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VARIABILITY IN HCN CONTENT OF WHITE CLOVER GROWING IN DIFFERENT ALTITUDINAL ZONES OF NEPAL

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ABSTRACT

Plants contain cyanogenetic glucoside and also the appropriate enzyme which hydrolyses this glucoside and produces HCN. There are different types of plants which contain: the glucoside but not the enzyme; the enzyme but not the glucoside; and neither the glucoside nor the appropriate enzyme. *Trifolium repens* L. (White clover) is polymorphic for cyanogenesis, with both cyanogenic and acyanogenic plants occurring in nature. The study was done in eight different varieties of *Trifolium repens* L. collected from different altitudinal zones such as subtropical representing Kirtipur (1245-1300 masl), subtropical to temperate representing Dhunche (1962 -2012masl) and temperate to sub-alpine representing Chandanbari (3400-3500masl). Plants collected from these areas were tested for HCN contents by "Feigl spot test" method. The results showed that highest quantity of HCN was present in WNML, medium in WPML and minimum in LPML variety growing at Kirtipur. Medium quantity of HCN was present in WNML, WPML, WPNML and PNML varieties growing at Dhunche. PML and LPNML varieties have no HCN and were suggested as the best fodder plants for livestock. HCN contents in plants growing in Chandanbari were very low or absent. Similarly, the transplant from Chandanbari to Kirtipur also did not show HCN except a medium amount in PNML variety. The study showed a marked variation in HCN content among the varieties of white clover. It also revealed that the higher amount of HCN was present in white coloured flower than pink coloured flower; HCN content of plant is lesser in colder and higher altitudinal zone and is higher in plant with larger size of the leaves as well as in plants growing in lower moisture and higher phosphorous containing soil.

Keywords: HCN, White clover, Flower colour, Altitude, Moisture, Phosphorous

INTRODUCTION

Varieties of white clover in Nepal *Trifolium repens* L. (white clover), is an allotetraploid, from the cross of putative parents T. pallescenes and T. occidentale (Richards, 2011). *Trifolium repens* L. is a legume of moist temperate pastures and one of the most important forage crops containing high protein and minerals than any other grass and other species of *Trifolium*. It is commonly used in renovation of permanent pastures lacking legumes in temperate regions, Mediterranean areas and some cool subtropical parts of the world (Frame et al., 1998). It fixes atmospheric nitrogen due to the presence of nitrogen fixing bacteria in the root nodules and improves feed quality. *Trifolium repens* was first introduced in Nepal during 1860 AD. Subsequently it was introduced in different parts of the country as a ground fodder for pasture land in government as well as in private dairy farms due to its value as a good fodder. Different white clover varieties from eight different countries have so far been introduced and believed that they had freely crossed with one another. Eight varieties of white clover were presently identified in Nepal (Shrestha, 2013; Table1).

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HCN content of plants

The release of HCN from damaged leaves is known as Cyanogenesis. White clover leaves contain two cyanogenic glucosides e.g. linamarin and lotaustralin. It also contains the enzyme known as linamerase. When the leaves are damaged e.g. on grazing by animals, the glucosides are hydrolysed by linamarase to produce glucose and an unstable aglycone. The aglycone dissociates to release HCN and acetone or 2-butanone (Hart, 1987). The level of glucosides in the foliage may be sufficiently high for grazing animals to suffer from cyanide poisoning. Cyanogenesis is regarded as a protection against predators, herbivores, especially insect pests, slugs and snails. Cyanogenic glucosides in plants act as a defensive mechanism against feeding by some herbivores (Richard, 1973). Acyanogenic white

clover may be favoured under certain climatic or edaphic conditions. Cyanogenesis is controlled by two independently segregating genes: Ac/ac controls the presence/absence of cyanogenic glucosides; and Li/li controls the presence/absence of their hydrolysing enzyme, linamarase. Whereas Li is well characterized at the molecular level, Ac has remained unidentified. The nature of these polymorphisms may reflect white clover's evolutionary origin as an allotetraploid derived from cyanogenic and acyanogenic diploid progenitors (Kenneth et. al., 2008; Kenneth et al., 2013).

MATERIALS AND METHODS

Study areas

Different varieties of white clover were collected from different altitudinal areas of Nepal such as Kirtipur in Kathmandu district, Dhunche and Chandanbari in Rasuwa district. Kirtipur represents the sub-tropical zone at 1245-1300 metre at sea level (masl) altitude. Dhunche represents sub-tropical to sub-temperate zone at 1962-2012 masl altitude. Chandanbari represents temperate to sub-alpine zone at 3400-3500 masl altitude.

Plant collection

Fresh and healthy plants were collected for HCN test from Kirtipur. Different varieties of white clover were brought from Dhunche and Chandanbari and grown in pots containing soil from Kirtipur at 1300 m altitude. The growth and flowering of every transplant were monitored for three consecutive years. So, fresh and healthy transplants from Dhunche and Chandanbari were collected for HCN test. HCN test was carried out in Kirtipur as well as in Dhunche.

Method of HCN test

Hydrocyanic acid (HCN) test was carried out in different varieties of white clover by "Feigl spot test" method (Feigl and Anger, 1966).

Preparation of test paper

100gm. each of Copper (II) ethyl acetoacetate and 4, 4'- Methylenebis (N, N-dimethylaniline), were dissolved separately in 10 ml of chloroform in two beakers. These two solutions were mixed together. The test paper was made by saturation of a sheet of filter paper (Whatman No.1) with the mixed (1:1) solution and was dried at room temperature. If the colour of the paper did not change, it was cut into small strips measuring 6 cm in length and half cm width. The test papers were preserved in a clean and dry amber bottle with lid for few months.

Testing plants for HCN

Three healthy and similar sized leaflets of each variety of white clover were collected for the test. It was kept at the centre of the sterilized dry vial. The leaflets were crushed and squeezed by a sterilized dry glass rod. Immediately a strip of test paper was inserted into the vial, without touching the leaf sample and kept hanging with the help of rubber lid of the vial. The vial was then kept in a dark place or away from the sunlight and the time was noted down. The procedure was repeated five times for each variety of white clover. The time when the colour of the test paper changed was noted. If the colour of test paper is changed into blue, it indicated a positive result for HCN content i.e. the plant was cynophoric or cyanogenic, while the test paper with no colour change indicated a negative result or absence of HCN content i.e. the plant was non-cynophoric or acyanogenic (Photograph 1).

Calculations of HCN

The HCN content calculation is shown in table 2.

RESULTS AND DISCUSSION

The presence or absence of hydrocyanic acid (HCN) content was tested in different varieties of *Trifolium repens* growing in different altitudinal zones of Nepal such as Kirtipur , Dhunche and Chandanbari (Table 3, Photograph 1). The results based on calculations as on table 2 (Table 3, figure 1) are shown below:

- WML variety of white clover growing at Kirtipur and Dhunche do not contain HCN while it showed maximum quantity of HCN when the plant transplanted from Dhunche to Kirtipur were tested. However, the specimen transplanted at Kirtipur from Chandanbari also did not show HCN.
- WNML variety of white clover from Kirtipur and the same variety transplanted at Kirtipur from Dhunche showed maximum quantity of HCN content while it is at a medium range in the same type growing at

Dhunche. However, the transplants from Chandanbari did not contain HCN.

- WPML variety of white clover growing at Kirtipur and Dhunche contained a medium quantity of HCN while those varieties transplanted at Kirtipur from Dhunche and Chandanbari did not contain HCN.
- WPNML variety of white clover growing at Kirtipur did not contain HCN while it contained medium quantities of HCN at Dhunche. The same variety when transplanted at Kirtipur from Dhunche and Chandanbari did not show HCN content.
- PML variety of white clover growing at Kirtipur and Dhunche as well as those varieties transplanted at Kirtipur from Dhunche and Chandanbari did not contain HCN at all.
- PNML variety of white clover from Kirtipur did not contain HCN while this variety in Dhunche showed a medium amount. PNML variety transplanted at Kritipur from Dhunche also did not show HCN while those transplanted from Chandanbari contained a medium amount of HCN.
- LPML and LPNML varieties of white clover, only from Kirtipur were analysed. LPML contained a minimum amount of HCN while LPNML did not contain it at all.

HCN content of different varieties of white clover at Kirtipur and Dhunche shows significant variations among themselves. WML and PML varieties of white clover growing at Kirtipur and Dhunche do not show HCN content. So these varieties are referred to be good fodder plants for livestock. Acyanogenic plants have advantage of being less injured by frost than cyanogenic plants (Daday, 1965) and therefore this could be an adaptation of plants growing in colder and higher areas where frost is frequent.

The transplant of WML variety from Dhunche to Kirtipur showed HCN content. It might be due to water stress because Kirtipur is drier than Dhunche while this variety was growing in the soil of low phosphorous (Shrestha Malla, 2002). HCN content in white clover were found to increase by moisture stress, low light intensity, cool grazing condition and low soil-phosphorous supply (Vickery et al. 1987). The transplant of PML from Dhunche and Chandanbari to Kirtipur has no HCN content. PML variety does not contain HCN at all. So PML variety is one of the best fodder plants for livestock. It might be due to smaller size of leaflets. There is a positive association between increasing leaf size and cyanogenesis levels for most of the New Zealand white clover cultivars (Caradus et al., 1996).

Maximum quantity of HCN was present in WNML variety growing at Kirtipur and medium quantity at Dhunche. The transplant from Dhunche to Kirtipur showed maximum HCN, however the transplant from Chandanbari to Kirtipur does not show HCN. Maximum HCN present in WNML growing at Kirtipur was due to moisture stress, low soil phosphorous on which it grows and due to large size of leaflets. Similarly medium content of HCN present in WNML variety at Dhunche was due to medium content of phosphorous (Table 4). Cyanogenic white clover populations were larger leaved, medium productive, and more persistent than acyanogenic populations (Doak, 1933; Foy and Hyde, 1937). On the other hand, an American study of diverse germplasm showed cyanogenic plants to be larger in diameter but shorter, earlier flowering and less persistent than acyanogenic plants (Paim and Dean, 1976).

WPML variety of white clover has medium content of HCN at Kirtipur and Dhunche areas due to large size of leaflets. WPNML variety actually is a good fodder plant for Kirtipur, as it has no HCN content. However WPNML variety has medium content of HCN at Dhunche. It might be due to cool grazing condition of the area. PNML variety also seems good due to absence of HCN at Kirtipur because it has small leaflets size and growing in soil with low phosphorous and high moisture content. However it has medium content of HCN at Dhunche, it might be due to larger leaflets compared to Kirtipur, growing in soil with low moisture and medium amount of phosphorous (Table 4). HCN content in LPML is negligible or low and LPNML variety has no HCN content at all. So, it showed that these varieties were good for cattle feedings.

HCN study in White clover at Kirtipur and Dhunche revealed that maximum HCN was found at Kirtipur and medium at Dhunche and to be low or absent at Chandanbari, but HCN study could not be conducted at Chandanbari. Meanwhile, the transplant of white clover from Chandanbari to Kirtipur also did not show HCN except for the PNML variety. The study also showed that HCN content in plants was

higher in lower altitude zone while it was lower in higher altitude zone. HCN content of white clover showed that it also depends upon altitude i.e. there was a negative correlation between HCN content of plants and altitude. The gene frequencies for the genes for glucoside and enzyme were 100% in warm regions and decreased to 0 % in cold northern latitudes or at high elevations (Leffel and Gibson, 1973). De Araujo, and Aldo (1976); Brighton and Horne (1977) also showed that the frequency of cyanogenic plants decreased at high altitudes. The proportion of cyanogenics showed a striking reduction with increasing altitude. Plants growing below 100 m, north-facing sites contained more cyanogenics than south-facing sites, but aspect did not effect on higher sites. Grassland management had no effect on the proportion of cyanogenics. Grazing by large herbivores does not favour cyanogenesis, but some invertebrate herbivore may do so (Richards et al., 2001).

TABLES

Table 1. : Different varieties of *Trifolium repens* in Nepal (Shrestha, 2013)

Flower colour	Leaf marking	Name of varieties	Symbol
White	Marked	White flower with marked leaf	WML
White	Non-marked	White flower with non-marked leaf	WNML
White with pink tinged	Marked	White with pink tinged flower and marked leaf	WPML
White with pink tinged	Non-marked	White with pink tinged flower and non-marked leaf	WPNML
Pink	Marked	Pink flower with marked leaf	PML
Pink	Non-marked	Pink flower with non-marked leaf	PNML
Light pink	Marked	Light pink flower with marked leaf	LPML
Light pink	Non-marked	Light pink flower with non-marked leaf	LPNML

Table 2: Calculation for HCN content

	Colour change	Time taken	Symbol	HCN Content
1	Plants whose colour changes	Within 5 mins.	+4	Maximum
2	Plants whose colour changes	Between 5 mins. to 2 hrs.	+3	Medium
3	Plants whose colour changes	Between 2 to 6 hrs.	+2	Medium
4	Plants whose colour changes	Between 8 to 24 hrs.	+1	Minimum
5	Plants whose colour changes	After 24 hrs.	0	Negative results

Table.3: HCN test on different varieties of *Trifolium repens* L.

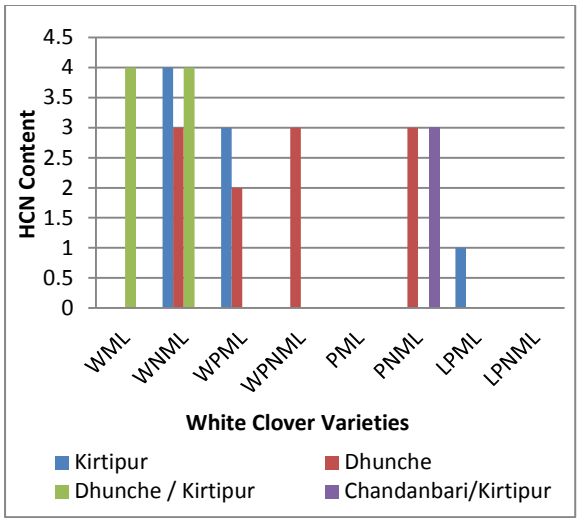
Varieties	Kirtipur	Dhunche	Dhunche / Kirtipur	Chandanbari/Kirtipur
WML	0	0	+4 (Max)	0
WNML	+4 (Max)	+3 (Med)	+4 (Max)	0
WPML	+3 (Med)	+2 (Med)	0	0
WPNML	0	+3 (Med)	0	0
PML	0	0	0	0
PNML	0	+3 (Med)	0	+3 (Medium)
LPML	+1 (Min)			
LPNML	0			

Table 4: Comparative study of leaflet size, soil moisture and soil phosphorous among different varieties of *Trifolium repens* L. (Shrestha, 2002)

Parameters	Study Areas	WML Var.	WNML Var.	WPML Var.	WPNML Var.	PML Var.	PNML Var.	LPML Var.	LPNML Var.
Leaflet size (sq. mm)	Kirtipur (1245-1300masl)	1.40	2.04	1.46	0.76	0.99	0.24	1.22	1.20
	Dhunche (1962-2012 masl)	0.62	0.54	0.58	0.55	0.49	0.49		
	Chandanbari (3400-3500 masl)	1.07	0.32	0.19	0.53	0.91	0.44		
Soil moisture % on which the plant grows	Kirtipur	14.82	14.96	12.99	14.17	14.26	13.44	13.44	12.47
	Dhunche	30.72	28.73	29.89	6.19	32.5	6.40		
	Chandan Bari	46.4	24.08	40.31	19.66	25.85	23.17		
Soil phosphorous % on which the plant grows	Kirtipur	7.41	7.99	1.09	1.19	5.07	1.36	1.09	6.04
	Dhunche	0.003	0.002	0.002	0.002	0.001	0.002		
	Chandan Bari	0.001	0.002	0.000	0.001	0.000	0.001		

Photograph 1: HCN testing with strips in sealed bottles

Figure 1: HCN content in different varieties of *Trifolium repens* L. growing on different altitude.



CONCLUSIONS

In Kirtipur, the maximum quantity of HCN was present in WNML variety of white clover, medium in WPML variety and minimum in LPML variety. In Dhunche, medium quantity of HCN was present in WNML, WPML, WPNML and PNML varieties of white clover. In Chandanbari, the content of HCN in plants might be very low or absent because the transplant of plants from Chandanbari to Kirtipur did not show HCN except a medium amount in PNML variety. It can be concluded that the maximum amount of HCN was present in plants with white coloured flower than pink coloured flower.

Furthermore, the amount of HCN was lesser in colder regions and higher altitude than in plants of warmer regions and lower altitude i.e. the frequency of cyanogenic plants decreased. Acyanogenic WML and PML from Kirtipur and Dhunche have an advantage of being less injured by frost than cyanogenic plants. However, the slugs and snails consume these acyanogenic plant types rather than cyanogenic ones (Crawford- Sidebotham, 1972). The ability to produce free cyanide would therefore be more advantageous to plants in warmer habitats, where slugs and snails remain active during the winter. Cyanogenesis would be less valuable to plants in areas where winter weather controls herbivore populations.

HCN content of plants also depended on moisture and phosphorous content of soil; low light intensity; cool grazing condition of areas and size of the leaf.

No HCN content was found in LPNML growing at Kirtipur and PML variety growing at Kirtipur, Dhunche , transplant from Dhunche and transplant from Chandanbari at all, so these varieties are recommended as a best fodder plant for livestock.

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REFERENCES

Brigton F and Horne MT, 1977. Influence of temperature on cyanogenic polymorphisms. Nature. UK. 265:437-438.

Cahn MG and Harper JL, 1976. The biology of leaf mark polymorphism in *Trifolium repens* L. 2. Evidence for the selection of leaf marks by rumen fistulated sheep. Heredity. 37: 327-333.

Caradus JR, Woodfield DR and Hay RJM, 1996. The poisoning of White clover cultivars in New Zealand. In: Woodfield, D. R. (Ed). White clover. New Zealand's competitive Edge. Grassland Research and Practice series No. 6, New Zealand Grassland Association, Palmerston North, pp. 45-49.

Crawford- Sidebotham TJ, 1972. The role slugs and snails in the maintenance of the cyanogenesis polymorphisms of Lotus corniculatus and *Trifolium* repens. Heredity.28: 405-411.

Daday H, 1965. Gene frequencies in wild populations of *Trifolium repens* L. IV. Mechanism of natural selection, Heredity. 20: 355-365.

De Araujo AM, 1976. The relationship between altitude and cyanogenesis in white clover (*Trifolium repens* L.). Heredity. 37: 293.

Doak BW, 1933. A chemical method for determination of type in white clover. New Zealand Journal of science and Techn. 14: 359-365.

Feigl and Anger, 1966. "The Analyst", American Journal. 91: 282-284.

FoyNR and Hyde EOC, 1937. Investigation of the reliability of the picric-acid test for distinguishing strains of white clover in Zealand. New Zealand Journal of Agriculture. 55: 219-224.

Frame J, Charlton JFL and Laidlaw AS, 1998. White clover. Temperate Forage Legumes, CAB International, Willingford, Oxon ox10 8DE, UK.198.

Hart AL, 1987. Physiology In: White clover. M.J. Baker and W.M. Williams (Ed). CAB international. UK.pp.125-152.

Kenneth MO, Nicholas JK, Lindal S and Shih-chung H, 2008. Evidence on the Molecular Basis of the AC/ac Adaptive Cyanogenesis Polymorphism in White clover *Trifolium repens* L. Genetics. 179 (1):517-526.

Kenneth MO, Nicholas JK and Lindal S, 2013. Recurrent gene deletions and the evolution of adaptive cyanogenesis polymorphism in white clover (*Trifolium repens* L.). Molecular Ecology.22 (3):724-738.

Leffel RC and Gibson PB, 1973. White clover. Forage grasses and legumes. 3 rd. Ed. Agricultural Research Service, USDA. Iowa State University Press. pp. 167-176.

Paim NR and Dean CE, 1976. Evaluation of cyanogenic and acyanogenic clones and progenies of *Trifolium repens* L. Agronomy Abstracts. 59.

Richard AJ and Fletcher A, 2001. The effects of altitude, aspect, grazing and time on the proportion of cyanogenics in neighbouring populations of *Trifolium repens* L. (white clover). Heridity.

Richard JW, 1973. Herbivore feeding and Cyanogenesis in *Trifolium repens* L. Heridity. 30(2): 241-245.

Richards R, 2011. A survey of the genetic diversity in populations of white clover, *Trifolium repens* with a focus on South-western Europe: M Sc. thesis in Plant Molecular Biology at Massey University, Palmerston North, NewZealand.

Shrestha AM (Malla), 2002. The Study on The Diversity of *Trifolium repens* L. (white clover) In Nepal. Ph. D Dissertation. Central Department of Botany. T.U. Kirtipur. Kathmandu, Nepal.

Shrestha AM (Malla), 2013. Identification of Different Varieties of *Trifolium repens* L. In Nepal. Asian J. Agri Biol.1 (2):51-62.

Vickery PJ, Wheeler JL and Mulcahy C. 1987. Factors affecting the hydrogen cyanide potential of white clover (*Trifolium repens* L.). Australian J. of Agriculture Research.38:1053-1059.