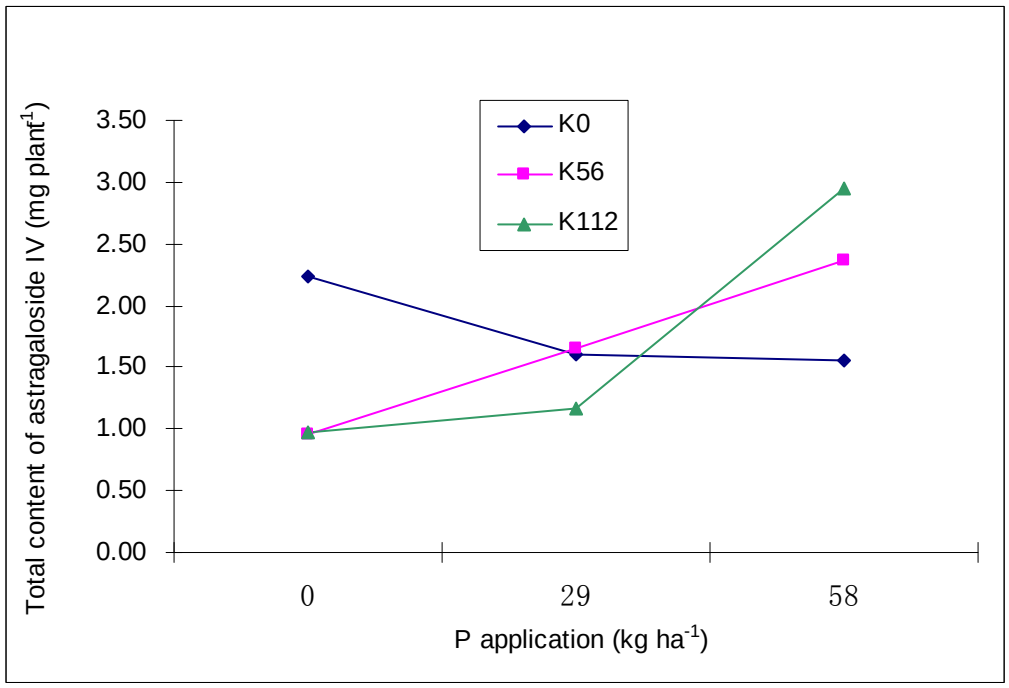

Fig. 3-8. Effects of P and K application on total content of astragaloside IV in 7-month plants

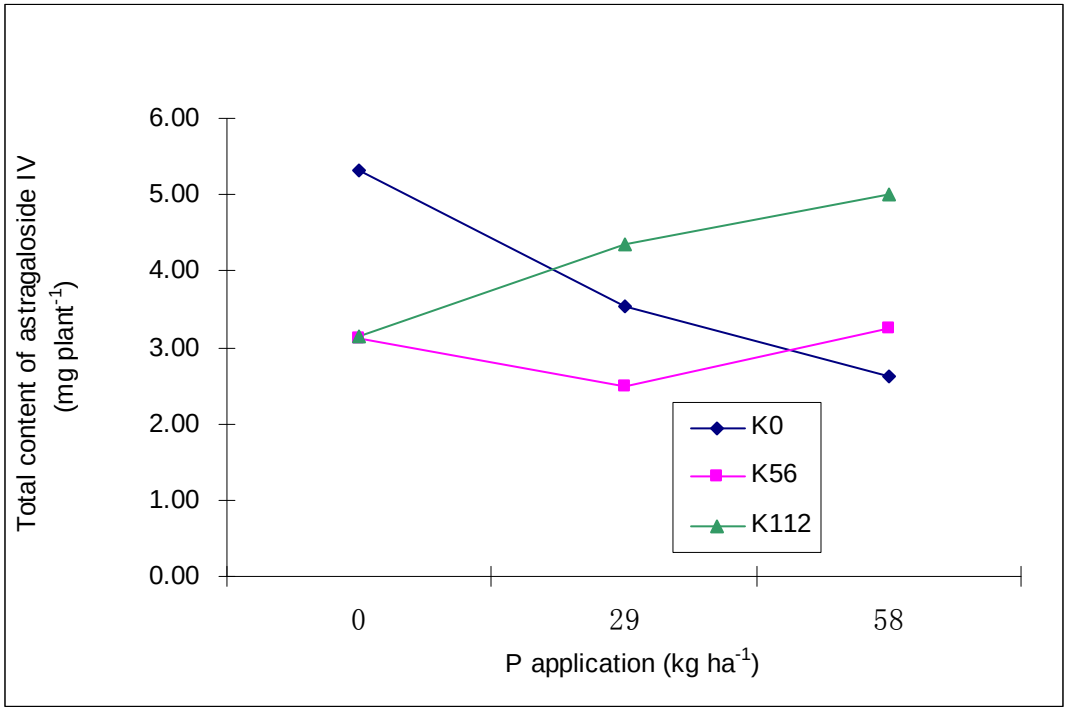

Fig. 3-9. Effects of P and K application on total content of astragaloside IV in 13-month plants

IV. SUMMARY

In September 2006, three field experiments were carried out (1) deep tillage (subsoiling vs no subsoiling), (2) variety trial (seven variety from difference areas), and (3) P (0, 29, and 58 kg ha^{-1}) and K (0, 56, and 112 kg ha^{-1}) fertilizer and lime. The percent of germination for different varieties ranged from 20.1 to 89.5 except AM5. The percent recovery after winter ranged from 81% to 95% for different varieties.

Plant mortality was most severe with small seedlings in spring and summer, but was observed throughout the season. Soil-borne pests, such as white fringe beetle, fed on the root and crown of the seedlings, which caused the roots to break. They can be controlled by appropriate insecticide. In spring, the soil can be treated by pesticide to kill eggs and larvae; in fall, special gas can be used to induce adults to go to other places to lay eggs. Under high soil moisture and summer heat, *Astragalus* is susceptible to root rot caused by fungi. Since insects may be easily controlled, disease may be more important constraint to overcome in SE US. Land must be well drained for *Astragalus*. Loose soil and raise beds can be used to control soil moisture.

A. membranaceus failed to nodulate with indigenous rhizobia. Inoculation with rhizobium specific to *Astragalus* spp. is highly recommended.

Aastragaloside IV and the internal standard were clearly separated and resolved using HPLC-ELSD. This method was precise, accurate and sensitive for the quantitative determination of astragaloside IV in *A. membranaceus*.

Small roots had higher concentrations of astragaloside IV than big root for the same growing period and variety. The higher concentration of astraloside IV was found in 13-month plants than in 7-month plants.

In-row subsoiling management (in SS treatment) reduced the soil strength, which enabled roots to grow longer and lateral roots to grow farther from the crown. However, subsoiling didn't improve those characteristics on shoot weight, root weight, shoot height, root diameter and root branches. Subsoiling significantly increased the concentration and the total content of astragaloside IV in the roots. Although deep tillage was somewhat beneficial for *A. membranaceus*, it did not sufficiently loosen soil to yield straight and unbranched roots.

The results of the fertility trial did not conclusively demonstrate a benefit from application of P and K. The lack of response may have been related to the inability of the plants to fix nitrogen, because of the failure to form nodules. There was no lime effect on root weight, root to shoot ration, shoot height, root diameter, root length and root branch at 29kg P + 56kg K ha^{-1} for either 7-month or 13-month plants. Lime had no effect on astragaloside IV.

Accessions AM3, AM4 and AM5 had good adaptability, highest root weight (yield) and root quality (relatively higher concentrations of astragaloside IV) and can be chosen to plant in southeast US as alternative crop. Further research on disease and pest control, variety selection and fertility for *A. membranaceus* are needed in order to develop recommendations for its cultivation in the southeastern U. S..

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