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Effects of cyanogenesis on morphology and estimated leaf flavonoid content in 51 white clover accessions

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ABSTRACT

Context. Plant secondary metabolites are of increasing interest for agriculture due to their diverse beneficial ecological functions. The forage crop white clover (*Trifolim repens* L.) has been intensively studied for its heritable polymorphism in the production of hydrogen cyanide (HCN), a toxic defense phytochemical. In fodder production, white clover accessions are selected for biomass production, whereby HCN production is an unwanted trait. **Aim.** Although white clover is a legume crop species of global importance, little is known about the linkage between cyanogenesis and growth traits, in particular in combination with resistance-related phytochemicals, such as flavonoids. We aimed to identify differences in biomass production, estimated leaf flavonoid content, and trait correlations in cyanogenic (HCN-producing) and acyanogenic (not HCN-producing) individuals and accessions of white clover. **Methods.** We analysed 51 white clover accessions from a German germplasm collection for variability in selected traits: cyanogenesis as equivalent electrode potential, estimated leaf flavonoid content, root and shoot production, leaf area, specific leaf area, and number of leaves produced. **Key results.** Most accessions considered as cyanogenic were heterogeneous for HCN production. Chemical–morphological trait correlations differed between cyanogenic and acyanogenic plants. Acyanogenic individuals and accessions produced more and larger leaves compared to cyanogenic ones. Within cyanogenic accessions, the higher the HCN level of a plant, the fewer but larger leaves were produced. **Conclusions.** Our results highlight the variation in HCN production within the selected accessions, which calls for a consistent approach for cyanogenesis-based categorisation. **Implication.** This study demonstrates the potential of combining phytochemical traits with biomass production in white clover when selecting material in a breeding program.

Keywords: cyanogenesis, forage legume, genotypic diversity, leaf flavonoids, legume chemistry, morphological traits, plant breeding, Trifolium repens, white clover germplasm.

Introduction

Cyanogenesis, or the ability to produce hydrogen cyanide (HCN), is a common trait in certain plant families. Most plants emit low levels of toxic HCN as a by-product of ethylene biosynthesis. Over 3000 higher plant species, including many important food plants, release HCN via cyanogenesis (Conn 1981). In cyanogenic plants, cell rupture enables the reaction of cyanogenic glycosides (CNglcs) with the corresponding enzymes (β-glucosidase, α-hydroxynitrile lyase) (Conn 1981; Seigler 1998; Gleadow and Møller 2014). The released HCN is toxic, which explains the major biological function as two-component defence against herbivores, primarily arthropod generalists (Nahrstedt 1985; Hughes *et al.* 1992; Ballhorn *et al.* 2010*a*). The effectiveness of reducing herbivore damage thereby depends on multiple factors, such as the concentration of the cyanogenic precursors, the amount of tissue damaged (Provenza *et al.* 1992; Gleadow and Woodrow 2002) and, as described by Ballhorn *et al.* (2005), the cyanogenic capacity (HCNc), a measurement of the amount of hydrogen cyanide released per minute. In addition, the production of CNglcs is affected by abiotic stresses, notably extreme temperatures, soil salinity, nitrogen supply and drought (Daday 1965; Vickery *et al.* 1987;

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Ballhorn and Elias 2014). However, cyanogenesis comes along with both advantages and disadvantages in the context of plant breeding for agricultural application. It has been shown that low levels of HCN production, leading to increased plant fitness, outweigh the negative effects of its toxicity. Cyanogenic plants showed higher yields, increased drought tolerance and were more resistant to pests and herbivores (Daday 1965; Foulds and Grime 1972; Ennos 1981). On the other hand, a direct trade-off between cyanogenesis and defence against fungal pathogens was shown in rubber tree and lima bean (Lieberei 1988; Ballhorn *et al.* 2010*b*). Cyanogenic white clover (*Trifolium repens* L., Fabaceae) plants perform even better under moderate persistent drought stress compared to those that are not able to produce HCN (acyanogenic morph) (Kooyers *et al.* 2014). This may be one aspect explaining why many cyanogenic crops, such as manioc, sorghum, and white clover are still cultivated for human consumption and animal forage production (Jones 1998).

On the other hand, high levels of CNglcs are detrimental since the consumption of cyanogenic plants entails risks for both human and livestock by inhibiting cellular respiration (Cheeke 1995; Leavesley *et al.* 2008; Hamel 2011). Among cyanogenic plant species, white clover is one of the most widely grown perennial pasture legumes of the temperate zones. Due to its high nutritive value, ability to fix nitrogen, and long seasonal growth, white clover is used as a pasture legume and forage crop, mainly for sheep and cattle (Frame and Newbould 1986; Caradus *et al.* 1996). Because of the ability to adapt to a wide range of climatic conditions, it is used in farming systems all over the world (Frame and Newbould 1986). Nevertheless, its competitiveness and growth is reduced under drought stress (Thomas 1984). Its beneficial properties, especially in crop rotation systems, makes it one of the major pasture legumes in New Zealand, Great Britain (UK), Ireland, the US, and Denmark (Mather *et al.* 1995). Its economic status in these countries explains the large number of accessions in regional seed collections and the continued research effort put into breeding programs to improve resilience and yield (Annicchiarico 2003; Widdup and Barrett 2011; Jahufer *et al.* 2012; Egan *et al.* 2021). White clover accessions have been therefore well characterised for agriculturally relevant traits (Caradus 1986; Caradus and Woodfield 1997; Aasmo Finne *et al.* 2000; Oliveira *et al.* 2013). The published checklists of varieties by Caradus and Woodfield (1997) compiles characteristics such as tolerance and susceptibility to pests, growth traits and HCN production of numerous accessions used for breeding (Caradus *et al.* 1989; Caradus and Woodfield 1997).

The two-gene system for cyanogenesis in white clover is one of the best-studied polymorphisms in plants (Coop 1940; Corkill 1942; Hughes 1991; Hayden and Parker 2002). Cyanogenic plants need to possess at least one dominant allele for substrate and enzyme (Olsen *et al.* 2007). Assessment in white clover plants has been commonly performed using a colorimetric test for either a simple 0/1 categorisation or a combination of colorimetric and spectrophotometric methods for quantitative analyses of HCN (Egan *et al.* 1998; Bradbury 2009). Although widely accepted and advantageous in its usage, the colorimetric test suffers from limitations such as the comparison among studies with regard to the differentiation of the cyanogenic level. When considering the intraspecific variation, plant breeding practices require reliable quantitative measurements that allow for consistent assessment of the level of cyanogenesis. Among other techniques, this could be achieved by using a cyanide (CN) ion-selective electrode (ISE) (Gillingham *et al.* 1969). These ion-selective (IS) measurements to assess cyanogenesis are based on measuring the number of effective cyanide ions (ionic activity) as proportional output potential.

Besides avoiding unwanted phytochemicals, breeders also target for beneficial biochemical properties. Particularly, breeders favour accessions with tolerance to abiotic stressors. The production of flavonoids, for example, enhances resistance to drought, one major cause of crop losses (Nakabayashi *et al.* 2014; Li *et al.* 2021). Furthermore, flavonoids serve as bioactive compounds mediating consumer-specific interactions with herbivores or pathogens (Mierziak *et al.* 2014; Whitehead *et al.* 2021). In addition, they play a key role as stimulants and chemoattractants in establishing and maintaining root colonisation by growth-promoting symbioses with mycorrhizal fungi and rhizobia (Siqueira *et al.* 1991; Larose *et al.* 2002; Eckardt 2006). These agriculturally relevant symbioses make flavonoids an additional target for breeding approaches (D'Amelia *et al.* 2018). However, it is as yet unknown if and how flavonoid content trades off with HCN production in white clover. Furthermore, when multiple traits together make up the quality of a breeding line, assessing their correlations is indispensable to breeders in order to avoid unwanted positive and negative correlations (Yan and Wallace 1995; Yan and Frégeau-Reid 2008; Chen and Lübberstedt 2010). Whether correlations among growth traits and secondary metabolite production follow similar trajectories in cyanogenic and acyanogenic white clover accessions/individuals is as yet unknown.

Due to their agronomic value, many white clover accessions have been deposited in germplasm collections worldwide. Several studies analysed the trait and genetic diversity of accessions from collections; notably from countries with extensive white clover farming e.g. New Zealand, Great Britain (UK), Ireland, the US, and Denmark (Mather *et al.* 1995; Zhang *et al.* 2010; Wu *et al.* 2021). Equally detailed information on growth and chemical traits of white clover cultivars in European gene bank collections is scarce. The characterisation of these collections is important for the identification of genetic sources for white clover breeding. In this study, we screened 51 white clover accessions from a German gene bank of the Leibnitz Institute of Plant Genetics and Crop Plant Research (IPK) for two chemical traits, cyanogenesis and total leaf

flavonoid content, combined with growth traits. The aim was to identify positive and negative trait correlations among these traits. For this, we examined accessions used in agriculture for their cyanogenic status using a quantitative ion-selective electrode, and determined traits related to biomass production. Our work addressed the following questions: (1) Do cyanogenic and acyanogenic accessions differ in morphological traits, and which of those are the main drivers of variation in biomass production? (2) Do morphological traits and estimated leaf flavonoid production differ between cyanogenic and acyanogenic individuals? (3) Do traits correlate within cyanogenic and acyanogenic subsets and do these correlations differ between the two groups? We were thereby able to assess which agro-morphological traits best explain biomass production and whether growth and flavonoid production differ consistently among cyanogenic and acyanogenic plants.

Materials and methods

Plant material and cultivation

Seeds of 51 high agronomic performance white clover accessions were obtained from the Leibnitz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland, Gatersleben, Germany. Seeds were surface sterilised by soaking them in 10% H_2O_2 for 10 min. Per accession, 15–30 seeds were germinated in a climate chamber (21°C, 50% relative humidity; 16:8 h photoperiod) on glass beads with water for 14 days. To ensure a comparable level of development among the replicates of each accession, we selected five seedlings with the first true leaf unfolded. Each replicate was planted in pots filled with a sand/soil mixture (1:1) and grown for 63 days in the greenhouse at 25°C (16 h day) and 21°C (8 h night) at ambient relative humidity. All pots were randomised and fertilised with half-strength Hoagland solution weekly (Hoagland and Arnon 1938).

Morphological traits

A total of three leaf traits (total number of leaves, leaf area, specific leaf area) and six biomass traits were measured and analysed to evaluate correlations between growth and chemical traits in cyanogenic and acyanogenic plants. At the end of the growth phase, at 9 weeks after transplanting the seedlings to the pots, the total number of leaves (TNL) was determined. Six fully unfolded trifoliate leaves from three ramets of two stolons per plant were harvested. Leaves were placed on a white paper and scanned immediately. Leaf areas (LAs) were determined using Adobe Photoshop CS6 and ImageJ (ver. 1.53, http://imagej.nih.gov/ij0). After freeze drying (−80°C, 800 Pa, 72 h) leaves were weighed and the specific leaf area (SLA) was calculated as average mm^2 leaf area per mg dry weight. After assessing root fresh weight and shoot fresh weight, the plant materials were oven-dried (60°C, 72 h) to determine root dry weight (RDW) and shoot dry weight (SDW). Finally, the root:shoot ratios for dry biomass (RSD) were calculated.

Sample preparation

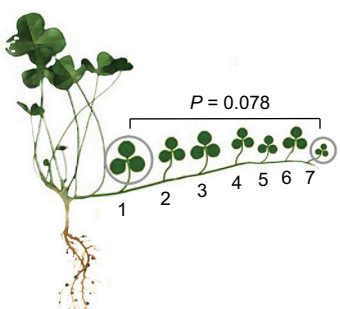
For chemical analyses, including the measurement of cyanogenic levels (CNG) and estimated total leaf flavonoid content (TFC), 5–7 additional fully unfolded trifoliate leaves were collected from each plant one day before the final harvesting. With regard to the leaf sampling procedure, the effect of leaf age on HCN production was tested in a pilot study. Cyanogenesis level along stolons was measured using a Cyanide Selective Electrode as also used in the main study (see section Determination of cyanogenic level). Seven leaves along stolons were sampled and measured for HCN production. Although there was only weak evidence of differences between the youngest and oldest leaves along stolons (P = 0.078, Table 1), we took this tendency into account when sampling leaves for the main experiment. The youngest fully enfolded leaves of one or two ramets of three randomly chosen stolons were selected. Freeze dried leaves (−80°C, 0.008 bar, 72 h) were ground two times for 10 min at 30 Hz using a ball mill (Mixer Mill MM 200, RETSCH GmbH, Haan, Germany).

Reagents and chemicals

Trichloromethane, potassium dihydrogen phosphate, sodium hydroxide, quercetin dihydrate, and aluminum chloride

Table 1. Electrode potential in mV (with ±s.e.m.) of different aged leaves along white clover stolons.

Leaf position from basal to apical	Electrode potential (mV)	N
1	−114.0 ± 6.42	5
2	−116.4 ± 4.48	5
3	−117.6 ± 4.02	5
4	−117.8 ± 2.69	5
5	−125.4 ± 3.17	5
6	−127.8 ± 3.65	5
7	−134.0 ± 7.11	5



hexahydrate were obtained from Carl Roth GmbH and Co. KG (Karlsruhe, Germany). Copper ethyl acetoacetate and 4,4′-methylenebis(N,N-dimethylaniline) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Methanol was purchased by VWR International GmbH (Darmstadt, Germany). Water for the preparation of reagents and extracts was purified by an ultrapure purification system (Thermo Scientific Barnstead GenPure, Fisher Scientific GmbH, Schwerte, Germany).

Determination of cyanogenic level

Hydrogen cyanide levels were measured with a Cyanide Solid State Half-Cell Ion Selective Electrode (CN-ISE), Cat. No. 9406BN combined with a double junction reference electrode, Cat. No. 900200 (Fisher Scientific, Schwerte, Germany). Per sample, 20 mg of dried and finely ground leaf material were extracted in 1 mL 0.2 M KH_2PO_4. The solution was incubated at 37°C for 30 min and the reaction stopped by adding 200 µL 10 M NaOH. After shaking (1 min, 30 Hz) and centrifuging for 10 min at 10.000 rpm (11.180*g*) at room temperature, the supernatant was taken up with a pipette and combined with 19.8 mL Millipore water in a new tube. The HCN measurement was conducted under stirring at room temperature by placing the electrode in the extract. Relative HCN release was expressed as electrode potential (mV).

Before testing the accessions, the electrode read-out was aligned with the result of a sensitive colorimetric test, a modified Feigl-Anger HCN assay (Feigl and Anger 1966; Olsen *et al.* 2007). Filter paper was soaked in a solution of 5 mg 4,4′-methylenebis(N,N-dimethylaniline) and 5 mg copper ethyl acetoacetate in 2 mL trichloromethane. After drying, the test paper was stored until further use at 4°C. For a subset of genotypes, 4–5 medium-sized leaves were harvested, transferred into two 2 mL tubes, and immediately stored for at least 30 min at −80°C. One tube with leaf samples was subsequently defrosted for 10 min at 37°C and the thawed leaf material was crushed with a pestle directly in the tube. Freshly prepared test filter paper was placed on top of the open tube and the lid was closed to fix the paper. The tubes with the test paper were incubated for another 90 min at 37°C. Samples for which the paper turned blue were classified as cyanogenic. The resulting data was combined with CN-ISE measurements to determine at which electrode potential a colour reaction was detected. Based on this combination, samples from the main experiment with a value below −58.5 mV were considered as cyanogenic. Accessions were categorised as cyanogenic when at least one out of five replicates per accession tested positive for HCN production. On the basis of our results and the range of the measured electrode potentials (EP), we categorised the selected accessions within our study in low (EP ≥ −58.5 mV), medium (EP < −80.0 mV), and high (EP < −100.0 mV) cyanogenic.

Determination of the estimated total flavonoid content

To estimate total flavonoid content (TFC) in white clover leaves, we used the aluminum chloride colorimetric method (Chang *et al.* 2002). Quercetin was used to prepare a standard calibration curve. For this, we prepared a stock solution of 5.595 mg quercetin dihydrate dissolved in 50 mL 80% methanol. Serial dilutions of 5 µg and 10–100 µg (in steps of 10 µg) were prepared. Of each solution, 0.5 mL was mixed 1:1 with a solution of 2% $AlCl_3$ in 1.5 mL tubes. Per sample, 10 mg dried ground leaf material was extracted in 80% methanol, sonicated for 45 min at 40°C and centrifuged for 10 min at 2555*g* (room temperature). The supernatant was stored at −20°C until measurement. A solution of 0.5 mL 2% $AlCl_3$ was combined with the extract. Both the standard dilutions and the extracts were incubated for 60 min before measuring the absorbance against a blank at 420 nm on a UV-Vis spectrophotometer using 1 mL cuvettes (Jasco V-620, Pfungstadt, Germany). Based on the readings of the quercetin dilution series, we calculated TFC from the calibration curve ($Y = 0.0325x + 0.32$, $R^2 = 0.9256$) as mg quercetin equivalent (QE) per g of dried leaf material.

Statistical analysis

All statistical analyses were performed using R v4.0.2 (R Core Team 2020) using the packages 'Rmisc' (Hope 2013), 'ggplot' (Wickham *et al.* 2016), 'car' (Fox and Weisberg 2019), 'multcomp' (Hothorn *et al.* 2008), 'corrplot' (Wei and Simko 2017), 'ggbiplot' (Vu 2011), and 'nlme' (Pinheiro *et al.* 2018). To identify significant intercorrelations ($P < 0.05$) we calculated Pearson correlation coefficients for all measured traits. Considering the potential impact of cyanogenesis on these correlations, the analysis was conducted for cyanogenic and acyanogenic individuals separately. For distance measures between the accessions, we conducted a principal component analysis (PCA) on the standardised means of all morphological traits. To identify the impact of each morphological trait and TFC on the variability we calculated the mean of the coefficients of variance (CV; significant differences at a level of $P < 0.05$; Supplementary Table S1) among all cyanogenic and acyanogenic accessions. We tested further differences between cyanogenic and acyanogenic individuals and accessions for each measured trait. Shapiro–Wilk and Levene's test were performed to test for normality of residuals and homogeneity of variances. After ensuring that the requirements for parametric analyses were met, analyses of variances (ANOVAs) were run and pairwise analyses based on multiple comparisons of means (Tukey's HSD) were performed. In case of non-parametric data, we used Dunn multiple comparisons with *P*-values adjusted with the Benjamin Hochberg method after a Kruskal–Wallis test. Least significant differences were identified at a level of $P < 0.05$.

Results

Cyanogenesis on the individual and the accession level

In order to classify the accessions according to their HCN production, we examined how many of the tested individuals produced HCN per accession and to what extent (Table 2). HCN production was tested for each replicate (single individual). Over all tested accessions ($n = 51$), 28 were homogenous with regard to cyanogenesis, since all replicates per accession were either positive or negative for HCN production. Of these, 26 were acyanogenic and only two were fully cyanogenic (TRIF 1291, TRIF 1283). The remaining 23 were heterogenous as we identified cyanogenic and acyanogenic replicates within these accessions. In total, we identified 199 acyanogenic and 56 cyanogenic individuals.

Growth traits and estimated total flavonoid content among and within cyanogenic and acyanogenic accessions

The measured growth variables were analysed for acyanogenic and cyanogenic accessions and individuals (Table 3). On the level of accessions, specific leaf area was higher in accessions classified as acyanogenic ($P_{SLA} = 0.019$). Estimated total flavonoid content of leaves was higher in cyanogenic plants on both the individual ($P = 0.025$) and the accession level ($P = 0.0002$).

Comparing acyanogenic and cyanogenic individuals, we found differences in the measured growth traits and estimated total flavonoid content in leaves. The number of leaves was higher in acyanogenic plants ($P_{TNL} = 0.033$).

Trait variation on accession level

On the basis of the previously described results we were able to determine the trait variation for the acyanogenic and cyanogenic accessions. We found low variability in leaf area (LA), specific leaf area (SLA), estimated total flavonoid content (TFC) of leaves, and total number of leaves (TNL), as shown by the coefficient of variance (CV; CV < 25%, Fig. 1). Growth parameters, notably root weight (RDW), explained most variability (CV > 50%). LA, SLA, TFC and TNL thereby differed from the growth traits each ($P = 0.000$). Also, among growth traits CVs differed between SDW and both RDW and RSD ($P = 0.000$), as well as between RDW and RSD ($P = 0.008$). There was no evidence for an effect of cyanogenesis (Table S1). The first two principal component axes explained 69.6% of the variation in the nine measured morphological traits (Fig. 2). PCA axis 1 explained 43.4% of this variation and was positively associated with SDW, RDW, RSD and TNL, whereas axis 2 (26.2%) was positively associated with SLA and LA. Mean values of all measured traits for each accession are provided in Table S2.

Trait correlation on the individual level

Since most of the cyanogenic accessions turned out to be heterogenous for HCN production (see above), we could only perform trait correlations on the individual level. All measured variables were analysed for correlations between biomass traits, leaf traits, and chemical traits for cyanogenic and acyanogenic plants separately. For acyanogenic plants ($n = 199$), we found positive and negative correlations between the measured leaf traits and biomass data (Fig. 3*a*). Leaf area (LA) correlated positively with shoot dry weight (SDW; $P_{SDW} = 0.025$). Specific leaf area (SLA) showed a negative correlation to root dry weight (RDW), and dry root/shoot ratio ($P_{RDW} = 0.020$, $P_{RSD} = 0.039$). The total number of leaves per plant (TNL) correlated strongly and positively with all biomass traits (P_{SDW}, P_{RDW} and $P_{RSD} = 0.000$). For the estimated total leaf flavonoid content (TFC) we could detect a positive correlation to SDW, SLA, and TNL ($P_{SDW} = 0.003$, $P_{SLA} = 0.004$ and $P_{TNL} = 0.053$).

Contrary to the results for acyanogenic plants, we found no evidence for correlations between either LA or SLA with the selected biomass traits (Fig. 3*b*). As in acyanogenic plants,

Table 2. Electrode potential in mV (with ±s.e.m.) of 25 cyanogenic white clover.

Accession ID	Electrode potential (mV)	N	Accession ID	Electrode potential (mV)	N	Accession ID	Electrode potential (mV)	N
TRIF 1291	−133.38 ± 4.61	5	TRIF 1270	−86.70 ± 14.27	3	TRIF 1284	−91.3 ± n.a.	1
TRIF 1283	−104.82 ± 10.62	5	TRIF 285	−85.63 ± 9.00	3	TRIF 1213	−86.9 ± n.a.	1
TRIF 1288	−138.98 ± 5.13	4	TRIF 1267	−109.15 ± 8.25	2	TRIF 246	−82.10 ± n.a.	1
TRIF 1276	−127.75 ± 3.18	4	TRIF 1258	−82.90 ± 4.20	2	TRIF 1279	−79.6 ± n.a.	1
TRIF 1281	−120.73 ± 1.95	4	TRIF 1178	−81.30 ± 13.70	2	TRIF 1286	−77.3 ± n.a.	1
TRIF 1277	−78.25 ± 10.74	4	TRIF 1320	−128.1 ± n.a.	1	TRIF 1231	−59.2 ± n.a.	1
TRIF 1155	−125.03 ± 1.52	3	TRIF 166	−115.70 ± n.a.	1	TRIF 1285	−58.90 ± n.a.	1
TRIF 1180	−104.70 ± 5.60	3	TRIF 1199	−107.9 ± n.a.	1			
TRIF 1266	−93.13 ± 8.55	3	TRIF 1293	−100.6 ± n.a.	1			

n.a., no calculation for s.e.m. is applicable due to sample size of $N = 1$.

Table 3. Means (±s.e.m.) of measured biomass and leaf traits and estimated total flavonoid content for acyanogenic and cyanogenic individuals and accessions.

Measured trait	Accession level			Individual level		
	Acyanogenic	Cyanogenic	*P*	Acyanogenic	Cyanogenic	*P*
SDW (mg)	1389.30 ± 283.59	1323.15 ± 254.03	0.739	1341.62 ± 95.10	1405.17 ± 187.77	0.511
RDW (mg)	528.61 ± 107.90	429.12 ± 81.65	0.436	482.93 ± 34.23	459.98 ± 61.46	0.689
RSD	0.36 ± 0.07	0.31 ± 0.06	0.429	0.34 ± 0.02	0.32 ± 0.04	0.506
TNL	19.81 ± 4.04	19.19 ± 3.79	0.180	19.76 ± 1.40	18.55 ± 2.47	0.033
LA (mm^2)	1036.36 ± 211.54	1014.42 ± 197.03	0.543	1010.45 ± 92.24	1078.71 ± 187.77	0.223
SLA (mm^2/mg DW)	53.08 ± 10.83	49.40 ± 9.91	0.019	52.04 ± 4.75	48.14 ± 8.38	0.058
TFC (mg of QE/g of DW)	2.06 ± 0.42	2.19 ± 0.42	0.000	2.10 ± 0.14	2.24 ± 0.30	0.025

Significance at a probability level of 0.05.
SDW, shoot dry weight (mg); RDW, root dry weight (mg); RSD, root shoot ratio dry; TNL, total number of leaves; LA, leaf area (mm^2); SLA, specific leaf area (mm^2/mg dry weight); TFC, estimated total flavonoid content of leaves (mg of quercetin equivalent/g of dry weight).

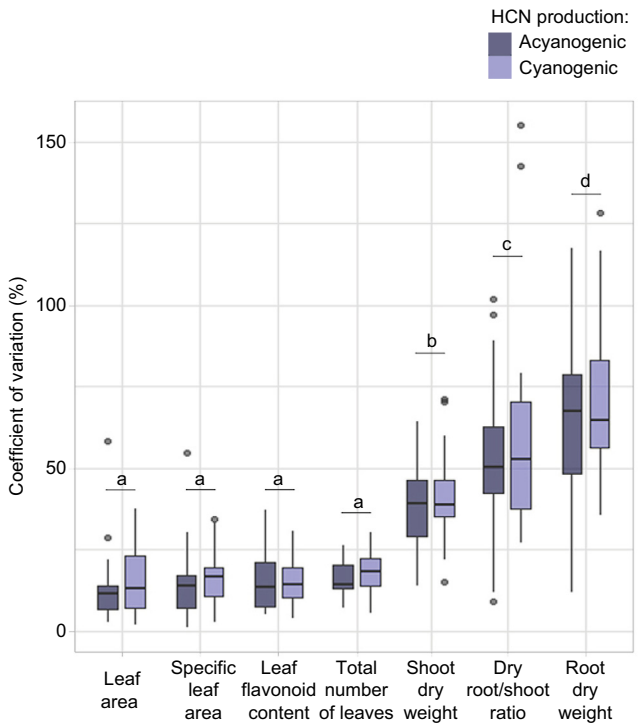


Fig. 1. Mean coefficients of variance (%) ± s.e.m. of morphological traits and estimated total flavonoid content in shoots (TFC) compared between acyanogenic (n = 26) and cyanogenic (n = 25) *Trifolium repens* (L.) accessions, measured on five replicates each. a–d – different letters indicate statistical significance at a probability level of 0.05.

TNL correlated mostly strongly with all biomass traits (P_{SDW} and P_{RDW} = 0.000, P_{RSD} = 0.001). Cyanogenic level (CNG) correlated positively with LA (P = 0.000) and negatively with TNL (P = 0.024). Furthermore, there was no correlation between the two measured molecular parameters, TFC and CNG (P = 0.857).

Discussion

In the study presented here, we examined 51 agriculturally relevant white clover accessions from Central Europe for HCN production levels. The selected accessions were variable for cyanogenesis; most accessions contained both cyanogenic and acyanogenic individuals. We found that overall growth traits did not differ between cyanogenic and acyanogenic accessions. On the individual level the number of leaves produced was lower and estimated leaf flavonoid content higher in cyanogenic compared to acyanogenic plants. We were also able to identify positive and negative trait correlations within the cyanogenic and acyanogenic subgroups. Additionally, these trait correlations differed between the two groups. The strength of the differences and direction of correlations found are discussed below with regard to agricultural usage of white clover accessions.

Accessions are mostly heterogenous for cyanogenesis

We tested all individuals of the selected accessions for their hydrogen cyanide (HCN) production. Most of the accessions that could be classified as cyanogenic were heterogenous for the production of HCN. This result is in line with previous studies (Caradus and Woodfield 1997). Among all tested accessions, 78% of the individual plants were acyanogenic. It was hypothesised that cyanogenesis is linked with altitude assuming a selection for the acyanogenic morph in regions with low winter temperature (Daday 1954; de Araújo 1976; Till-Bottraud *et al.* 1988). The low frequency of cyanogenic accessions could reflect this trait's status as mostly undesirable in breeding practice e.g. when using white clover as forage or green manure (Bjarnholt *et al.* 2008).

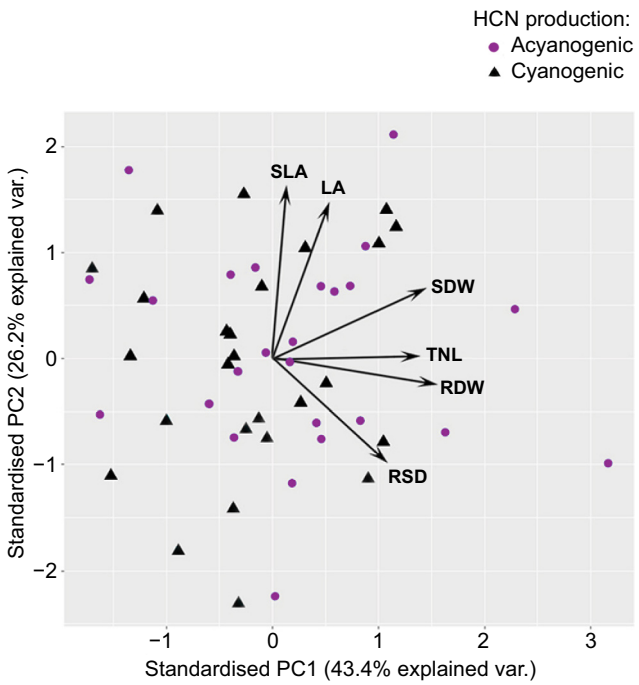


Fig. 2. Principal component analysis (PCA) using six morphological traits including three biomass traits and three leaf traits measured in 51 *Trifolium repens* (L.) accessions). Different symbols indicate acyanogenic (dot, $n = 26$) and cyanogenic (triangle, $n = 25$) accessions. SDW, shoot dry weight; RDW, root dry weight; RSD, root shoot ratio dry; TNL, total number of leaves; LA, leaf area; SLA, specific leaf area.

Differences of qualitative and quantitative HCN production of accessions among studies

Over the decades of white clover breeding, HCN production has always been taken into account when characterising accessions. These classifications are partly inconsistent, both between previously published data and in comparison with those presented here. Caradus (1986) and Caradus and Woodfield (1997) collected data from different studies including qualitative HCN production (cyanogenic/acyanogenic), the level (low, moderate, high), and the frequency (percentage) of cyanogenic plants. As a measure of the incidence of cyanogenic activity, we report in the present work the number of positive replicates, expressed as a percentage. In contrast, previous studies reported an average of the intensity of cyanogenesis across individual samples. Nevertheless, the comparison reveals a comparable rating for most of the cyanogenic accessions; indicating a similar relationship in the classification despite different levels of analysis (Table 4). An apparent limitation of the method described here is its unsuitability for determining the exact cyanotype. The classification as acyanogenic does not consider the presence of cyanogenic glycosides in the absence of the corresponding enzyme. The use of a linamarase enzyme assay would have completed the results. Nevertheless, this limitation should not crucially affect the outcome of the study.

Four accessions have been previously stated as acyanogenic in at least one study while we identified one

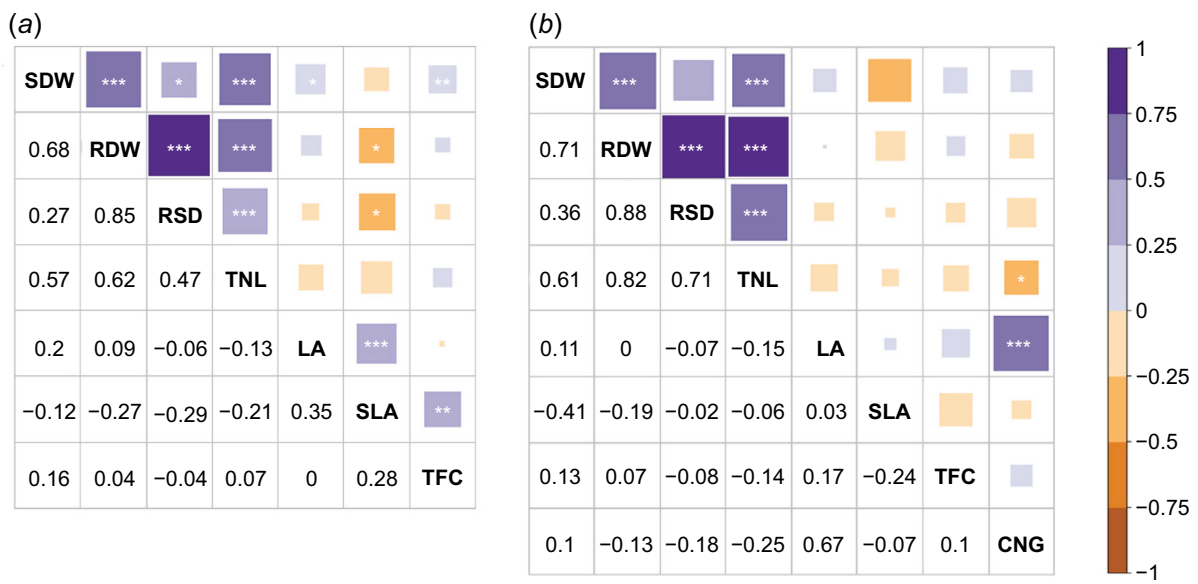


Fig. 3. Pearson correlation coefficients among the measured variables grouped in (*a*) acyanogenic and (*b*) cyanogenic *Trifolium repens* (L.) individuals. Significance at a probability level of *– of 0.05. **– of 0.01. ***– of 0.001. n.s., no significant correlation; SDW, shoot dry weight; RDW, root dry weight; RSD, root shoot ratio dry; TNL, total number of leaves; LA, leaf area; SLA, specific leaf area; CNG, cyanogenesis level.

or more cyanogenic replicates. For eight accessions no positive result for HCN production could be detected in any of the replicates whereby those were described as cyanogenic (Table 4).

Due to the variable number of replicates between studies, data on the frequency of cyanogenic plants can only illustrate the rarity of fully cyanogenic accessions. A replicate number of $n = 5$ seems to be insufficient to identify cyanogenic individuals in accessions in which the ability for cyanogenesis is a rare trait. Nevertheless, there is no clear indication how frequently cyanogenesis has to occur within an accession for being classified as cyanogenic. The same applies when categorising into low, medium or high cyanogenic accessions; which is additionally limited by the usage of different measurement methods. Crush and Caradus (1995) already stated that differences in the method can cause quantitative discrepancies. Nevertheless, they conceded similarities in the ranking of accessions within studies.

Chemical–morphological trait correlations are affected by the cyanogenic status

We evaluated the importance of agro-morphological trait correlation with phytochemical properties in white clover on the level of the cyanogenic status of individuals. Correlations between leaf and biomass traits showed a few similarities among the acyanogenic and cyanogenic subsets. In both cyanogenic and acyanogenic plants, trait variability was mainly driven by root biomass. The total number of leaves per plant (TNL) was positively linked to biomass production regardless of the cyanogenic status, which corresponds to previous findings (Hawkins 1959; Caradus *et al.* 1989). Interestingly, leaf area (LA) and shoot biomass showed a positive correlation only for acyanogenic plants. Similarly, SLA correlated positively and significantly with LA in acyanogenic plants only, which means that leaf thickness increases with leaf size. There is contrasting information on the linkage between these two leaf traits from previous studies in other plant species (Cunningham *et al.* 1999; Fonseca *et al.* 2000). Our results indicate that LA and SLA are decoupled in cyanogenic but not in acyanogenic plants in white clover. This observation suggests that cyanogenic plants produce thicker leaves than acyanogenic ones, which might result from different growth strategies possibly related to adaptation to abiotic conditions. Furthermore, cyanogenesis was significantly linked with leaf traits. We found that individuals producing higher levels of HCN produced fewer leaves but with a greater leaf area. Possibly, the negative correlation between HCN level and leaf number reflects a cost of being highly cyanogenic. Indeed, previous experiments found that acyanogenic plants perform better in mixed and pure stands (Kakes 1989; Noitsakis and Jacquard 1992). Leaf size may compensate for having fewer leaves to some extent. Since the leaves of cyanogenic plants are better defended, they might need to compensate for herbivory resistance less with leaf number.

SLA, as a characteristic trait used to estimate biomass production, increases with increasing latitude, which can be related to differences in efficiency in the use of light energy (Dolph and Dilcher 1980; Gong and Gao 2019). Along a latitudinal and altitudinal gradient, HCN production is lower with more abundant acyanogenic populations in colder regions and in higher altitude (Daday 1954; de Araújo 1976). Our results support these findings on the accession level since SLA was higher in acyanogenic plants.

Estimated total leaf flavonoid content was higher in cyanogenic accessions and individual plants (Table 3). A distinct trade-off between cyanogenesis and phenolic compounds was previously described for the legume species *Phaseolus lunatus* (Ballhorn *et al.* 2010*b*). However, estimated total flavonoid levels significantly increased with specific leaf area and the number of leaves in acyanogenic plants whereas the direction was inverse in cyanogenic plants. In cyanogenic plants estimated flavonoid production seems to come at the expense of leaf area per dry matter. A trade-off effect between flavonoid content and biomass production has been described for white clover. Low levels of quercetin correlated with increased plant growth (Hofmann and Jahufer 2011). The cyanogenic accessions we analysed may have originated from populations occurring in stressed habitats since flavonoids are involved in stress resistances. At the same time cyanogenic plants produced less biomass supporting the described negative trade-off. Since we measured TFC representatively as quercetin equivalent, it is not possible to determine which flavonoids are affected in their synthesis with reduced biomass accumulation in cyanogenic plants. A targeted HPLC (high-performance liquid chromatography) measurement would have allowed a precise determination of leaf flavonoid content. Nevertheless, our results underline the previously suggested potential of flavonoid content as a selection target in white clover breeding.

Cyanide ion-selective electrode measurements for identifying cyanogenic plants

For our analyses we used cyanide ion-selective electrode measurements. The reliability of electrode measurements for forage crops had been proven by combined photometric determination with alkaline picrate, as this method was considered sensitive, specific and reproducible (Gillingham *et al.* 1969). In contrast to the colorimetric methods, the IS measurement is less complex and avoids the use of instable or toxic colour-forming reagents. Nevertheless, electrochemical methods have limitations. Measurements are not specific due to interferences from other substances in the plant tissue (Blaedal *et al.* 1971). We found that it is a valuable alternative to conventional methods when assessing intraspecific variance in HCN production, which allowed us to make quantitative trait correlations. After

Table 4. Comparison of the results on the percentage of cyanogenic plants and categorisation of the cyanogenic level for accessions that have been previously described.

Accession ID	Cultivar/ accession name	Country of origin[A]	Percentage of cyanogenic plants	*N*	Categorisation of the cyanogenic level	Percentage of cyanogenic plants (*N* variable)[B]	Categorisation of the cyanogenic level[B]
TRIF 1155	Aberystwyth S.100	United Kingdom	75	4	High	–	Moderate[a]
TRIF 1180	Karina 1180	Germany	40	5	High	60[a]	Moderate[a]
TRIF 1199	Pronitro 1199	Netherlands	25	4	High	70[a]	Cyanogenic[a]
TRIF 1267	Kersey	United Kingdom	40	5	High	78[a]	Moderate[a]
TRIF 1276	Major	France	80	5	High	100[a]	Cyanogenic[a]
TRIF 1281	Nesta	United Kingdom	80	5	High	70[a]	Very high
TRIF 1283	Olwen	United Kingdom	100	5	High	100[a]	High[a,b]
TRIF 1288	Sabeda	United Kingdom	80	5	High	85[a]	High[a]
TRIF 1291	Tamar	Netherlands	100	5	High	100[a]	Cyanogenic[a]
TRIF 1293	Trifo Daehnfeldt	Denmark	20	5	High	–	High
TRIF 1320	Radzikowska Radi	Poland	20	5	High	–	Low[a]
TRIF 166	Dutch	Netherlands	20	5	High	5–13[a]	Cyanogenic[a]
TRIF 1258	Donna	United Kingdom	40	5	Medium	70[a]	High[a]
TRIF 1266	Karina 1266	Germany	60	5	Medium	–	(same as Karina 1180)[a]
TRIF 1270	Linda	United Kingdom	60	5	Medium	50[a]	Moderate[a]
TRIF 1284	Ovcak	Czech Republic	20	5	Medium	–	Acyanogenic[a] to low[a]
TRIF 246	Mecklenburger Weißklee	Germany	20	5	Medium	–	Acyanogenic[a]
TRIF 285	Pronitro 285	Netherlands	60	5	Medium	–	(same as Pronitro 1199)[a]
TRIF 1213	Steinacher Weißklee	Germany	20	5	Medium	15[a]	Cyanogenic[a]
TRIF 1231	Liblanc	Germany	20	5	Low	–	–
TRIF 1277	Menna	United Kingdom	80	5	Low	–	Moderate[a]
TRIF 1279	Milkanova	Denmark	20	5	Low	–	Acyanogenic[a], moderate[a], low[a,c]
TRIF 1285	Pastevec	Czech Republic	20	5	Low	25[a]	Low[a]
TRIF 1286	Radzikowska 1286	Poland	20	5	Low	25[a]	(same as Radzikowska Radi)[a]
TRIF 1165	Pertina	Netherlands	0	5	Acyanogenic	–	Acyanogenic[a], low[a], moderate[a]
TRIF 1169	Rivendel	Denmark	0	5	Acyanogenic	30[a]	Moderate[a]
TRIF 1181/ 1260	Gandalf	Denmark	0	5	Acyanogenic	–	Acyanogenic[a] to low[b]
TRIF 1183/ 231	Zerno	Germany	0	5	Acyanogenic	–	Acyanogenic[a]

(*Continued on next page*)

Table 4. (*Continued*).

Accession ID	Cultivar/ accession name	Country of origin[A]	Percentage of cyanogenic plants	*N*	Categorisation of the cyanogenic level	Percentage of cyanogenic plants (*N* variable)[B]	Categorisation of the cyanogenic level[B]
TRIF 1197	Fries-Groninger	Netherlands	0	5	Acyanogenic	–	Acyanogenic[a]
TRIF 1200	Vígl'asská	Czech Republic	0	5	Acyanogenic	–	Low[a]
TRIF 1253	Astra	Poland	0	5	Acyanogenic	–	Moderate[a]
TRIF 1254	Blanca	Belgium	0	5	Acyanogenic	75[a]	High[a]
TRIF 1256	Cultura	Netherlands	0	5	Acyanogenic	–	Acyanogenic[a], low[a]
TRIF 1257	Daeno	Denmark	0	5	Acyanogenic	–	Acyanogenic[a]
TRIF 1278	Milka Pajbjerg	Denmark	0	5	Acyanogenic	25[a]	Low[a]
TRIF 1282/ 1179	NFG Gigant	Germany	0	5	Acyanogenic	–	Acyanogenic[a], moderate[a]
TRIF 1287	Retor	Netherlands	0	5	Acyanogenic	–	Acyanogenic[a], low[a]
TRIF 1306/ 284	Podkowa	Poland	0	5	Acyanogenic	–	Acyanogenic, low[c], moderate
TRIF 229	Szarvasi 4	Hungary	0	5	Acyanogenic	–	Low[a]
TRIF 286	Armada	Netherlands	0	5	Acyanogenic	–	Acyanogenic[a]
TRIF 1271	Lirepa	Germany	0	5	Acyanogenic	–	Moderate[a]
TRIF 1273	Luclair	France	0	5	Acyanogenic	–	Low[a]
TRIF 1178	Angeliter Milka	Denmark	0	5	Acyanogenic	25[a]	(same as Milka Pajbjerg)[a]
TRIF 1194/ 1195	B 6-73	Poland	0	5	Acyanogenic	–	–
TRIF 1156	Babinska	Poland	0	5	Acyanogenic	–	–
TRIF 1162	Liganta	Germany	0	5	Acyanogenic	–	–

[A]According to information from the Leibnitz Institute of Plant Genetics and Crop Plant Research (IPK).
[B]According to published data from reviews by [a]Caradus and Woodfield (1997), [b]Viette *et al.* (2000), [c]Stochmal and Oleszek (1997).
– , no data available.

proper calibration, we found this method to be fast and straightforward, which made it suitable for large numbers of samples with almost similar reproducibility to colorimetric methods (Gillingham *et al.* 1969). By measuring leaves at different positions along stolons, we found weak evidence that there is variation for HCN even within one individual (Table 1). Potentialintraindividual variation should definitely be considered in future assessments, for example this can be achieved by consistent sampling procedures such as harvesting leaves from different stolons and the same position, as presented in this study. Considering that HCN genotypes may be more resilient under drought, breeders may want to keep this trait at a level that is low enough for using white clover as a forage crop. Based on our experiences, we recommend this fast and easy method as a semi-quantitative way of identifying low cyanogenic genotypes that isrelevant for crop breeding due to its accuracy and easy handling.

The study presented here supported the detailed categorisation of registered white clover accessions into cyanogenic and acyanogenic types, and documented the heterogeneity therein. We based our classification on an easy-to-use method and integrated it with a multi-trait approach, combining biomass production and relevant phytochemical properties. We thereby resolved some discrepancies among previous studies in the categorisation of cyanogenic accessions. Our results also identified agriculturally relevant trait correlations in cyanogenic and acyanogenic white clover plants over all accessions. This provides valuable information for breeders aiming to combine biomass productivity with optimised levels of cyanogenesis and flavonoids.

Supplementary material

Supplementary material is available online.

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