International Journal of Food Science and Nutrition
ISSN: 2455-4898; Impact Factor: RJIF 5.14
Received: 04-01-2020; Accepted: 05-02-2020; Published: 03-03-2020
www.foodsciencejournal.com
Volume 5; Issue 1; 2020; Page No. 14-19

Effect of ecotype and phenological phase on protein fractions and *in vitro* digestibility of *Leucaena* sp. from southern México

Irene Karen Abrego Solis[1], Valeria Nazareth Herrera Merced[2], Luis Corona Gochi[3], Águeda García Pérez[4], Silvino Carrillo Pita[5], Humberto Hernández Hernández[6], Régulo Jiménez Guillén[7], Francisco Alejandro Castrejón Pineda[8]

[1, 2] Faculty of Chemistry - Food Chemistry. UNAM, Mexico

[3, 4, 8] Faculty of Veterinary Medicine and Zootechnics, Department of Animal Nutrition and Biochemistry, National Autonomous University of Mexico, Mexico

[5-7] CSAEGRO-SAGARPA, Cocula, Gro, Mexico

[7] INIFAP, Tuxpan, Iguala, Gro, Mexico

Abstract
To evaluate *Leucaena* ecotypes native to Mexico, its phenological state and the relationship of protein fractions with *in vitro* dry matter digestibility (*IV*DMD) were evaluated using an experimental design of divided plot, large plot: ecotype (nine native); small plot: phenological stage (regrowth vs. tender green bean). Crude protein (CP), protein fractions A, B1, B2, B3 and C, and *IV*DMD were analysed. There was an ecotype × phenological stage effect ($P < 0.05$) on the protein fractions. Fraction B2 was the most abundant, and *IV*DMD was greater in the regrowth stage vs. green bean. *IV*DMD was negatively correlated with B3, C and positively correlated with CP, A, B1 and B2. Estimation *IV*DMD = 53.46 + 2.078 (%A) - 2.31 (%C), $R^2 = 0.80$, $P < 0.05$. *IV*DMD increased as fraction a content increase and decrease fraction C. The outstanding ecotypes are 2012-11-29, H-1, 2013-01-27, A-1 and 2013-03-01, A-6.

Keywords: Leucaena, ecotypes, protein fractions, digestibility

1. Introduction
In the sub-humid tropics, because of poor forage quality, an alternative to achieve livestock sustainability is the use of tree legumes that supplement the nutritional value of grasses (Piñeiro-Vázquez *et al*. 2017; Ramírez-Avilés *et al*. 2018) [23, 25]. Legumes increase livestock productivity by three- to five-fold (Bacab *et al*. 2013; Best *et al*. 2017; Solorio *et al*. 2017) [2, 3, 28]. The legume yielding superior results has been *Leucaena*, notably *Leucaena leucocephala* leaves, which contain high concentrations of raw protein, energy, digestibility and palatability when compared with tropical grasses. This species provides a high forage rate with high leaf production. Pruning every 30 to 50 days represents 1620 and 2450 kg of dry matter (DM) ha^{-1}, respectively (de los Santos-Mayo *et al*. 2018) [7]. These characteristics combined with the low-cost production of *Leucaena leucocephala* confer its high potential for livestock production (Lagunes *et al*. 1999; Ibrahim *et al*. 2007) [18, 13], occupying a preponderant space in animal feed, even in Asia and Australia (Iraola *et al*. 2009; Ku *et al*. 2009; Meena Devi *et al*. 2013; Milera 2013; Hopkins *et al*. 2019) [14, 16, 20, 21, 12]. It mainly includes ecotypes that develop in regions of adverse conditions with high temperatures, extreme rainfall and low-fertility soils. In Mexico, only a few studies indicate changes in protein fractions and *in vitro* digestible dry matter (*IV*DDM) of *L. leucocephala* var. Cunningham (Ku *et al*. 2018) [17]. Moreover, there are currently no studies of these fractions in native ecotypes of *Leucaena* and their variation due to the phenological stage. This study sought to evaluate and compare the content of protein crude and protein fractions in leaves of nine native *Leucaena* sp. ecotypes from southern Mexico, and analyse their relationship with *IV*DMD in two phenological stages.

2 Materials and Methods
2.1 Origin of the samples and experimental design. Samples of leaves for the study were obtained from the Iguala Experimental Field (CEIGUA) of the National Institute of Agricultural and Livestock Forestry Research (INIFAP), located 2.5 km of the Iguala-Tuxpan Highway, northern Guerrero state (18°20'52.9" N latitude, 99°30'24.3" W longitude, 753 m above sea level). The climate of the region is classified as *Aw0*, warm with rains in summer (García 1981) [9]. It has a short rainy season from June to October, and the rest of the year is the dry period. The average annual rainfall is 1045 mm, which is obtained from an average of 87 days with rain. However, the annual evaporation is 2175 mm. The average maximum, minimum and average temperatures are 44.5, 18.0 and 33.7 °C, respectively. The soil is sandy clay texture, slightly compact, with 1% organic matter and pH 7.9–8.2 (Sánchez *et al*. 2018) [26]. Two years before this investigation in three states of southern Mexico, the seeds from nine native ecotypes of *Leucaena* sp.: H-15, F-10: Tuxtepec, Oaxaca (N 18° 5.474', W 96° 12.10'); 2012-12-19, A-1: Tuxtla-Gutiérrez, Chiapas (N 16° 43.987', W 93° 2.790'); 2012-11-29, A-1: Chiapas (N 16° 8.316', W 93° 48.164'); 2012-11-21, E-3: Huajintlan, Morelos (N 18° 36.267', W 99° 23.601'); 2013-03-21, A-6: Chilapa, Guerrero (N 17° 35.825', W 99° 8.821'); 2013-01-27, A-1: Guerrero (N 18° 35.679', W 99° 22.521'); 2012-11-30, 5-1: Riviera de la presa, Chiapas (N 16° 28.678', W 92° 50.967'); 2013-03-01, A-6: San Marcos, Guerrero (N 16° 46.352', W 99° 27.395'); 2012-11-29, H-1: Chiapas (N 16° 8.895', W 93° 11.440');

were collected. First, the seeds were germinated in storage. Once the seedling reached 15 cm in height, it was transplanted to a plastic bag (10 × 60 cm). After reaching a growth of 50 cm, 36 plants of each ecotype were transplanted to the previously prepared soil furrows with 1.5 m distance between them, and the same distance (2 m) between plants, in an experimental design of randomized blocks with three repetitions (plots of 8 m long × 5 m wide). Each plot had three rows and four *Leucaena* plants in each.

2.2.1 Field sampling
For the experiment, a pruning of the stems to 1.5 m height was previously carried out. Subsequently, after 4 months in the phenological phase of regrowth, the first sampling was made by cutting two branches of the central plants of each plot. Thus, approximately 400 g of wet forage composed of the petiole (<3 mm) and the leaflets were obtained at random from different, randomly-cut leaves on eight different branches per plot. After another 6 months, during February, when the plants manifested the phenological stage of green beans, another similar amount of leaves cut in different plants was harvested from those used in the previous sampling. The samples were obtained completely randomly in eight branches of the plants located in the centre of each plot.

2.3 Procedure and analysis
The identified samples were immediately transferred in coolers (approx. –3 °C) to the Bromatology Laboratory of the Animal Nutrition and Biochemistry Department, FMVZ-UNAM. Upon arrival at the laboratory, the samples were frozen (–20 °C) before dehydration by lyophilisation (EDWARDS Super Modulyo: Thames TW16 6AS United Kingdom). After drying, the particle size was reduced using a Thomas Wiley mill (1 mm sieve). Ground samples from each phenological phase were analysed for crude protein (CP) using the Kjeldahl method (AOAC 2000) [1]. In addition, the Cornell University net carbohydrate and protein system (CNCPS) fractionation was undertaken (Sniffen *et al*. 1992) [27] to determine the insoluble protein (IP) and true soluble protein (TSP) (Krishnamoorthy *et al*. 1982) [15], the protein insoluble in the neutral detergent fibre residue (NDFCP) and the protein insoluble in the acid detergent fibre residue (ADFCP) (Van Soest *et al*. 1991) [30]. With the results of these analyses, protein fractions A, B1, B2, B3 and C, were estimated according to the protocol proposed by Licitra *et al*. (1996) [19]. Samples were also analysed for *IV*DMD (%) using the two-phase technique of Tilley and Terry (1963) [29].

2.4 Statistical analysis
The results were analysed by ANDEVA for the experimental design mentioned above using a split-plot arrangement in which the large plot corresponded to the ecotype and the small plot to the different phenological stages. The following model was used:

$$Yijk = \mu + Bk + \alpha i + \delta ik + \beta j + (\alpha\beta)ij + \varepsilon ijk \quad (1)$$

Where, i is 1,2,3,..., 9 (ecotypes); j is 1.2, (phenological stages); k is 1,2,3 (repetitions); $Yijk$ is the response variable; αi is the effect of the ith major plot; Bk is the effect of the k-block; δik is the experimental error of the major plot (ecotypes) βj is the effect of the jth minor plot (phenological stages); $(\alpha\beta)ij$ is the effect of the major plot × minor plot interaction; εijk is the experimental error of the minor plot.

When a difference between treatments was observed ($P \leq 0.05$), the means were compared using Tukey's test. Pearson's multiple correlation test and linear regression analysis (SAS v. 9.2; SAS Institute, Cary, NC, USA) were also performed.

3. Results and discussion
3.1 Protein fractions
The amounts of CP, IP, TSP, NDFCP and ADFCP are shown in Table 1. In all these protein determinations, there was an effect ($P < 0.05$) of the ecotype × phenological stage interaction, indicating that these are the determining factors for protein content in each ecotype studied. Most of the ecotypes showed a higher amount of CP in the regrowth stage, and a lower concentration in the green bean stage ($P < 0.05$), except for the 2012-11-29, A-1 ecotype, which registered a higher CP value in this last stage (29.08%). In addition, it was the ecotype that manifested the highest ($P < 0.05$) amount of CP, indicating that there is always a determining genetic effect in each ecotype. This genetic expression results from the evolution of the original environment (nutrient-poor soil) the plant experienced, enabling them to develop in fertile soil, such as the Experimental Field INIFAP-SAGARPA, Iguala, Gro, Mexico. The lower amount of protein with increasing age, recorded by the majority of the native ecotypes of *Leucaena*, is similar to that reported by Olivos (2015) [22], who evaluated changes in the CP content of *L. leucocephala* var. Cunningham in crops at 6, 9 and 12 weeks in "La Posta" INIFAP, Paso del Toro, Veracruz, Mexico, and concluded that the CP content decreased linearly (P <0.05; 23.12%, 20.69% and 19.44%, respectively) as the legume aged. However, in the present investigation, the greater or similar amount of CP between ecotypes 2012-11-29, A-1 and 2012-11-21, E-3, in the mature stage of the plant indicates that the answer is genetically mediated in each ecotype, and is probably different because of the soil and environmental conditions in which each ecotype evolved. Regarding the IP content, all the ecotypes registered a greater amount in the green bean stage ($P < 0.05$) than the regrowth stage. The reason for the relatively greater amount of IP in the green bean stage is that this fraction contains the protein associated with the structural compounds of the plant cell wall, which increases as the legume matures. Regarding the TSP equivalent of fraction B1, all nine ecotypes displayed a greater amount of in the regrowth stage ($P < 0.05$), compared with the tender green bean stage. Again, Olivos (2015) [22] observed a similar trend of decrease in the amount of TSP with increasing age of the plant. It should be noted that the amount of TSP was lowest in the native *Leucaena* ecotypes, indicating that the cells of the leaves of this legume retain very little soluble protein. Preston and Leng (1990) [24] noted that 100% is used quickly in the rumen, giving rise to the branched-chain volatile fatty acids (VFAs) isobutyric acid, isovaleric acid, 2-methyl butyrate acid and valerate. The level of these acids in the rumen liquid is an indicator of the degradation of amino acids in the rumen because these are normally derived from the fermentation of valine, leucine, isoleucine and proline. Branched-chain VFAs are used by bacteria as growth factors. In temperate legumes, such as alfalfa, 100% of

fraction B1 is used in the rumen by microorganisms at a rate of degradability of 200–300%/h, based on the CNCPS sub-model (Sniffen *et al*. 1992) [27]. Chamorro (2005) [5] asserted that the inclusion of tree legumes in silvopastoral systems directly helps to improve the production and nutritional quality of associated grasses, mainly because it raises protein and mineral levels. The greatest amount of true protein is immediately gathered in the intracellular structures (organelles) of the leaves, which, when consumed by cattle, are digested by taking full advantage of the passage to the abomasum and small intestine of ruminants, except when the protein content bound to the cell wall (NDFCP) or the acid detergent fibre residue (ADFCP), in all the ecotypes, the amount was greater ($P < 0.05$) in the tender green bean stage relative to the regrowth stage (Table 2). In the tender green bean stage, the average proportion of NDFCP was 63% of the CP present in the ecotypes, whereas in the regrowth stage, it was only 13% of the CP. Both proportions are similar to those that Chamorro (2002) [4] reported in various tree legumes studied in Colombia. The importance of this fraction is that in the rumen, a high amount of this protein escapes ruminal degradation because it is shielded by the cell wall and passes to the intestine without modifications (that is why it is called protected or overpass protein). This protein, together with the microbial protein and the ration proteins, which due to their high solubility are not modified in the rumen and omasum, pass to abomasum and intestine where they are digested by the action of HCl and enzymes (pepsin, trypsin, chymotrypsin, carboxypeptidase and aminopeptidases), and constitute the metabolizable protein for the ruminant. According to Chamorro (2002) [4], 10–25% of the true fibre-bound protein in neutral detergent is digested in the rumen and the rest, as a protected or excess protein, passes to the small intestine where intestinal proteases digest 80% of that protein. As for the amount of ADFCP, which is equivalent to fraction **C** of the CNCPS, the average proportion of this fraction of protein in green beans was 28.39% (expressed as the percentage of the CP), and in the regrowth stage, it was much lower (4.65%). The importance lies in the fact that the ADFCP corresponds to the completely indigestible protein in ruminants, so it is not convenient to use the *Leucaena* native ecotype leaves when the plants have reached the stage of tender green beans. The amounts corresponding to the green bean stage were similar to the average ADFCP amounts recorded in *Trichanthera gigantea* and *Enterolobium cyclocarpum* of 8.81% and 8.83%, respectively, in a study by Chamorro (2002) [4] of several legumes used in Colombia. Olivos (2015) [22] determined ADFCP values of 3.90%, 3.13% and 2.34% in *L. leucocephala* var. Cunningham at 6, 9 and 12 weeks of regrowth, respectively, amounts slightly lower than those obtained at the regrowth stage of the present investigation. Table 3 presents the results of the protein fractions A, B1, B2, B3 and C, in the different native ecotypes of *Leucaena*. The statistical analysis revealed an effect ($P < 0.05$) of the ecotype × phenological stage interaction on all fractions recorded, but the main effects were be discussed separately to highlight important differences in the fractions that affect their use as a source of nutrients. For fraction A that represents the amount of non-protein nitrogen (NPN), all ecotypes recorded a greater amount ($P < 0.05$) in the regrowth stage than the tender green bean stage, it indicates the difference that exists among ecotypes to convert NPN into true protein, probably as a result of a genetically-regulated mechanism of greater adaptation to the geo-ecological conditions in which each ecotype develops. The results in the regrowth stage of the present study are similar to those that Olivos (2015) [22] recorded in *L. leucocephala* var. Cunningham. The relevance of this fraction is that when in the rumen, the microorganisms have at the same time an adequate amount of energy, so almost all this NPN is harnessed and transformed to microbial protein, which, when passed to the intestine, is digested and absorbed, constituting the metabolizable protein (amino acids), a comparatively more important protein source for ruminants. The proportion of fraction A (expressed as a percentage of the CP) in the present study was 38.03% in the regrowth stage and 2.84% in the green bean stage. In comparison, Fontenot (2002) [8] described young forages in the growth stage as containing up to 30% of the protein in the form of NPN, and Chamorro (2005) [5] in a study conducted in Colombia in *L. leucocephala* reported 21.50% NPN in fraction A. Regarding fraction B2 that corresponds to the true protein of slow ruminal degradation, the results (Table 3) indicate a decrease in the tender green bean stage compared with the amount in the regrowth stage ($P < 0.05$), except for the ecotype 11/29/2012, H-1, whose behaviour differed from the others. In the regrowth stage, the amount of B2 was equivalent to 40–50% of the total protein (CP), a proportion similar to that in *L. leucocephala* (Lam.) De Wit of 53.7%, which Gaviria *et al*. (2015) [10] detected when establishing the legume in a silvopastoral system together with two grasses. Sniffen *et al*. (1992) [27] reported, between 70 and 85% of fraction B2 is used in the rumen, and the remaining protein passes to the abomasum and small intestine where it is completely digested. Fraction B3 corresponds to the true protein that is not degraded in the rumen because of a shield or "protection" that constitutes the neutral detergent fibre of the plant cell wall, but when passing to the intestine, part of that neutral detergent fibre is solubilised by the acidic pH of the abomasum and then the alkaline pH of the duodenum, and, as a result, it releases B3 protein for intestinal digestion (Licitra *et al*. 1996) [19]. In the present investigation, differences in the concentration of B3 was observed among some of the *Leucaena* ecotypes (Table 2). A general trend of higher ($P < 0.05$) concentration in the tender green bean stage than the regrowth stage occurred, except for the ecotype 11/29/2012, H-1, which showed a low concentration in both phenological stages, confirming that the ecotypes show different B3 protein contents because of adaptation towards the geo-ecological conditions in which each one develops. In the present study, the proportion of B3 (expressed as a percentage of the CP) fluctuated between 11.41% and 33.67%. This proportion is lower than Chamorro (2002) [4] found in *L. leucocephala* in the Colombian tropics, which averaged 43.10% in all the species analysed. In contrast, Gaviria *et al*. (2015) [10] detected 8.2% B3 (expressed as a percentage of the CP) in *L. leucocephala*. According to Chamorro (2005) [5], in immature forages, between 10 and 25% of this fraction is degraded by ruminal microorganisms, and the remainder is passed to the abomasum and small intestine where the proteases digest 80% of this protein.

Table 1: Effect of ecotype and phenological stage on the total protein, insoluble protein, true soluble protein, the protein linked to the neutral detergent fibre residue and that linked to the acid detergent fibre residue, in leaves of nine *Leucaena* ecotypes of southern Mexico.

Ecotype	Phenological stage	CP g/100 g	IP g/100 g	TPS g/100 g	NDFCP g/100 g	ADFCP g/100 g
H-15, F-10	regrowth	$28.02^{a,b,c,A}$	$16.10^{f,B}$	$1.78^{a,A}$	$4.26^{e,B}$	$1.40^{d,B}$
	green bean	$26.12^{c,d,B}$	$22.52^{a,b,A}$	$0.59^{b,B}$	$15.91^{a,A}$	$7.88^{a,A}$
2012-12-19, A-1	regrowth	$28.67^{a,b,A}$	$17.44^{d,e,f,B}$	$1.66^{a,A}$	$4.96^{e,B}$	$1.28^{d,B}$
	green bean	$26.51^{b,d,c,B}$	$21.82^{a,b,A}$	$0.67^{b,B}$	$14.44^{a,b,A}$	$7.26^{a,A}$
2012-11-29, A-1	regrowth	$26.25^{c,d,B}$	$16.93^{e,f,B}$	$1.44^{a,A}$	$6.56^{d,e,B}$	$1.57^{d,B}$
	green bean	$29.08^{a,A}$	$22.75^{a,b,A}$	$0.53^{b,B}$	$13.83^{a,b,A}$	$8.05^{a,A}$
2012-11-21, E-3	regrowth	$27.65^{a,b,c,A}$	$16.86^{e,f,B}$	$1.61^{a,A}$	$5.08^{e,B}$	$1.52^{d,B}$
	green bean	$27.51^{a,b,c,B}$	$23.65^{a,A}$	$0.44^{b,B}$	$15.05^{a,b,A}$	$6.94^{a,A}$
2013-03-21, A-6	regrowth	$28.20^{a,b,c,A}$	$17.76^{d,e,f,B}$	$1.61^{a,A}$	$4.32^{e,B}$	$1.23^{d,B}$
	green bean	$23.01^{f,B}$	$19.37^{c,d,A}$	$0.53^{b,B}$	$9.69^{c,d,A}$	$5.25^{b,A}$
2013-01-27, A-1	regrowth	$26.60^{b,c,d,A}$	$17.32^{d,e,f,B}$	$1.66^{a,A}$	$5.07^{e,B}$	$1.40^{d,B}$
	green bean	$25.28^{d,e,B}$	$20.59^{b,c,A}$	$0.35^{b,B}$	$7.08^{d,e,A}$	$7.00^{a,A}$
2012-11-30, 5-1	regrowth	$25.68^{d,e,f,A}$	$15.75^{i,B}$	$1.27^{a,b,A}$	$4.37^{e,f,B}$	$1.22^{c,B}$
	green bean	$24.26^{d,e,f,B}$	$20.18^{bc,d,e,}$	$0.29^{c,B}$	$15.52^{a,b,A}$	$7.35^{a,b,A}$
2013-03-01, A-6	regrowth	$26.79^{b,c,d,A}$	$18.81^{c,d,e,B}$	$1.52^{a,A}$	$5.07^{e,B}$	$1.22^{d,B}$
	green bean	$23.61^{e,f,B}$	$19.51^{c,d,A}$	$0.17^{b,B}$	$11.72^{b,c,A}$	$3.79^{c,A}$
2012-11-29, H-1	regrowth	$26.92^{a,b,c,d,A}$	$16.13^{f,B}$	$0.55^{b,A}$	$3.50^{e,B}$	$1.22^{d,B}$
	green bean	$19.36^{g,B}$	$18.32^{d,e,f,A}$	$0.47^{b,B}$	$4.78^{e,A}$	$2.62^{c,d,A}$
SEM*		0.0052	0.0054	0.0002	0.0158	0.0022

CP - crude protein, IP - insoluble protein, TPS - true soluble protein, NDFCP - protein bound to neutral detergent fibre residue, ADFCP - protein bound to acid detergent fibre residue. * Standard error of the average. A–g - different letters per column indicate differences by ecotype effect ($P < 0.05$). A, B – different letters per column indicate differences by phenological stage effect ($P < 0.05$).

Table 2: Effect of ecotype and phenological stage on protein fractions and *in vitro* dry matter digestibility in leaves of nine *Leucaena* ecotypes of southern Mexico.

Ecotype	Phenological stage	Protein fraction g/100 g					*IV*DMD g/100 g
		A	B1	B2	B3	C	
H-15, F-10	regrowth	$10.1^{a,A}$	$1.78^{a,A}$	$11.8^{a,b,c,d,e,A}$	$2.9^{d,B}$	$1.4^{d,B}$	$66.6^{a,A}$
	green bean	$3.0^{e,f,B}$	$0.59^{b,B}$	$7.7^{e,B}$	$7.6^{a,b,c,A}$	$7.9^{a,A}$	$41.2^{c,d,e,B}$
2012-12-19, A-1	regrowth	$9.6^{a,A}$	$1.66^{a,A}$	$12.5^{a,b,c,d,A}$	$3.7^{c,d,B}$	$1.3^{d,B}$	$68.3^{a,A}$
	green bean	$3.4^{e,f,B}$	$0.67^{b,B}$	$8.3^{d,e,B}$	$5.5^{a,b,c,d,A}$	$7.3^{a,A}$	$40.7^{c,d,e,B}$
2012-11-29, A-1	regrowth	$7.9^{a,b,c,A}$	$1.44^{a,A}$	$10.4^{a,b,c,d,e,A}$	$5.0^{a,b,c,d,B}$	$1.6^{d,B}$	$68.4^{a,A}$
	green bean	$5.8^{c,d,e,B}$	$0.53^{b,B}$	$8.9^{c,d,e,B}$	$5.8^{a.b.c.d.A}$	$8.1^{a,A}$	$52.3^{b,B}$
2012-11-21, E-3	regrowth	$9.2^{a,b,A}$	$1.61^{a,A}$	$11.8^{a,b,c,d,e,A}$	$3.6^{c,d,B}$	$1.5^{d,B}$	$66.8^{a,A}$
	green bean	$3.2^{e,f,B}$	$0.44^{b,B}$	$9.2^{b,c,d,e,B}$	$8.3^{a,A}$	$6.9^{a,A}$	$44.9^{b,c,d,B}$
2013-03-21, A-6	regrowth	$8.8^{a,b,A}$	$1.61^{a,A}$	$13.5^{a,b,c,A}$	$3.1^{d,B}$	$1.2^{d,B}$	$69.9^{a,A}$
	green bean	$3.2^{e,f,B}$	$0.53^{b,B}$	$8.6^{d,e,B}$	$6.2^{a.b.c.d.A}$	$5.3^{b,A}$	$50.6^{b,c,B}$
2013-01-27, A-1	regrowth	$7.6^{a,b,c,A}$	$1.66^{a,A}$	$12.3^{a,b,c,d,A}$	$3.7^{c,d,B}$	$1.4^{d,B}$	$70.2^{a,A}$
	green bean	$3.4^{e,f,B}$	$0.35^{b,B}$	$11.9^{a.b.c,d,e.B}$	$4.2^{a,b,c,d,A}$	$7.0^{a,A}$	$40.9^{c.d,e,B}$
2012-11-30,5-1	regrowth	$8.7^{a,b,cA}$	$1.27^{a,b,A}$	$11.4^{a,b,c,d,e,A}$	$3.2^{b,c,d,B}$	$1.2^{c,B}$	$68.8^{a,A}$
	green bean	$3.8^{d,e,B}$	$0.29^{c,B}$	$4.7^{f,B}$	$8.2^{a,A}$	$7.4^{a,b,A}$	$48.4^{b,c,d,B}$
2013-03-01, A-6	regrowth	$6.5^{b,c,d,A}$	$1.52^{a,A}$	$13.7^{a,A}$	$3.9^{b,c,d,B}$	$1.2^{d,B}$	$70.4^{a,A}$
	green bean	$3.9^{d,e,B}$	$0.17^{b,B}$	$8.7^{d,e,B}$	$7.9^{a,b,A}$	$3.8^{c,A}$	$37.3^{d,e,B}$
2012-11-29, H-1	regrowth	$10.2^{a,A}$	$0.55^{b,A}$	$12.6^{a,b,c,d,A}$	$2.3^{d,B}$	$1.2^{d,B}$	$74.7^{a,A}$
	green bean	$3.6^{e,f,B}$	$0.47^{b,B}$	$10.5^{a,b,c,d,e,B}$	$2.8^{d,A}$	$2.6^{c,d,A}$	$32.5^{e,B}$
SEM*		0.0088	0.0002	0.0223	0.0186	0.0022	0.0015

*IV*DMD - *in vitro* dry matter digestibility. * Standard error of the mean. A–f – different letters per column indicate differences by ecotype effect ($P < 0.05$). A, B - different letters per column indicate differences by phenological stage effect ($P < 0.05$).

Table 3: Summary of the correlation between *IV*DMD and protein fractions in leaves of nine native *Leucaena* ecotypes from southern Mexico.

Variable	*IV*DMD		Variable	*IV*DMD	
	R^2	*P*		R^2	*P*
CP	0.3729	0.0055	A	0.7934	<0.0001
ADFCP	–0.8306	<0.0001	B1	0.7559	<0.0001
NDFCP	–0.7321	<0.0001	B2	0.5327	<0.0001
IP	–0.7749	<0.0001	B3	–0.3664	0.0064
TPS	0.7559	<0.0001	C	–0.8306	<0.0001

*IV*DDM - *in vitro* dry matter digestibility, CP - crude protein, ADFCP - protein bound to acid detergent fibre residue, NDFCP - protein bound to neutral detergent fibre residue, IP - insoluble protein, TPS - true soluble protein. A, B1. B2. B3, C - protein fractions.

3.2 In vitro dry matter digestibility

Table 2 shows the effect of the ($P < 0.05$) ecotype × phenological stage interaction on the *IV*DMD. In general, all ecotypes registered a higher percentage of *IV*DMD in the regrowth stage than in the green bean stage. It should be noted that when the leaves of the native *Leucaena* ecotypes pass from the regrowth stage to the green bean stage, their digestibility decreases approximately 28%. In the regrowth stage, the variation ($P < 0.05$) in *IV*DMD of the different native ecotypes of *Leucaena* is similar to the results of Guzmán *et al*. (2014) [11] in *L. leucocephala* var. Cunningham. In that study, *IV*DMD decreased linearly ($P < 0.001$) by an overall 8% when harvested at 6, 9 and 12

weeks of regrowth in "La Posta" INIFAP, Paso del Toro, Veracruz, Mexico (65.64%, 60.89% and 57.84%, respectively). A digestibility of over 65% indicates that the forage is not only a rich source of protein but also provides energy in an amount greater than needed for ruminant maintenance (Gaviria *et al*. 2015; de los Santos-Mayo *et al*. 2018) [10, 7]. The results of the correlation analysis between the *IV*DMD and protein fractions evidenced a positive correlation ($P < 0.05$) with CP, TSP, A, B1 and B2 fractions, indicating that the increase in the soluble fractions of the CP, including NPN (fraction A) increases *IV*DMD (Table 3). On the contrary, by increasing the age of the plant and moving from the regrowth stage to that of green beans, the IP, NDFCP, B3 and C fractions decrease digestibility. This last relationship is reasonable because these proteins are linked to the secondary cell wall, especially fraction C (ADFCP). This fraction mainly comprises crude lignin-bound protein in a series of compounds with a *p*-hydroxyphenyl nucleus, which, when presented in high quantity by themselves, reduces the digestibility (Church *et al*. 2010) [6]. The linear regression analysis indicated that it is possible to estimate the *IV*DMD efficiently ($P < 0.01$) from the following equation, with a high correlation coefficient ($R^2 = 0.8022$):

$$IVDDM\ \% = 53.46 + 2.08\ (\%\ A) - 2.31\ (\%\ C) \quad (2)$$

*IV*DMD increased as fraction a content increase and decrease fraction C. This equation better predicts digestibility (*IV*DMD) compared with the equation proposed by Chamorro (2002) [5] in a study that covered 19 species of fodder trees. When separately analysing fraction B3, the regression equation was $IVDMD\ (\%) = -0.721x + 83.93$, with a correlation coefficient (R^2) of 0.3827 ($P < 0.01$), but in the present study, the correlation with this protein fraction was $R^2 = -0.3664$ ($P < 0.001$). Conversely, when that researcher used fraction C of the protein, the *IV*DMD (%) was equal to $-1.4047C\% + 62{,}589$, with $R^2 = -0.4914$, $P < 0.001$), whereas in the present study, fraction C showed an R^2 of –0.8022 ($P < 0.01$).

4. Conclusion

In nine native *Leucaena* ecotypes of southern Mexico, the ecotype × phenological stage interaction was responsible for the changes in the contents of the protein fractions and *IV*DMD. The soluble fractions of the protein (A, B1, B2) decreased when advancing from the regrowth stage to the green bean stage. On the contrary, the insoluble fractions (B3 and C) increased with plant development towards maturity. In general, the most abundant fraction in the *Leucaena* ecotypes was fraction B2, corresponding to the protein of slow rumen degradability. In all ecotypes, the CP and fractions A, B1 and B2 positively affected *IV*DMD. Fractions B3, C, ADFCP, NDFCP and IP negatively affected *IV*DMD. The linear regression analysis indicated ($P < 0.01$) that in *Leucaena* ecotypes, it is possible to estimate *IV*DMD using the equation: $IVDMD = 53.46 + 2.08\ (\%A) - 2.31\ (\%C)$, with $R^2 = 0.8022$. The outstanding ecotypes to evaluate their productive behaviour in ruminants are 2012-11-29, H-1; 2013-01-27, A-1; and 2013-03-01, A-6.

5. Acknowledgments

Proyect IN226216 PAPIIT-DGAPA-UNAM

6. References

1. AOAC. Official methods of analysis of AOAC International. 17th ed. Horwitz W, editor. Gaithersburg (MD): AOAC International, 2000, 27-32.
2. Bacab HM, Madera NB, Solorio FJ, Vera F, Marrufo DF. Los sistemas silvopastoriles intensivos con *Leucaena leucocephala*: una opción para la ganadería tropical [The intensive silvopastoril [sic] systems with *Leucaena leucocephala*: tropical livestock option]. Rev Investig Agropecu. 2013; (17):67-81.
3. Best DA, Lara-Lara PE, Aguilar-Urquizo E, Cen-Chuc FE, Ku-Vera JC, Sanginés-García JR, *et al*. *In vivo* digestibility and nitrogen balance in sheep diets with foliage of fodder trees in substitution for soybean meal. Agrofor Syst. 2017; (91):1079-1085.
4. Chamorro D. Importancia de la proteína en la nutrición de rumiantes con énfasis en la utilización de proteínas de especies arbóreas. In: Memorias del Seminario Taller Internacional sobre manejo de la proteína en producción de Ganado bovino. Colombia: CORPOICA/ACCI/Ministerio de Agricultura y Desarrollo Rural, 2002.
5. Chamorro D. Producción de carne y leche con sistemas silvopastoriles en Colombia. Rev ACOVEZ, 2005; (34):5-9.
6. Church DC, Pond WG, Pond KR. Fundamentos de Nutrición y Alimentación de Animales. 2nd ed. México: Limusa, 2010.
7. de los Santos-Mayo S, Casanova-Lugo F, Díaz-Echeverria V, Chay-Canul AJ, González-Valdivia N, Piñeiro-Vázquez A, *et al*. Rendimiento y calidad del forraje de *Leucaena leucocephala* en dos sistemas silvopastoriles y dos frecuencias de poda [Forage yield and quality of *Leucaena leucocephala* in two silvopastoral systems and under two pruning frequencies]. In: Camacho JH, Canul AJC, Lugo FC, Vázquez ÁP, Benavides LM, Ferreyra ES, Menocal JA, editors. Avances de la investigación sobre producción animal y seguridad alimentaria en México. 1st ed. Morelia (México): Universidad Michoacana de San Nicolás de Hidalgo, 2018, pp. 579-584.
8. Fontenot J, Metabolismo y nutrición de los compuestos nitrogenados en los rumiantes. In: Church P, editor. Fisiología digestiva y nutrición de los rumiantes. México: Limusa, 2002, 211-217.
9. Garcia E. Modificaciones al Sistema de Clasificación Climática de Köppen (para adaptarla a las condiciones de la República Mexicana). 4th ed. México: UNAM, 1981.
10. Gaviria X, Rivera JE, Barahona R. Calidad nutricional y fraccionamiento de carbohidratos y proteína en los componentes forrajeros de un sistema silvopastoril intensivo [Nutritional quality and fractionation of carbohydrates and protein in the forage components of an intensive silvopastoral system]. Pastos y Forrajes. 2015; 38(2):194-201.
11. Guzmán SS, López GI, Mendoza LAH, Olivos AP, Corona GL, Castrejón PFA, *et al*. Composición nutrimental y digestibilidad *in vitro* de la materia seca en tres leguminosas por efecto de época y edad al corte. Memorias del XXXVIII Congreso Nacional de Buiatría; Jul 31–Aug 2; Villahermosa, Tabasco, México: AMVEB, 2014.
12. Hopkins K, Bowen M, Dixon R, Reid D. Evaluating

crude protein concentration of leucaena forage and the dietary legume content selected by cattle grazing leucaena and C_4 grasses in northern Australia. Trop Grasslands. 2019; 7(2):189-192.
13. Ibrahim M, Villanueva C, Casasola F. Sistemas silvopastoriles como una herramienta para el mejoramiento de la productividad y rehabilitación ecológica de paisajes ganaderos en Centro América. Arch Latinoam Prod Anim. 2007; 15(S1):74-88.
14. Iraola J, Muñoz E, Hernández JL, Moreira E. Efecto de una siembra simultanea de gramíneas y Leucaena sobre la producción y calidad de biomasa en un sistema silvopastoril. In: Memorias II Congreso sobre Sistemas Silvopastoriles Intensivos; Nov 3–5; Morelia y Tepalcatpec, México. Morelia (México): Fundación Produce Michoacán, 2009, pp. 31-37.
15. Krishnamoorthy U, Muscato TV, Sniffen CJ, Van Soest PJ. Nitrogen fractions in selected feedstuffs. J Dairy Sci. 1982; 65(2):217-225.
16. Ku VJ, Ramírez AL, Ayala BA, Cliay CA, Contreras HM, Pifieiro VA, *et al*. La nutrición animal con árboles forrajeros y la cartera de investigación en SSPi. In: Memorias II Congreso sobre Sistemas Silvopastoriles Intensivos; Nov 3–5; Morelia y Tepalcatpec, México. Morelia (México): Fundación Produce Michoacán, 2009, pp. 191-200.
17. Ku Vera JC, Piñeiro Vázquez AT, Valencia Salazar SS, Solorio Sánchez FJ, Aguilar Pérez CF, Ayala Burgos AJ, *et al*. Mitigación de las emisiones de metano entérico en rumiantes alimentados con leguminosas tropicales [Mitigation of enteric methane emissions in ruminants fed with tropical legumes]. In: Camacho JH, Canul AJC, Lugo FC, Vázquez ÁP, Benavides LM, Ferreyra ES, Menocal JA, editors. Avances de la investigación sobre producción animal y seguridad alimentaria en México. 1st ed. Morelia (México): Universidad Michoacana de San Nicolás de Hidalgo, 2018, pp. 303-312.
18. Lagunes FIJ, Fox DG, Blake RW, Pell AN. Evaluation of tropical grasses for milk production by dual-purpose cows in tropical Mexico. J Dairy Sci, 1999; (82):2136-2145.
19. Licitra G, Hernandez TM, Van Soest PJ. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim Feed Sci Technol. 1996; 57(4):347-358.
20. Meena Devi VN, Ariharan VN, Nagendra Prasad P. Nutritive value and potential uses of *Leucaena leucocephala* as biofuel–a mini review. Res J Pharm Biol Chem Sci. 2013; 4(1):515-521.
21. Milera M. Contribución de los sistemas silvopastoriles en la producción y el medio ambiente [Contribution of silvopastoral systems to production and environment]. Av Investig Agropecu. 2013. 17(3):7-24.
22. Olivos AP. Efecto de época y edad al corte sobre las fracciones de la proteína en leguminosas introducidas a Veracruz. Tesis de Licenciatura. México: FVMZ, UNAM, 2015.
23. Piñeiro-Vázquez AT, Jiménez-Ferrer GO, Chay-Canul AJ, Casanova-Lugo F, Díaz-Echeverría VF, Ayala-Burgos AJ, *et al*. Intake, digestibility, nitrogen balance and energy utilization in heifers fed low-quality forage and *Leucaena leucocephala*. Anim Feed Sci Technol, 2017; (228):194-201.
24. Preston TR, Leng RA. Ajustando los sistemas de producción pecuaria a los recursos disponibles: Aspectos básicos y aplicados del nuevo enfoque sobre la nutrición de rumiantes en el trópico. Colombia: CONDRIT, 1990.
25. Ramírez-Avilés L, Sánchez FJS, Pérez CFA, Burgos AJA, Ku Vera JC. Sistemas ganaderos sostenibles en el trópico: retos y oportunidades [Sustainable tropical livestock systems: challenges and opportunities]. In: Camacho JH, Canul AJC, Lugo FC, Vázquez ÁTP, Benavides LM, Ferreyra ES, Menocal JA, editors. Avances de la investigación sobre producción animal y seguridad alimentaria en México. 1st ed. Morelia (México): Universidad Michoacana de San Nicolás de Hidalgo, 2018, Pp: 171-176.
26. Sánchez A, Hernández H, Carrillo S, Jiménez R, Corona L, Rosiles R, *et al*. Correlación entre contenido de elementos minerales y digestibilidad *in vitro* de la MS en ecotipos nativos de leucaena del sur de México [Correlation between mineral elements content and *in vitro* digestibility of dry matter in native ecotypes of Leucaena of southern Mexico]. In: Camacho JH, Canul AJC, Lugo FC, Vázquez ÁP, Benavides LM, Ferreyra ES, Menocal JA, editors. Avances de la investigación sobre producción animal y seguridad alimentaria en México. 1st ed. Morelia (México): Universidad Michoacana de San Nicolás de Hidalgo, 2018, Pp: 585-589.
27. Sniffen CJ, O'Connor JD, Van Soest PJ, Fox DG, Russell JB. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J Anim Sci. 1992; (70):3562-3577.
28. 28 Solorio SFJ, Wright J, Franco MJA, Basu SK, Sarabia SL, Ramírez L, Ayala BA, Aguilar PC, Ku VJC. Silvopastoral systems: best agroecological practice for resilient production systems under dryland and drought conditions. In: Ahmed M, Stockle CO, editors. Quantification of climate variability, adaptation and mitigation for agricultural sustainability. Cham (Switzerland): Springer. 2017. Pp: 233–250.
29. Tilley JMA, Terry RA. A two-stage technique for the *in vitro* digestion of forage crops. Grass Forage Sci. 1963; 18(2):104-111.
30. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 1991; 74(10):3583-3597.