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Chemical Composition and *in vitro* Ruminal Fermentation Characteristics of Tetraploid Black Locust (*Robinia pseudoacacia* L.)

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ABSTRACT
The aim of this study was to investigate nutritive value of tetraploid black locust (*Robinia pseudoacacia*). Chemical composition and *in vitro* fermentation characteristics by using *in vitro* gas production technique were stated for tetraploid black locust, birdsfoot trefoil (*Lotus corniculatus* L.), alfalfa (*Medicago sativa* cv.) and galega (*Galega orientalis* Lam.). Tetraploid black locust showed high crude protein (16.02%), ether extract (2.85%), crude ash (13.16%) and Ca (3.85%) content and low neutral detergent fibre (21.63%), acid detergent fibre (19.91%) and acid detergent lignin (6.52%) content. However, fermentation characteristics, such as cumulative gas production of 48 h, dry matter degradability, neutral detergent fibre digestibility, total volatile fatty acid of tetraploid black locust, were lower than that of birds foot trefoil and alfalfa ($p<0.05$). Tetraploid black locust fermentation resulted in reducing the molar proportions of acetate and butyrate and increasing that of propionate ($p<0.05$). In conclusion, according to the chemical composition, tetraploid black locust was a high-quality feed resources. However, utilization of tetraploid black locust by ruminal microorganisms *in vitro* was limited. Further *in vivo* studies could be required to expand the knowledge of the nutritive value of tetraploid black locust.

Key words: Tetraploid black locust, *in vitro* gas production, nutritive value, ruminal microorganism, degradability

INTRODUCTION
In Xinjiang, ruminant farming is an important part of the husbandry industry. The development of livestock needs high-quality forage. Alfalfa hay is usually the best feedstuff for sheep and cattle (Maheri-sis *et al*., 2007). It has not only good palatabihty, but also high nutritional value. However, the average production level of alfalfa hay was only 3.75 t ha^{-1}. The annual output was about 50 million tons of dry matter in China (Wang and Liu, 2004). This can not yet meet the domestic demand for livestock development. Recently, the development of animal husbandry has being accelerated in Xinjiang (Hu, 2004). Under this condition, the contradiction between supply and demand will be further intensified. So, it is important to find a new feed resource which can replace alfalfa hay to solve the high-quality forage shortage challenge.

Tetraploid black locust (*Robinia pseudoacacia*) bred through doubling the chromosomes of the diploid *R. pseudoacacia*. This tetraploid species was introduced to China in 1997 (Jiang *et al*., 2009). A number of studies investigated on its cultivation, seed germination characteristics, flowering habits and provision (Wang, 2003; Li and Jiang, 2005; Zhang *et al.*, 2010). Those studies confirmed its cultivation value as a forage species and thousands of hectares of tetraploid black locust have been planted in China.

The browse of black locust is highly preferred by goats (Pande *et al.*, 2002). Compared with alfalfa pellets, the live weight gain of goats was not affected by black locust consumption (Papachristou *et al.*, 1999). Jiang *et al.* (2009) also reported that the tetraploid black locust leaves and stems were suitable feed for dairy cows and goats. However, some studies found that black locust meal was not a satisfactory alternative to alfalfa meal as a livestock feed (Singh *et al.*, 1997). It is not yet clear what cause such difference. Furthermore, few researches were conducted to evaluate utilization of tetraploid black locust by ruminal microorganisms.

The aim of this study was to determine chemical composition and comparison of nutritive value of tetraploid black locust and other leguminous forages including birdsfoot trefoil (*Lotus corniculatus* L.), alfalfa (*Medicago sativa* cv.) and galega (*Galega orientalis* Lam.) by using *in vitro* gas production technique.

MATERIALS AND METHODS

Experimental design: Two experiments were performed. Experiment 1 analyzed the chemical compositions of tetraploid black locust and other forages, including birdsfoot trefoil, alfalfa and galega and nutritive value was compared with each other. In experiment 2, the *in vitro* gas production technique was adopted to determine the fermentation of tetraploid black locust, alfalfa and birdsfoot trefoil by mixed ruminal microorganisms. All of the experiments were performed in the Laboratory of Animal Nutrition, Xinjiang Agricultural University, P.R.China (Mar, 2010 to Aug, 2010).

Forage samples: Tetraploid black locust shoots were harvested on September, 2009 (1st cut) and were dried naturally in cool place as primary samples. Birdsfoot trefoil (2nd cut, midbloom), alfalfa (2nd cut, prebloom) and galega (1st cut, prebloom) were kindly provided by professor Aishan. All the samples were hammer milled with 1 mm screen and preserved in dried bottles for latter analysis.

Experiment 1: The Dry Matter (DM) content was determined by drying in a forced air oven at 105±1°C for 3 h and ash was determined by incineration in a muffle furnace at 550°C for 3 h. The Ether Extract (EE) was determined by extraction with petroleum ether. The N content was determined by using the Kjeldhal method; those values were converted to Crude Protein (CP) by multiplying by a factor of 6.25. The Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) analyses were performed by the sequential procedure of AOAC (1999). The NDF was assayed with sodium sulphite and without alpha amylase. Phosphorus (P) was analyzed by the method of Parks and Dunn (1963). Calcium (Ca) was determined by a colorimetric method (Michaels *et al.*, 1949).

Experiment 2: *In vitro* fermentation was performed according to the method of Menke and Steingass (1988) with some modifications. Samples of rumen fluid were collected from four ruminally fistulated male Kazak sheep fed 1.26 kg day^{-1} (DM basis) of a wheat straw-based diet (71.20% wheat straw, 21.55% ground corn, 4.62% cottonseed meal, 1.54% soybean meal, 0.34% limestone, 0.24% calcium phosphate, 0.12% urea, 0.34% salt, 0.05% premix). Rumen fluid was strained through four layers of cheese cloth into a prewarmed, insulated bottle. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2.

The *in vitro* fermentations were in 125 mL serum bottles containing 100 mL of dilute rumen fluid and 1.00 g of forage sample and the blanks contained only buffered ruminal fluid. Incubation was carried out in 5 repeats within each run. Bottles were placed in a water bath at 39°C and maintained at slow continuous agitation (100 rpm) for 48 h. Gas production was recorded at 2, 5, 8, 12, 18, 24, 36 and 48 h. At the end of incubation, the pH of each bottle was measured immediately with a pH-meter (model FE20; METTLER TOLEDO, China) and the fermentation was stopped by swirling the bottles on ice. The culture of each bottle was strained through nylon bag (100 μm pore size). The substrate residues were collected quantitatively for determination of DM and NDF. DM Degradability (DMD) and NDF degradability (NDFD) of samples were calculated according to Van Soest and Robertson (1985). A 10 mL sample of each filtered liquid was collected from each bottle for subsequent analysis of ammonia N (NH_3-N) and Volatile Fatty Acid (VFA). NH_3-N was measured by colorimetric method (Chaney and Marbach, 1962). VFA were determined according to the procedures described by Getachew *et al.* (2001) using a gas chromatograph (model GC-2010; SHIMADZA Corporation, Kyoto, Japan). Crotonic acid was used as internal standard.

During the incubation, gas production was determined by using a water displacement apparatus (Fedorah and Hrudey, 1983). To give a more precise estimate of the gas production throughout the duration of *in vitro* fermentation, a nonlinear equation was used to analyze the kinetic data by the method described by Orskov and McDonald (1979). The parameters were estimated by the NLREG (version 6.3) software (Brentwood, TN).

The Metabolism Energy (ME) of substrates was calculated by the model of Menke *et al.* (1979).

Statistical analysis: The PASW Statistics 18.0 software package was used for statistical analysis (Norusis, 2009). Data were analyzed using a one-way ANOVA, followed by mean comparisons using the Duncan's new multiple range test.

RESULTS

Chemical composition: As showed in Table 1, the CP content in tetraploid black locust was higher than that of birdsfoot trefoil ($p<0.05$), but significant lower than that of alfalfa and galega ($p<0.05$). The EE content was highest for tetraploid black locust (2.85%), followed by birdsfoot trefoil (2.27%) and lowest for alfalfa and galega ($p<0.05$). Compared with other forages, tetraploid black locust contented lower ADF and NDF. However, ADL content in tetraploid black locust was significant higher than that in alfalfa and galega, lower than that in birdsfoot trefoil. The contents

Table 1: Chemical composition of four forages (% on DM basis)

Chemical composition	Tetraploid black locust	Birdsfoot trefoil	Alfalfa	Galega
Moisture (%)	5.31±0.14[a]	4.61±0.16[b]	4.14±0.06[c]	4.57±0.29[bc]
CP	16.92±0.33[c]	13.72±0.11[d]	18.61±0.47[b]	21.90±0.91[a]
EE	2.85±0.02[a]	2.27±0.23[b]	1.96±0.03[c]	1.80±0.08[c]
NDF	21.63±0.50[d]	53.29±0.47[a]	34.31±1.57[c]	41.47±0.01[b]
ADF	19.91±0.64[d]	47.20±0.39[a]	27.93±0.18[c]	33.68±0.95[b]
ADL	6.52±0.41[b]	9.75±0.36[a]	5.45±0.29[c]	4.75±0.01[c]
Ash	13.16±0.22[a]	10.44±0.20[b]	10.75±0.00[b]	11.08±0.43[b]
Ca	3.85±0.18[a]	1.21±0.18[b]	1.30±0.01[b]	1.33±0.03[b]
P	0.22±0.02[b]	0.18±0.00[c]	0.22±0.00[b]	0.31±0.02[a]

a,b,c,d, in each row for differences ($p<0.05$) between groups: Data presented as Mean±SD. CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fibre, ADL: Acid detergent Ligin, ADF: Acid detergent fibre, Ca: Calcium, P: Phosphorus of ash and Ca in tetraploid black locust were the highest among the four forages ($p<0.05$) and there was no difference among birdsfoot trefoil, alfalfa and galega. The P content in tetraploid black locust was higher than that of birdsfoot trefoil ($p<0.05$) and lower than that of galega ($p<0.05$).

Gas production characteristics: Gas production volumes (mL 1.0 g^{-1} DM) in different incubation time are presented in Table 2. The gas volumes for tetraploid black locust in different incubation time were significantly lower than that of alfalfa ($p<0.05$). Compared with birdsfoot trefoil, there was no significant change in gas production of tetraploid black locust in the first 8 h of incubation. Later, however, the gas production of tetraploid black locust was significantly lower than that of birdsfoot trefoil. At the end of incubation, the highest cumulative gas production was observed in alfalfa (123.9 mL 1 g^{-1} DM) which was followed by birdsfoot trefoil (104.00 mL 1 g^{-1} DM). Tetraploid black locust produced the lowest cumulative gas (74.19 mL 1 g^{-1} DM).

Kinetics of gas production: Gas production parameters of tetraploid black locust, birdsfoot trefoil and alfalfa are shown in Table 3. The gas production of the insoluble fraction (b) and the potential extent of gas production ($|a|+b$) of tetraploid black locust were significant lower than that of birdsfoot trefoil and alfalfa ($p<0.05$). Rate of gas production expressed in mL h^{-1} (c) of tetraploid black locust (0.1129) and birdsfoot trefoil (0.1187) significantly lower than that of alfalfa (0.1481) ($p<0.05$). Proportion of variance explained (R^2) ranged from 0.9795 to 0.9878 for the three substrates and all p-values of curve fitting were smaller than 0.01. This indicated that gas production curve of the three forages fitted well the mathematical model.

***In vitro* DM and NDF digestibility:** DM degradability (DMD) and NDF degradability (NDFD) of tetraploid black locust were 45.52 and 15.53%. They were significantly lower than that of birdsfoot trefoil and alfalfa ($p<0.05$).

Estimated metabolism energy: The ME of tetraploid black locust was 5.13 MJ kg^{-1} DM and was significantly lower than that of birdsfoot trefoil (5.74 MJ kg^{-1} DM) and alfalfa (6.53 MJ kg^{-1} DM) (Table 3).

Fermentation characteristics: *In vitro* fermentation characteristics of tetraploid black locust, birdsfoot trefoil and alfalfa are displayed in Table 4. When mixed ruminal microorganisms were incubated with different substrate, the final pH value and ammonia nitrogen (NH_3-N)

Table 2: Cumulative gas production volume (mL 1 g^{-1} DM) at different incubation time of tetraploid black locust, birdsfoot trefoil and alfalfa

Incubation time (h)	Tetraploid black locust	Birdsfoot trefoil	Alfalfa
2	19.92±9.47^b	27.74±10.06^b	39.13±3.13^a
5	40.68±10.28^b	45.37±11.73^b	61.76±5.97^a
8	48.92±11.26^b	60.14±12.13^b	87.30±8.58^a
12	54.09±12.57^c	85.31±13.20^b	109.96±11.01^a
18	63.80±13.22^b	100.53±14.44^a	118.46±12.32^a
24	72.27±13.60^c	101.55±13.89^b	120.08±12.60^a
36	73.26±13.44^c	103.02±13.16^b	122.86±13.75^a
48	74.19±13.42^c	104.00±13.21^b	123.94±13.80^a

a,b,c, in each row for differences ($p<0.05$) between groups: Data presented as Mean±SD

Table 3: Kinetics of gas production, DMD, NDFD and estimated metabolism energy from *in vitro* fermentation of tetraploid black locust, birdsfoot trefoil and alfalfa

Items	Tetraploid black locust	Birdsfoot trefoil	Alfalfa
b (mL g^{-1} DM)	65.88±7.53^b	104.60±12.05^a	119.10±12.05^a
|a|+b (mL g^{-1} DM)	74.43±13.54^b	110.62±16.23^a	124.83±13.71^a
c (mL h^{-1})	0.1129±0.0123^b	0.1187±0.0071^b	0.1481±0.0099^a
R^2	0.9835	0.9795	0.9878
p-value of curve fitting	<0.01	<0.01	<0.01
DMD (%)	45.52±1.24^c	62.00±4.18^b	70.51±2.38^a
NDFD (%)	15.53±8.85^b	46.68±4.51^a	47.24±5.05^a
ME (MJ kg^{-1} DM)	5.13±0.37^c	5.74±0.38^b	6.53±0.34^a

a,b,c, in each row for differeuces (p<0.05) between groups: Data presented as Mean±SD. DMD: Dry matter degradability, NDFD: Neutral detergent fibre degradiability, ME: Methanol extract, b: Gas production of insoluble fraction, c: Rate of gas production

Table 4: *In vitro* fermentation characteristics of tetraploid black locust, birdsfoot trefoil and alfalfa

Items	Tetraploid black locust	Birdsfoot trefoil	Alfalfa
pH	6.54±0.07^a	6.41±0.02^b	6.42±0.02^b
NH_3-N (mmol L^{-1})	17.46±1.92^b	20.35±1.45^a	22.26±0.64^a
TVFA (mmol L^{-1})	7.76±0.99^b	9.45±0.40^a	8.83±0.25^a
Acetate (mol%)	56.89±0.19^c	57.37±0.47^b	58.52±0.18^a
Propionate (mol%)	26.83±0.22^a	25.85±0.21^b	25.24±0.06^c
Butyrate (mol%)	16.28±0.18^b	16.78±0.28^a	16.24±0.16^b
A:P	2.12±0.02^c	2.22±0.04^b	2.32±0.01^a

a,b,c, in each row for differeuces (p<0.05) between groups; Data presented as Mean±SD. TVFA: Total volatile fatty acid, A:P: Ratio of acetate to propiorate

concentration in culture showed significant difference (p<0.05). Tetraploid black locust showed lower pH value (6.54) and NH_3-N (17.46 mmol L^{-1}) than of all the substrates tested. In contrast, tetraploid black locust presented the lowest concentration of Total Volatile Fatty Acid (TVFA) (7.76 mmol L^{-1}). For final pH, NH_3-N and TVFA, there were no significant difference between birdsfoot trefoil and alfalfa. Alfalfa fermentation resulted in the highest NH_3-N concentration (22.26 mmol L^{-1}) and birdsfoot trefoil fermentation produced the highest TVFA (9.45 mmol L^{-1}). In the three groups, tetraploid black locust fermentation resulted in reducing the molar proportions of acetate and butyrate and increasing that of propionate (p<0.05). The molar proportions of acetate, propionate and butyrate in this study were 56.89, 26.83 and 16.28 mol%, respectively. As a result, the highest A:P (the ratio of acetate to propionate) was observed in alfalfa (2.32) which, was followed by birdsfoot trefoil (2.22). Tetraploid black locust showed the lowest A:P (2.12).

DISCUSSION

Black locust is a nitrogen-fixing leguminous tree with alternate, pinnately compound leaves native to southeastern North America and naturalized in the temperate regions of Europe and Asia (Moshki and Lamersdorf, 2011). It has been used for livestock feed in countries around the world. Tetraploid black locust bred through doubling the chromosomes of the diploid black locust. Results of several researches revealed that tetraploid black locust is a nutrition-rich fodder for livestock (Jiang *et al*., 2009; Zhang *et al*., 2010). In the present study, tetraploid black locust showed higher CP, EE and ash content than other forages. The findings were similar to those reports by Wang *et al*. (1999) and Li *et al*. (2006). Additionally, tetraploid black locust had moderate CP content (16.92%). Furthermore, the percentages of NDF, ADF and ADL in tetraploid black locust were 21.63, 19.91 and 6.52%, respectively and they were lower than that of other forages. This was not agreeing with study conducted by Sultan *et al.* (2008) who reported that contents of CP, NDF, ADF and lignin of black locust were 11.9, 51, 22 and 6.9%, respectively. This could be related to harvest season, cultivate place of feedstuffs and earth fertility. According to the chemical composition, tetraploid black locust was a high-quality feed resources in the present study.

In addition to nutrient content, the digestibility is an important factor affecting the nutritive value of feed. The *in vitro* gas production technique has been used as a method for determining the nutritive value of feedstuffs. It has provided better predictions of the *in vivo* digestibility and the energetic value of forages than other *in vitro* techniques (Herrero *et al.*, 1996). The gas production is basically the result of the fermentation of carbohydrates into VFA (Getachew *et al.*, 1998a). The kinetics of gas production could be affected by carbohydrate fraction (Deavill and Givens, 2001). The high fermentations of the insoluble fraction were observed in birdsfoot trefoil and alfalfa. This indicated that the carbohydrate fractions of birdsfoot trefoil and alfalfa were readily availability to the microbial population. Although tetraploid black locust was low in NDF, the cumulative gas productions were significantly lower than that of birdsfoot trefoil and alfalfa in most of time. This consisted with the report of Fay *et al.* (1980) who found that black locust would not cause bloat because it does not give rise to enough gas. This could be due to the fact that (1) tetraploid black locust is a relatively rich source of protein, which yields less gas than carbohydrates (Cone and Van Gelder, 1999); (2) some chemicals in tetraploid black locust could inhibit ruminal fermentation. The feed value of black locust leaves has been debated for some time mainly because of the presence of antiquality components such as condensed tannins and lectin (Burner *et al.*, 2008). Inclusion of tannins in diet consistently decreased both the rate of ruminal gas and methane gas production (Min *et al.*, 2005). This could be resulted from that some ruminal bacteria were sensitive to tannins. Several investigations found that condensed tannins inhibited growth and protease activity in *Butyrivibrio fibrisolvens*, *Streptococcus bovis* and *Butyrivibrio proteoclasticus* isolated from rumen (Jones *et al.*, 1994; Vasta *et al.*, 2010). This could also explain why the DMD and NDFD of tetraploid black locust were significantly lower than those of birdsfoot trefoil and alfalfa in the current trial. This agree with previously research reported by Salawu *et al.* (1997) who found that in sheep fed a grass hay with addition of condensed tannins, in sacco digestibility was decreased for DM, CP, NDF and ADF components of the diet. The decreased digestibilities associated with condensed tannins probably resulted from the formation of complexes between condensed tannins and dietary carbohydrates and proteins (Mohammadabadi *et al.*, 2009).

There is a positive correlation between DM loss or gas production and VFA concentration (Getachew *et al.*, 1998b). VFA mainly derive from dietary carbohydrates. In our study, TVFA produced from tetraploid black locust was significantly lower than from birdsfoot trefoil and alfalfa. This was very likely due to the lower DMD and NDFD of tetraploid black locust. In the case of tetraploid black locust, the molar proportion of acetate was lower and that of propionate was higher, the reason being that the content of NDF in tetraploid black locust was lower than in birdsfoot trefoil and alfalfa. As a consequence, A:P was lowed by tetraploid black locust fermentation. This implied that the fermentation characteristics were obviously different among the three substrates.

NH_3-N concentration in tetraploid black locust group was significantly lower than that of birdsfoot trefoil and alfalfa. This showed similarities with the reports of Horton and Christensen (1981).

There is little information about ME of tetraploid black locust. Sultan *et al.* (2008) reported that ME of black locust was 6.9 MJ kg^{-1} DM. In this study, the ME of tetraploid black locust was 5.13 MJ kg^{-1} DM and was significantly lower than that of birdsfoot trefoil and alfalfa. The difference could be related to harvest season, cultivate place and maturity of feedstuffs. Menke and Steingass (1988) reported a strong correlation between ME values measured *in vivo* and predicted from 24 h *in vitro* gas production and chemical composition of feed. The *in vitro* gas production method has also been widely used to evaluate the energy value of several classes of feed (Chumpawadee *et al.*, 2007; Aghajanzadeh-Golshani *et al.*, 2010; Saricicek and Kilic, 2011).

CONCLUSIONS

In summary, the results of this investigation indicate that the tetraploid black locust used in this study was a high-quality feed resources. However, utilization of tetraploid black locust by ruminal microorganisms *in vitro* was limited. Further *in vivo* studies could be required to expand the knowledge of the nutritive value of tetraploid black locust.

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