Short Communication
Range Mgmt. & Agroforestry 41 (1) : 173-177, 2020
ISSN 0971-2070

Effect of altitude on nutritional profile of white clover (*Trifolium repens* L.)

Rajan Katoch* and Ankur Tripathi
Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur-176062, India
*Corresponding author e-mail: rajankatoch@yahoo.com
Received: 28th February, 2019 Accepted: 8th March, 2020

Abstract
In the present study, white clover from different altitudes in mid Himalayas was investigated for nutritive profile. Protein content revealed variation from 18.66 to 24.38% with altitude. The lowest protein content was observed in white clover from low elevations. The fiber content (NDF and ADF) and cell wall constituents (ADL, hemicelluloses and cellulose) were observed inversely related to altitude. The cell content, total ash and reducing sugars increased from 32.47 to 40.37%, 8.23 to 10.46% and 89.10 to 53.32 mg/g, respectively with increase in elevation. Total phenols, simple phenols and tannins were also inversely related (1.84 to 0.82%, 0.81 to 0.31% and 1.03 to 0.51%, respectively) with altitude. However, hydrocyanic acid (HCN) content in white clover plants followed the reverse trend. HCN content increased from 0.360 to 1.044mg/g on dry matter basis from lower to higher elevation. The study revealed that white clover from higher altitudes is nutritionally superior as compared to lower altitudes; however, elevated levels of HCN content at higher altitudes affect the feeding value of white clover.

Keywords: Anti-nutrients, Forage legume, Hydrocyanic acid, Nutritive profile, White clover

Forage legumes are important constituents of ruminant diets due to high biomass yield, excellent source of proteins, carbohydrates, minerals, and other essential nutrients (Khatta *et al.,* 1999; Sharma *et al*., 2000; Ruckle *et al.,* 2017). The supplementation of forage legumes to low quality roughages enhances their nutritional status by providing sufficient quantity of essential nutrients. Among forage legumes, white clover (*Trifolium repens* L.) has been recognized as one of the excellent forage legume of temperate region, where it is contributing sustainability and reducing input costs for forage production (Roder *et al.,* 2007). Originated in Europe, white clover is extensively used forage legume in all temperate countries. Although white clover is a perennial legume component of temperate pasture systems (Nichols *et al.,* 2016), it also thrives well in cold subtropical regions. The optimum temperature for white clover growth is about 24°C, but growth rate remains fairly constant well below and above the optimum temperature (Mitchell, 1956). White clover has excellent nutrient profile with 25.87% crude protein, 10.32% ash, 20.93% cellulose, 9.70% hemicellulose, 76.05% reducing sugars (Rattray and Joyce, 1974; Hamdy *et al*., 2009). In temperate and cold subtropical regions, white clover has potential to revitalize the permanent pasture system (Frame *et al.,* 1998). Despite the excellent nutritional potential, presence of certain aversive factors inhibit the action of digestive enzymes, form insoluble protein complexes, decrease the bioavailability of essential nutrients (phytates, tannins, polyphenols) and impair hydrolytic functions of enzymes. Cyanogenesis, the ability of plant to synthesize cyanogenic glucosides has major concern with white clover feeding (Papalauskiene and Sprainaitis, 2003). These glucosides constitute a relatively polar, water soluble group of secondary metabolites which liberate noxious hydrocyanic acid (HCN), glucose, and aldehydes on hydrolysis with â-glucosidase in the gastrointestinal tract of ruminants (Stochmal and Oleszek, 1997; Patel *el al.,* 2013). These degradation products have toxic effects on ruminants as they suffer from direct and indirect effects of cyanide on various metabolic processes especially electron transport chain (Lehmann *et al.,* 1991; Crush and Caradus, 1995). Excessive consumption of cyanide ions inhibits the action of cytochrome oxidase in respiratory chain and lead to death due to unavailability of oxygen and energy (Montgomery, 1980). Several reports indicated that forage quality of white clover depends upon plant maturity and prevailing environmental conditions during active growth (Panahi *et al.,* 2012; Milosevic *et al.,* 2013). On maturity, the nutritional quality and digestibility of forages decreases due to accumulation of low energy digestible dry matter. The environmental factors especially geographical position and prevailing temperature during growth have significant influence on forage quality. High temperature during active growth alters the levels of fiber content, cell wall constituents and soluble carbohydrates which in turn decrease the forage digestibility (Pearson and Ison, 1997). Buxton and Fales (1994) suggested that each 1°C rise in temperature generally decreases the forage digestibility from 0.3% to 0.7%. In hilly areas, with the increase in elevation, the temperature of region decreases and vice versa. Therefore, forages grown in higher altitudes are more nutritious and digestible in comparison to those from lower altitudes (Tenikecier and Ates, 2019). Considering the importance of white clover in ruminant feeding in hilly regions, present study was undertaken to investigate the effect of altitude on nutritional composition of white clover in mid Himalayas of Himachal Pradesh.

Fresh white clover samples were collected from different elevations during second week in the month of February. The samples were oven dried at 50ºC, ground to fine powder and stored in airtight bags to avoid direct contact with moisture. Nitrogen content was measured by micro-kjeldahl method (AOAC, 1970). The crude protein was determined by multiplying the %N with the factor 6.25. This is from the assumption that nitrogen is derived from protein containing 16% nitrogen. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), hemicellulose and cellulose content in white clover plants were determined according to the method of Van Soest and Wiens (1967). The ash content was determined following AOAC method (2000). Phenolic content was estimated by the method given by Makkar (2003) using tannic acid (0.5 mg/ml) as standard. Reducing sugars were calculated by the method given by Miller (1959). The level of hydrocyanic acid (HCN) in white clover plants was determined by the procedure described in AOAC (1990). 1gm powdered sample was soaked in 10 ml of distilled water for 4hr and then filtered. The resulting solution was steam distilled into dilute NaOH solution (2.5%) and then treated with dilute KI (5%) and ammonium hydroxide (6N). The distillate was titrated against 0.02N $AgNO_3$ [1ml of 0.02N $AgNO_3$ = 1.08mg of HCN (1Ag equivalent to 2CN)] to observe faint but permanent turbidity against a black background as end point.

The present study revealed the influence of altitude on nutritional composition of white clover collected from different locations under mid hill regions of Himachal Pradesh (Table 1). The nutritive value of forage largely determines the daily performance of livestock by providing essential nutrients such as proteins. In the study, crude protein revealed variation from 18.66 to 24.83% in white clover sampled from different altitudes. The lower levels of crude protein content at lower altitudes probably attributed to increased proportion of stem to whole plant (Mohebi *et al.*, 2016). Marten (1970) also observed that increase in temperature at lower altitude reduced the crude protein concentration in alfalfa (*Medicago sativa*).

The ADF and NDF contents in white clover revealed variations with altitude. The ADF content was high (49.73%) at lower altitude as compared to higher altitude (46.60%). Similarly, white clover from lower altitude revealed high NDF content (67.53%) as compared to those from higher altitudes (59.06%). At lower altitude, high temperature increases the synthesis of different cell wall constituents which ultimately affects forage digestibility (Sarwar *et al.*, 1991; Buxton and Fales, 1994; Buxton, 1995). Study revealed that ADL content in white clover plants increased from 1.3 to 4.73%, while hemicellulose content increased from 14.57 to 17.80% at lower altitudes. This might be associated with the increased partitioning of cellulose and hemicelluloses content for lignification (Cone and Engels, 1990; da Silva *et al.*, 1987). Wilson *et al.* (1991) also reported that tropical and temperate forage species under high temperature regime showed increased rate of lignification. Deinum *et al.* (1981) suggested that at higher altitudes, lignification in forages may not keep pace with stem development, thereby results in higher digestibility. White clover collected from higher altitudes revealed higher levels of cell content (40.37%) as compared to those from lower altitude (32.47%). At lower elevations, higher temperature reduces the concentration of water soluble carbohydrates and affects dry matter digestibility (Marten, 1970). Nelson and Moser (1994) also reported that at higher altitudes, cell content (highly digestible carbohydrates) produced from photosynthesis accumulates due to low temperature sensitivity of photosynthesis.

Ash content represents inorganic residues in forages and provides a measure of total amount of minerals. McDowell (2003) reported that concentration of minerals in forages depends on the interaction of a number of factors including soil, plant species, maturity stage, yield, and climatic conditions. In this study, total ash content in white clover reduced from 10.46 to 0.36% with the increase in altitude which might be attributed to high soil moisture content as well as to increased activity of phosphate solublizing bacteria in plant rhizosphere (Selvakumar *et al.*, 2009). The reducing sugar content was increased from 53.32 to 89.08 mg/g of dry matter

Table 1. Nutritional profile of white clover (*Trifolium repens* L.) from different altitudes of mid Himalayas

Location	Elevation (m)	CP (%)	ADF (%)	NDF (%)	ADL (%)	HC (%)	Cell content (%)
Kandi	1581	21.23±0.89	44.60±0.40	59.63±0.60	1.30±0.26	15.03±0.60	40.37±0.61
Kandbari	1471	20.18±0.92	44.86±0.50	59.74±0.60	1.93±0.23	14.87±0.11	40.26±0.60
Bandla	1392	20.41±1.28	45.20±0.93	60.13±0.13	2.20±0.35	14.93±2.60	39.87±0.13
Uttrala	1379	19.83±0.56	47.13±0.23	62.13±0.03	2.30±0.26	15.00±0.21	37.87±0.03
Jiya	1340	20.53±0.64	48.06±0.31	63.19±0.19	2.40±0.20	15.12±0.50	36.81±0.19
Chauntra	1305	19.13±0.88	48.13±1.00	63.39±0.30	2.53±0.31	15.26±1.19	36.61±0.26
Holta	1231	24.38±1.06	48.73±0.93	64.06±0.20	2.46±0.31	15.33±1.40	35.94±0.20
Jogindernagar	1198	22.86±0.26	48.80±0.87	64.46±0.20	3.40±0.20	15.66±1.80	35.54±0.20
Menjha	1093	23.45±0.77	49.06±0.31	64.79±0.50	3.56±0.21	15.72±0.40	35.21±0.26
Paprola	975	20.30±0.95	49.13±0.64	64.99±0.50	4.36±0.38	15.86±0.90	35.01±0.50
Malan	951	19.01±0.18	49.20±0.40	65.66±0.90	4.46±0.23	16.46±1.00	34.34±0.50
Mahakal	904	21.11±0.88	49.26±0.31	66.72±0.32	4.60±0.20	17.45±0.70	33.28±0.32
Nagrota	857	18.66±0.89	49.73±0.12	67.53±0.53	4.73±0.12	17.80±0.54	32.47±0.53

Location	Cellulose (%)	RS (mg/g)	Ash (%)	TP (%)	SP (%)	TT (%)	HCN (mg/g)
Kandi	43.30±0.17	89.08±3.28	10.46±0.31	0.82±0.03	0.31±0.01	0.51±0.03	1.04±0.06
Kandbari	42.93±0.70	84.39±1.46	10.20±0.20	0.93±0.01	0.33±0.01	0.60±0.03	0.86±0.11
Bandla	43.00±2.50	80.75±1.14	9.63±0.15	0.99±0.01	0.34±0.01	0.65±0.01	0.82±0.16
Uttrala	44.83±0.21	74.38±2.15	9.53±0.15	1.02±0.01	0.35±0.02	0.67±0.03	0.82±0.06
Jiya	45.66±0.50	73.78±1.15	9.46±0.31	1.06±0.08	0.36±0.04	0.70±0.08	0.79±0.06
Chauntra	45.60±1.06	70.14±3.70	9.36±0.15	1.16±0.01	0.51±0.09	0.65±0.02	0.76±0.11
Holta	46.26±1.79	64.69±1.39	9.33±0.31	1.27±0.01	0.53±0.05	0.74±0.02	0.76±0.19
Jogindernagar	45.40±1.40	59.39±2.92	8.66±0.06	1.32±0.02	0.54±0.01	0.78±0.02	0.65±0.19
Menjha	45.50±0.36	56.15±3.87	8.53±0.31	1.47±0.01	0.59±0.03	0.88±0.01	0.61±0.06
Paprola	44.76±0.55	55.90±1.64	8.46±0.35	1.50±0.01	0.65±0.04	0.85±0.01	0.58±0.12
Malan	44.73±0.61	54.23±5.69	8.36±0.15	1.49±0.02	0.69±0.01	0.80±0.03	0.54±0.11
Mahakal	44.66±0.31	53.63±1.37	8.33±0.15	1.62±0.04	0.71±0.01	0.91±0.05	0.50±0.06
Nagrota	45.00±0.36	53.32±2.68	8.23±0.25	1.84±0.03	0.81±0.03	1.04±0.01	0.36±0.12

Mean of 3 values ± standard deviation; ADF: Acid detergent fiber; ADL: Acid detergent lignin; CP: Crude protein; HC: Hemi-cellulose; HCN: Hydrocyanic acid; NDF: Neutral detergent fiber; RS: Reducing sugar, SP: Simple phenol; TP: Total phenol; TT: Total tannin

with altitude. The elevated level of reducing sugars at higher altitude indicates the importance of carbohydrate reserve for winter survival in white clover (Boller and Nosberger, 1983). Oleberg (1956) also suggested that low temperature initiates the transformation of starch into sugars for continuation of various metabolic processes in plants.

Anti-nutrients interfere with the nutritive value of the forages. Phenolics and tannins are the important anti-nutritional factors in forages, which lower the nutritional quality, digestibility, and absorption of nutrients from forages. In the present study, white clover collected from lower altitudes revealed high levels of total and simple phenols (1.84% and 0.80%, respectively). Tannins in forages are known to have adverse effect on the digestibility of dietary protein and to a lesser extent, on the availability of carbohydrates and lipids (Mosely and Griffiths, 1979). These also affect the palatability of forages by forming complexes with salivary glycoproteins. Total tannins in white clover ranged from 0.47 to 1.04% from higher to lower altitudes. The increased concentration of polyphenolic compounds in white clover at lower altitudes revealed increased activity of enzymes involved in the formation of shikimic acid pathway intermediates involved in alleviation of climatic stresses and to deter herbivores from feeding. Lees *et al*. (1994) reported that high temperature increased the concentration of tannins in the leaf tissue of big trefoil (*Lotus pedunculatus)*. The present study revealed a direct relationship between accumulation of hydrocyanic acid and altitude. At higher altitude, white clover revealed higher level of hydrocyanic acid content (1.044 mg/g of dry matter) as compared to those from lower altitude (0.36 mg/g of dry matter). The elevated level of HCN in white clover at higher altitude is the probable way of desisting insect-pest attack (Stochmal and Oleszek 1997). Stochmal and Oleszek (1997) also suggested that

HCN content in white clover decreased gradually with the increase in temperature. Lindroth *et al.* (2000) reported that at higher altitudes, ultraviolet-B radiation also increased the rate of hydrocyanic acid accumulation in plants.

The study revealed that environmental factors had significant impact on the nutritional quality of white clover. White clover plants grown in colder or higher altitudes zones had better nutritional profile as compared to those from lower altitudes. Anti-nutritional factors excluding HCN content had inverse relationship with altitude. White clover plants at higher altitude, exceeded the critical level (0.37 mg HCN/g of dry matter; Lehmann *et al.,* 1990), and therefore, excessive feeding of white clover from higher altitudes needs to be monitored to avoid cyanide poisoning on livestock.

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