Changes of Cyanogenic Glucosides in White Clover (*Trifolium repens* L.) during the Growing Season

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Cyanogenic glucosides have been determined in eight white clover cultivars during three growing seasons. Differences in total cyanogen content were found for different cultivars and also for the time of sampling during the vegetation season. A direct correlation was found between the total cyanogen content and mean air temperature for four days before sampling. At temperatures below 15 °C all varieties contained the highest cyanogen content; an increase in temperature during the summer time resulted in a drastic decrease in cyanogen synthesis. The linamarin/lotaustralin ratio ranged from 0.4 to 0.8 and was correlated with the total cyanogen content; cultivars with higher cyanogen level had a lower lin/lot ratio. It was concluded that white clover can be a good example of a plant species having a mechanism which can switch between growth-dominated and differentiation-dominated plant types depending on to the environmental conditions.

Keywords: *Cyanogenic glucosides; white clover; Trifolium repens; seasonal changes*

INTRODUCTION

Cyanogenic glucosides are the group of nitrogenous secondary compounds which during enzymatic hydrolysis release glucose, HCN, and appropriate lactones or aldehydes. These degradation products are toxic to animals, and the lethal dose for HCN ranges between 0.5 and 3.5 mg/kg of body weight (Montgomery, 1980; Solomonson, 1981). Prolonged feeding of animals with the diet containing many cyanogenic plants may influence the nervous system (Rosling, 1987; Howlett et al., 1990) or may disturb the selenium metabolism (Beilstein and Whanger, 1984; Elzubeir and Davis, 1988). Thus, high levels of cyanogens severely restrict the amount of such meals in animal rations. In Switzerland, white clover varieties which release on hydrolysis more than 370 mg of HCN/kg of dry matter are not recommended to be used as a component of pasture swards (Lehmann et al., 1990). In Eastern European countries, including Poland, until recently there was no problem with cyanogenesis in white clover as all varieties were bred from the local populations which are low in cyanogenic compounds. This low frequency was thought to be correlated to the January isotherm (Jones, 1972). However, breeders looking for better, pest resistant varieties often use breeding stocks containing genes responsible for high levels of cyanogen synthesis, and some varieties of this region are high in cyanogen content (Stochmal and Oleszek, 1995).

Cyanogen concentration in white clover population/varieties has been related to a number of environmental factors including altitude, temperature, water stress, low light intensity, plant age, and phosphorus fertilization (Daday, 1954a,b; Foulds and Grime, 1972; De Araujo, 1976; Baker and Williams, 1987; Vickery et al., 1987; Pederson et al., 1996). To the best of our knowledge no research so far has been performed on the seasonal patterns of cyanogen concentration in white clover plants. Thus, the aim of the present work was to trace the patterns of cyanide changes in eight white clover varieties during one vegetation season and to correlate these changes with the temperature. Moreover, the presented data is the first report on the determination of cyanogenic compounds in white clover with analytical liquid chromatography.

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MATERIALS AND METHODS

Plant Material. Seven Polish registered varieties of white clover, including Anda, Armena, Astra, Podkowa, Rema, Romena, and Santa, as well as reference variety Milkanova, seeds of which were obtained from Dr. Lehmann from Eidgenössische Forschungsanstalt für landwirtschaftlichen Pflanzenbau, Zürich, Swizerland, were planted on the 1.5 × 1.5 m plots in 1993 at Pulawy, Poland. Samples of the clovers (leaves and stems) were collected every week during three growing seasons 1993−1995. Collected samples were friezed at the field at −18 °C, lyophilized, powdered, and then stored at −15 °C until analyses were performed.

Extraction. Powdered samples (1 g) were extracted using 70% MeOH (10 mL) by blending in a Ultra-Turrax T25 (IKA, Labortechnik, Germany) blender, followed by sonication (20 min) at room temperature. The extracts were concentrated *in vacuo* (40 °C) until almost all of the MeOH was removed, and the resulting solution was passed through the preconditioned Sep-Pak C18 (Waters Associates, Milford, MA) cartridge, which was then washed with 20% MeOH (5 mL). The eluates were combined, freeze-dried, dissolved in 0.5 mL of HPLC mobile phase, and used for determination.

High-Performance Liquid Chromatography. The purified clover samples were analyzed according to procedure previously described (Stochmal and Oleszek, 1994). Samples (20 μL) were analyzed using an HPLC unit (Knauer, Berlin, Germany) equipped with a computer system to monitor chromatographic parameters and to process the data, and a differential refractometer detector. A Eurospher RP18 (Säulentechnik, Homburg, Germany) column (25 cm × 4.6 mm i.d., 5 μm particle size) was used with H_2O:MeOH:H_3PO_4 (85:15:0.05) as the mobile phase. The system was operating isocratically at a flow rate of 1 mL/min. Glucosides were identified by comparing their retention times with those of authentic linamarin and lotaustralin standards (Stochmal and Oleszek, 1994). Quantification was based on an external standard method.

Statistical Analyses. Analyses of regression were performed with IBM Statgraphics program.

S0021-8561(97)00435-4 CCC: $14.00

Table 1. Lowest and Highest Concentrations of Cyanogenic Glycosides in White Clover Varieties during the Vegetation Season

	lowest concentration		highest concentration	
variety	mg/g of dry matter	mg of HCN/ kg of dry matter	mg/g of dry matter	mg of HCN/ kg of dry matter
Anda	2.573	267	9.428	996
Armena	2.337	245	11.462	1204
Santa	2.169	229	7.620	804
Romena	1.708	181	6.754	716
Astra	0.847	90	5.161	547
Rema	0.846	89	4.276	453
Milkanova	0.522	56	4.156	440
Podkowa	traces	traces	traces	traces

RESULTS AND DISCUSSION

Preliminary studies of cyanogenic glucosides content in white clover samples showed pronounced differences between samples collected during different seasonal periods. The most distinct differences were observed during the season of 1994 when plants were at the second year of full utilization and at best physiological status. Thus, the data presented in this paper cover the full season of 1994 when samples were taken every week. Table 1 shows the lowest and the highest cyanogen concentrations registered for all varieties during the season. Only the oldest variety, Podkowa, selected from the local Polish populations, showed traces of cyanogens, which were below the detection limit of HPLC. Milkanova, which had in Swiss conditions 370 mg of HCN/kg (Lehmann et al., 1990), showed in the present studies the lowest concentration of 56 and the highest of 440 mg of HCN/kg of dry matter. Six other Polish varieties during the period favorable for low synthesis could be recognized as a low or intermediate cyanogenic, if we accept the Swiss level of 370 mg of HCN/kg of dry matter (Lehmann et al., 1990). But during the periods favorable for highest accumulation (spring and autumn), none of the tested varieties, including Milkanova, could be recognized as low or intermediate. All of them could be classified as high cyanogenic varieties. These data clearly show the impact of the season on the rate of cyanogen synthesis and also how important for the evaluation of these compounds is a period of sample collection. The results are generally in agreement with the data of Vickery et al. (1987), showing that variation in a HCN of 200−520 mg/kg of DM is possible for the range between winter and late spring for unfertilized white clover.

Detailed changes in the concentration of total cyanogen content (linamarin + lotaustralin) during the growing season are presented in Figures 1 and 2. The seven varieties accumulated cyanogens in a similar pattern. Highest concentration was observed during the spring months (May−June) with a rapid decrease in July and gradual increase during August to the spring level in autumn (September−October). This seems to support the view expressed by Baker and Williams (1987) that cyanogen concentration increases at the periods with low temperatures.

In the present study regressions relating cyanogen content to the air temperature (Figure 3) were calculated for seven cultivars. The highest correlation coefficients ($P < 0.01$) between cyanogen level and temperature were found for the case when temperature presented a mean value for four days preceding sampling (Table 2). These coefficients had the values of −0.63, −0.60, −0.72, −0.54, −0.57, −0.74, and −0.66 for Anda, Armena, Astra, Rema, Romena, Santa, and Milkanova, respectively; when temperature went up for four days, plants reacted by decreasing the biosynthesis of cyanogens. Comparison of the curve corresponding to the mean temperature for four days (Figure 3) with the

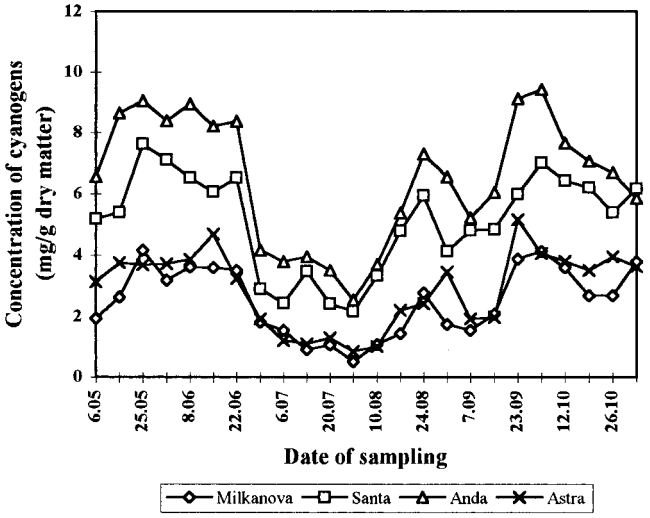

Figure 1. Seasonal changes (1994) in cyanogenic glucoside content in white clover cv. Milkanova, Santa, Anda, and Astra.

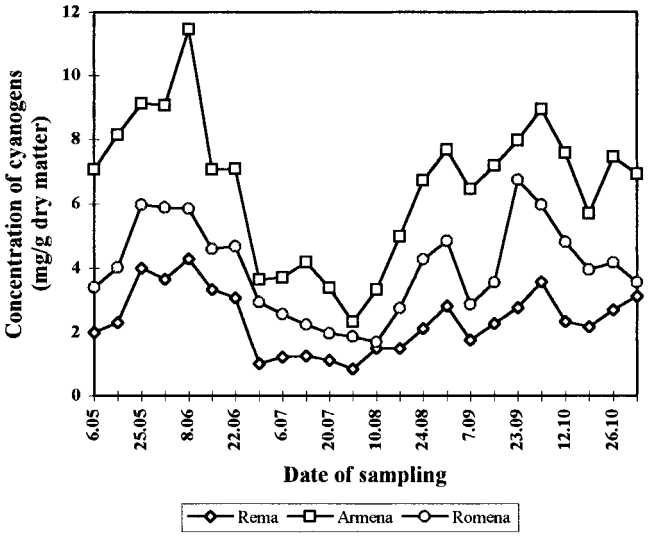

Figure 2. Seasonal changes (1994) in cyanogenic glucoside content in white clover cv. Rema, Armena, and Romena.

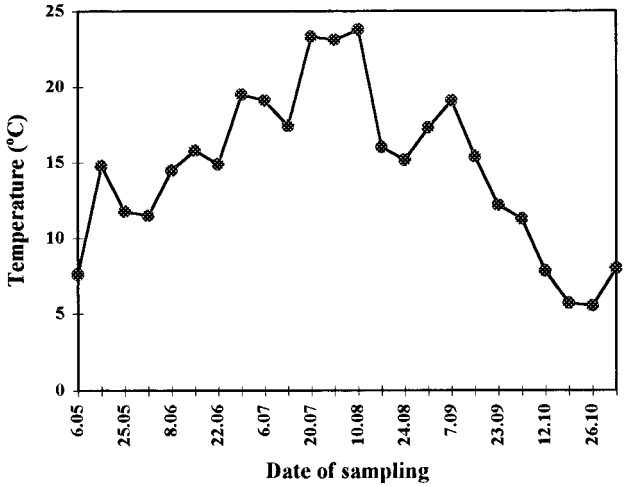

Figure 3. Mean air temperature (1994) for four days preceding clover sampling.

Table 2. Linear Correlation Coefficients of Cyanogen Concentration with Average Surface Daily Air Temperature ($P < 0.01$)

sample[a]	Anda	Armena	Astra	Rema	Romena	Santa	Milkanova
1	0.55	0.48	0.64	0.44	0.48	0.66	0.60
2	0.60	0.53	0.68	0.49	0.53	0.70	0.62
3	0.61	0.57	0.69	0.50	0.53	0.71	0.59
4	0.62	0.59	0.71	0.53	0.56	0.74	0.64
5	0.63	0.60	0.72	0.54	0.57	0.74	0.66
6	0.52	0.49	0.61	0.42	0.41	0.64	0.57
7	0.56	0.51	0.69	0.41	0.49	0.65	0.62

[a] (1) Average temperature for the sampling day. (2) Average temperature for the sampling and one preceding day. (3) Average temperature for the sampling and two preceding days. (4) Average temperature for the sampling and three preceding days. (5) Average temperature for the sampling and four preceding days. (6) Average temperature for the sampling and five preceding days. (7) Average temperature for the sampling and six preceding days.

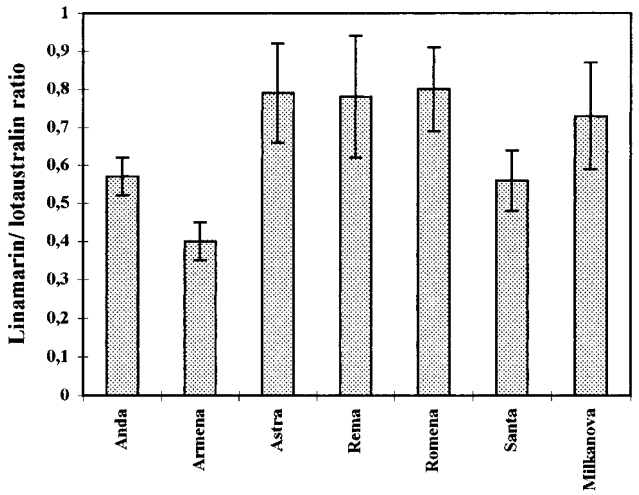

Figure 4. Linamarin/lotaustralin ratio in seven white clover varieties.

curves (Figures 1 and 2) presenting cyanogen changes shows that when the mean temperature was dropping down below 15 °C, the concentration of cyanogens was the highest. Increase of temperature above 15 °C resulted in a gradual decrease of cyanogen concentration to the minimal value obtained at the highest temperature of the season which was 23−24 °C. Plants reacted to the temperature changes in a similar manner in a different periods of the growing season. It was evident that even short warming in the middle of September resulted in drastic decrease in cyanogen concentration. Fluctuations in temperature at the range of 5−15 °C had no impact on cyanogen level.

Application of the analytical HPLC procedure for the determination of linamarin and lotaustarlin made it possible to determine their ratio (lin/lot) in different cultivars. Literature data which were so far based on the extraction efficiency indicated that lin/lot ratio may range between 0.50 and 0.67 (Butler, 1965), and in some cultivars lotaustralin can make up to 80% of total cyanogens (Blaim, 1965). Present findings fully support these data. The lin/lot ratio ranged from 0.4 (Armena) up to 0.8 (Romena) in a number of tested cultivars. Some regularity could be observed, however, in the presented data (Figure 4). Cultivars having high total cyanogen concentration (Anda, Armena, Santa) showed lower values of lin/lot ratio in comparison to the varieties with intermediate levels of total cyanogens (Astra, Rema, Romena, Milkanova). This means that high cyanogen varieties more preferably synthesize lotaustralin than linamarin.

The exact function of cyanogenic glucosides in plants has not been fully understood. However, there are many indications that HCN, acetone, and 2-butanone released during cyanogen hydrolysis are important factors of plant defense systems against herbivores (Jones, 1978; Nahrstedt, 1985, 1988; Selmar et al., 1988). But plant by itself has to solve the economic problem "to grow" or "to defend" (Herms and Mattson, 1992). The growth-differentiation balance (GDB) hypothesis has as its fundamental premise the existence of physiological trade-off between growth and differentiation, the later term including secondary metabolites synthesis (Tuomi et al., 1990). Two groups of plants can be recognized under this theory: growth-dominated with rapid growth and poor chemical defense and differentiation-dominated plants with a slow growth rate and good defense with high levels of toxins (Herms and Mattson, 1992; Harborne, 1992). The data obtained in the present research for white clover may indicate that one plant species can possess both mechanisms, switching between growth and differentiation type according to environmental conditions. During the periods when temperature is below 15 °C and vegetative growth is very slow, clover plants seem to defend themselves against herbivores by a drastic increase in synthesis of cyanogens. When environmental conditions for rapid regrowth improve and grazing creates little hazard for the plant, the synthesis of cyanogens is strongly reduced. To shed more light on to this hypothesis, more research is needed to study the rate of cyanogen synthesis in white clover under some other conditions not favorable for rapid regrowth.

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Received for review May 23, 1997. Revised manuscript received September 2, 1997. Accepted September 11, 1997.[⊗]

JF970435E

[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1997.