

Linking Metabolites in Eight Bioactive Forage Species to Their in Vitro Methane Reduction Potential Across Several Cultivars and Harvests

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Abstract

An *in vitro* study was conducted to analyze the fermentation end-products from 17 cultivars of eight polyphenol containing forage species. The polyphenol composition and proanthocyanidin (PA) structural features of all cultivars were analyzed with UPLC-MS/MS in leaves of vegetative or generative plants. All samples were incubated with and without polyethylene glycol (PEG, a tannin-binding agent) to separate the tannin-effect on methane (CH₄) production from that of the forage quality. Sulla and big trefoil, two particularly PA rich species, were found to have the highest CH₄ reduction potential of up to 47%. However, they showed simultaneous and almost equal reductions in gas production (GP; a proxy for digestibility). The addition of PEG led to an increase in both GP and CH₄ production, confirming the role of tannins on CH₄ reduction. Moreover, PA structural features and concentration were found to be an important source of variation for CH₄ production from PA containing species. Despite having low polyphenol concentrations, chicory and plantain were found to reduce CH₄ production without reducing GP. Generally, the variation across cultivars from the same species was found to be lower than interspecies variability, and the results were found to be consistent across growth stages, indicating the findings representativeness.

Introduction

One of the main environmental issues from ruminant livestock production are enteric methane (CH₄) emissions, which contribute to up to 45% of the total livestock sector emissions¹. The radiative forcing capacity of CH₄ is 63 times higher than that of CO₂ over the first 20 years, and is 27 times higher over the 100-year horizon. That makes CH₄ the second most important greenhouse gas regarding anthropogenic climate change, after CO₂². On the other hand, the very shorter atmospheric lifetime of CH₄ (around 12 years), results in a much higher potential compared to CO₂ and N₂O, to reduce the radiative forcing in the short term. Thus, it can provide a valuable opportunity to limit the extent of anthropogenic climate change until long term solutions are in place³. Although, CH₄ emissions from ruminants cannot be completely eliminated without losing the ability of ruminants to digest cellulose, the losses in form of CH₄ can amount up to approximately 3 to 6.5% of gross energy intake in ruminants⁴. Ruminants are particularly important for human nutrition, due to their ability to convert human inedible biomass into highly nutritious human-consumable food. However, this potential is achieved best with diets that contain high shares of forages or food wastes, as concentrates consist of variable and considerable amounts of cereals and other feed sources that could be digested more efficiently by humans directly⁵.

The inclusion of forages rich in plant specialized metabolites (PSMs) (e.g. tannins and saponins) in the animal diet can potentially reduce CH₄ emissions in the ruminants⁶. Particularly tannin-rich forages (TRFs) are considered a promising solution due to their multifunctionality and their beneficial effects on animal health and productivity. These TRFs can exert both, beneficial and anti-nutritional effects on ruminants, based on tannin type and concentration supplied in the feed⁷. Tannins are known to bind with feed constituents such as protein, fibre and carbohydrates. They can improve protein utilization of the forages by making protein-tannin complexes in the rumen (pH = 6-7), and transporting them to small intestine where they will eventually dissociate at the higher pH (pH > 7). This can reduce feed energy losses in form of CH₄ emissions⁸. This is especially relevant for ruminant production systems in temperate regions where despite having high nutritional value for ruminants, the conventional forages, such as lucerne (*Medicago sativa* L.) and white clover (*Trifolium repens* L.), have been associated with bloating and inefficient nutrient utilisation^{9,10}. The high extent of protein degradation in the rumen increases high urinary N excretion, leading to increased ammonia (NH₃) volatilization from the cattle manure and concomitantly negatively affecting the environment⁷.

Tannins can be broadly classified as proanthocyanidins (PAs; *syn.* condensed tannins), hydrolysable tannins (HTs) and phlorotannins, and the latter are mainly found in marine organisms. Hydrolysable tannins can be further subdivided into gallic acid derivatives, ellagitannins and gallotannins¹¹, while PAs consist of polymers and oligomers of flavan-3-ol subunits, most often catechin or epicatechin procyanidins (PCs), and gallo catechin and epigallocatechin (prodelphinidins, PD)¹². Procyanidins and PDs differ from each other in terms of their hydroxylation pattern of their flavan-3-ol B-ring, with PD subunits containing three hydroxyl groups and PC subunits containing two hydroxyl groups in the B-ring⁷. The structural traits are important, as particularly the percentage of PD subunits from total PAs (PD%) and the polymer size have been shown to effect the mode and extent of the desired bioactive properties from PAs¹³. Both forages rich in PAs, such as sainfoin (*Onobrychis viciifolia* L.) and birdsfoot trefoil (*Lotus corniculatus* L.), as well as forages rich in HTs, such as salad burnet (*Sanguisorba minor*), have emerged as promising species that can improve N utilisation in the ruminants while simultaneously curtailing CH₄ emissions^{14,15}. When supplied in low to moderate concentrations, tannins (both PAs and HTs) in forages can potentially improve animal performance, reduce NH₃ volatilization from manure by redirecting N excretion from urine to feces, lower CH₄ emissions

and decrease bloating incidences in cattle^{8,16}. Birdsfoot trefoil, one of the promising TRFs, has been found to increase milk yield and milk protein content compared to perennial ryegrass in Friesian cows, and improved wool growth in sheep¹⁷.

Several *in vivo* and *in vitro* studies have analyzed the antimethanogenic potential of TRFs; however, a high variability in the results from these studies makes it difficult to harness their benefit in the livestock systems¹⁸. This is likely due to the above mentioned high structural diversity of tannins, which makes it difficult to assess their bioactivity and understand their effect on animal physiology and nutrition. It has been widely recognized that PA structural characteristics are important determinants of bioactivity, however, in the majority of animal nutrition studies the structural characteristics or the composition of PAs and HTs are rarely considered. These structural characteristics are known to vary not only across different plant species but also within their cultivars and have been found to be affected by plant's phenological stage¹⁹. However, the variation within the cultivar for each species was found to be less than the variation across the species²⁰. To date, no study has identified the antimethanogenic effect of several cultivars each from multiple forage species grown under identical environmental conditions and across multiple harvests to determine not only the interspecies variability, but also the reproducibility of these findings within species and across harvests, which is ultimately a main prerequisite for their use in practice.

Consequently, the study aimed to assess the *in vitro* CH₄ reduction potential while minimizing the aforementioned variations and accounting the often-neglected PA structural features. To our knowledge, this is the first study to analyze the CH₄ mitigation potential of eight agronomically important temperate forage plants grown under identical environmental conditions and harvested at the same phenological stage. Main objectives of this study were, (i) to analyze the inter- and intraspecies variation of CH₄ mitigation potential of multiple forage species, (ii) to assess the tannin-effect on the CH₄ reduction from the TRFs, and (iii) determine the influence of PA structural features, such as PD% and PA polymer size, on the CH₄ production.

Results

Differences in gas and methane production were observed across species and their cultivars

The *in vitro* gas production (GP) from the forages differed significantly across the cultivars ($P < 0.0001$) with the GP ranging from 25 to 54 ml/200mg DM across both harvests (Figure 1, -PEG). At the species level, chicory had the highest GP (44 to 54 ml/200 mg DM), while sulla had the lowest GP (25 to 32 ml/200 mg DM). The variability within the cultivars of any species for GP was only observed among the cultivars of sainfoin and ribwort plantain, with the latter showing the largest differences as Svatojansky had produced 28% less gas compared to PLA60 in the first harvest. The effect of harvest ($P=0.63$) on GP was not significant; however, a significant interaction effect of cultivar and harvest ($P < 0.0001$) on GP was observed. This was apparent for ribwort plantain on one hand, where the cultivar Svatojansky produced 40% more gas in the second harvest compared to the first harvest and as a result its GP then exceeded that of the cultivar PLA60. For salad burnet on the other hand, the GP was reduced in the second harvest compared to the first harvest by approx. 26%, yet this difference was homogeneous across both the cultivars.

The CH₄ production was also influenced by the plant species and their cultivars ($P < 0.0001$) and the effect of harvest ($P=0.06$) was not significant (Figure 2, -PEG treatment). The lowest CH₄ production was observed for the ribwort plantain cultivar Svatojansky (5.6 ml/200 mg DM) and the sulla cultivar Sudda (5.6 ml/200 mg DM), followed by the big trefoil cultivar Lot 29 (6.8 ml/200 mg DM). Despite containing PAs, CH₄ production from birdsfoot trefoil (9-11 ml/200 mg DM) was the highest and was similar to lucerne (10.8 ml/200 mg DM). In general, the CH₄ production from the cultivars in the first harvest was lower compared to the second harvest and there was a significant cultivar-harvest interaction ($P < 0.0001$). Within species variation in CH₄ production was observed in the cultivars from birdsfoot trefoil, sainfoin and ribwort plantain. As seen for GP, the variability in CH₄ production between ribwort plantain cultivars was larger in the first harvest, with Svatojansky producing 35% less CH₄ than PLA60. In the second harvest, the variation between the cultivars decreased and Svatojansky produced 5% more methane than PLA60. In birdsfoot trefoil, Rocco generally produced lowest CH₄ and had on average 10% lower CH₄ formation compared to other birdsfoot trefoil cultivars (Bull and Lotar). Largely, GP and CH₄ production from the cultivars were stable across the harvests.

Despite comparatively less variable, the cultivars also differed significantly in the CH₄ percentage in total gas (MP) ($P < 0.0001$). The MP ranged from 17 to 25% in the first harvest and from 20 to 26% in the second harvest. The effect of harvest was not significant ($P=0.06$) on MP; however, the interaction between harvest and cultivar was found to be significant ($P < 0.0001$). Within species variability in MP was

again limited to cultivars from sainfoin, ribwort plantain and birdsfoot trefoil (Figure 3; -PEG treatment). Generally, the MP from tannin containing forages (20-26%) was found to be higher compared to ribwort plantain and chicory (17-21%). The lowest CH₄ produced per unit total gas production was found in ribwort plantain followed by chicory.

In general, there was a concomitant reduction of GP and CH₄ production from all the tannin containing species. It was observed that the decline in CH₄ production (7-47%) with TRFs (sulla, big trefoil, salad burnet and sainfoin), was higher than the GP reduction (5-43%) when compared to lucerne (Supplementary Table S2). Contrary to that, chicory cultivars were found to increase GP (7-21%) and reduce CH₄ production (5-25%) with respect to lucerne in both the harvests.

Forage quality and tannin composition

The forage chemical composition of the leaves varied extensively across the species. While general trends existed, and herbs had lower CP and higher NDF concentrations compared to legumes, there was a large variation across different functional groups. In general, highest CP concentration was found in big trefoil and birdsfoot trefoil cultivars (224-272 g/kg DM) when plants were harvested at the flowering stage (first harvest) (Table 1), while sainfoin cultivars had the lowest CP concentrations (157-180 g/kg DM) of all legumes and generally the highest NDF concentrations (518-548 g/kg DM). In the vegetative stage at the second harvest, however, two sainfoin cultivars (CPI 63750 and Esky) had the highest CP concentrations of all species. However, generally the differences in CP concentration decreased with the vegetative stage and no clear differences were observed between legumes and herbs at this stage. Additionally, differences between cultivars of same species were mainly observed for CP concentration and the species ribwort plantain, salad burnet and chicory.

Table 1
Chemical and polyphenol composition of the plants evaluated in the trial

Species	Cultivar	Harvest	CP	NDF	ADF	TP	TF	TT
Chicory	Plumato	1	181	346	212	1.5 ^{AB}	0.1 ^{ABC}	0 ^A
Chicory	Spadona	1	122	464	281	1.3 ^{ABC}	0.1 ^{ABC}	0 ^A
Sulla	Grimaldi	1	232	496	363	22.5 ^D	0 ^A	22 ^{BC}
Sulla	Sudda	1	186	495	366	25.5 ^D	0.1 ^{ABC}	25.2 ^B
Birdsfoot trefoil	Bull	1	224	469	202	3.1 ^B	0.7 ^{DEF}	2.3 ^D
Birdsfoot trefoil	Lotar	1	239	389	236	2.6 ^B	0.5 ^{BDE}	2.1 ^D
Birdsfoot trefoil	Rocco	1	272	349	239	2.4 ^{AB}	0.4 ^{ABCD}	2 ^D
Big trefoil	Lot 29	1	250	482	340	21.4 ^{DE}	1.5 ^{Fb}	19.9 ^{BCb}
Big trefoil	Wild type	1	266	518	376	16 ^D	0.9 ^{EF}	15.1 ^C
Lucerne	Galaxy	1	259	412	219	0.2 ^{Cb}	0.2 ^{ABCa}	0 ^{Aa}
Sainfoin	CPI 63750	1	157	519	351	20.2 ^{Db}	2.6 ^{Gb}	17 ^{BCb}
Sainfoin	Esky	1	206	530	407	18.4 ^{Db}	2.9 ^{Gb}	15 ^C
Sainfoin	Visnovsky	1	181	549	411	20.1 ^D	3.8 ^{Gb}	15.4 ^C
Ribwort plantain	PLA60	1	211	443	237	0.6 ^{AC}	0.1 ^{AC}	0 ^A
Ribwort plantain	Svatojansky	1	129	512	319	0.3 ^C	0 ^A	0 ^A
Salad burnet	PI 308861	1	163	523	95	43.8 ^E	0.4 ^{BCDE}	42.4 ^E
Salad burnet	Sang 10	1	110	426	112	42 ^E	0.3 ^{ABCD}	40.1 ^E
Chicory	Plumato	2	220	393	232	1.7 ^{AB}	0.1 ^{AB}	0 ^A
Chicory	Spadona	2	137	449	301	1.9 ^{AB}	0.2 ^{AB}	0 ^A
Sulla	Grimaldi	2	240	549	455	24.9 ^{CD}	0 ^A	24.5 ^B
Sulla	Sudda	2	224	467	393	27.9 ^C	0.1 ^{AB}	27.7 ^{BC}
Birdsfoot trefoil	Bull	2	229	418	247	2.3 ^A	0.6 ^{CD}	1.7 ^D
Birdsfoot trefoil	Lotar	2	232	408	234	2.2 ^A	0.5 ^{CDE}	1.8 ^D
Birdsfoot trefoil	Rocco	2	242	393	211	1.7 ^A	0.3 ^{BE}	1.4 ^D
Big trefoil	Lot 29	2	250	554	375	12.6 ^{DE}	0.8 ^{Ca}	11.8 ^{Ea}
Big trefoil	Wild type	2	229	493	344	12.4 ^E	0.8 ^{CD}	11.6 ^E
Lucerne	Galaxy	2	235	433	245	0 ^{Fa}	0 ^{Aa}	0 ^{Aa}
Sainfoin	CPI 63750	2	263	540	372	11.2 ^{Ea}	1.3 ^{Fa}	9.4 ^{Ea}

CP, crude protein (g/kg DM); NDF, neutral detergent fibre (g/kg DM); ADF, acid detergent fibre (g/kg DM); TP, total polyphenols (g kg⁻¹ DM); TF, total flavonols (sum of myricetin, quercetin and kaempferol concentration, g kg⁻¹ DM); TT, total tannins (g kg⁻¹ DM). Values identify mean concentration of the measured parameters. Within a column significant differences (P < 0.05) between the different cultivars for each harvest is represented by different uppercase letters and the significant differences within the harvests for each cultivar (P < 0.05) is represented by different lower-case letters. For CP, NDF and ADF, a pooled sample for each cultivar was used for analysis due to biomass limitation.

Species	Cultivar	Harvest	CP	NDF	ADF	TP	TF	TT
Sainfoin	Esky	2	273	520	385	11.5 ^{Ea}	1.7 ^{Fa}	9.5 ^E
Sainfoin	Visnovsky	2	216	554	426	13.8 ^E	2.8 ^{Ga}	10.6 ^E
Ribwort plantain	PLA60	2	157	419	239	0 ^{BFG}	0 ^A	0 ^A
Ribwort plantain	Svatojansky	2	122	511	342	0.2 ^G	0 ^A	0 ^A
Salad burnet	PI 308861	2	221	552	156	49.7 ^H	0.4 ^{DE}	47.9 ^C
Salad burnet	Sang 10	2	221	553	187	45.1 ^H	0.3 ^{BE}	43.3 ^C

CP, crude protein (g/kg DM); NDF, neutral detergent fibre (g/kg DM); ADF, acid detergent fibre (g/kg DM); TP, total polyphenols (g kg⁻¹ DM); TF, total flavonols (sum of myricetin, quercetin and kaempferol concentration, g kg⁻¹ DM); TT, total tannins (g kg⁻¹ DM). Values identify mean concentration of the measured parameters. Within a column significant differences ($P < 0.05$) between the different cultivars for each harvest is represented by different uppercase letters and the significant differences within the harvests for each cultivar ($P < 0.05$) is represented by different lower-case letters. For CP, NDF and ADF, a pooled sample for each cultivar was used for analysis due to biomass limitation.

Significant differences among cultivars were observed for total polyphenol (TP), total flavonols (TF, sum of myricetin, quercetin and kaempferol concentration), and total tannin (TT) concentration ($P < 0.0001$). The highest TF concentrations were found in sainfoin cultivars with a range from 1.3 to 3.8 g/kg DM, while ribwort plantain cultivars generally contained no flavonols. Salad burnet was the only HT containing forage in our study and had the highest TP concentrations ranging from 42 to 50 g/kg DM. Proanthocyanidin containing species were sainfoin, sulla, big trefoil and birdsfoot trefoil with concentrations ranging from 1 to 25 g/kg DM across both harvests. Proanthocyanidin structural features, PD% and mean degree of polymerisation (mDP), generally differed significantly ($P < 0.0001$) across species, while differences across the cultivars of a single species were restricted to birdsfoot trefoil (Table 2). In birdsfoot trefoil, Rocco was consistently found to have significantly higher mDP values (12-14) and PD% (48-52%) than Bull (mDP: 9-10, PD%: 30-33.3%) and Lotar (mDP: 9-11, PD%: 33-39%).

Table 2
Proanthocyanidin (PA) concentration and the structural traits of the PA containing species evaluated in the trial

Species	Cultivar	Harvest	PA	mDP	PD%
Sulla	Grimaldi	1	22 ± 4.9 ^{ADE}	17.2 ± 0.9 ^{ADE}	93.6 ± 0.5 ^{AD}
Sulla	Sudda	1	25.2 ± 2.8 ^A	19.3 ± 1.3 ^A	91.4 ± 0.9 ^A
Birdsfoot trefoil	Bull	1	2.3 ± 1.1 ^B	9.7 ± 1B	33.3 ± 7.9 ^B
Birdsfoot trefoil	Lotar	1	2.1 ± 0.5 ^{BC}	10.6 ± 1.3 ^{BC}	39 ± 4.1 ^B
Birdsfoot trefoil	Rocco	1	2.1 ± 0.8 ^{CD}	13.5 ± 0.8 ^{CD}	52.3 ± 3.3 ^C
Big trefoil	Lot 29	1	19.9 ± 2.4 ^{AE}	16.7 ± 0.2 ^{AE}	90.8 ± 1.7 ^{AD}
Big trefoil	Wild type	1	15.1 ± 4.1 ^{ADE}	15.8 ± 0.2 ^{ADE}	90.5 ± 0.8 ^{AD}
Sainfoin	CPI 63750	1	17 ± 1.2 ^{DEb}	14.3 ± 0.7 ^{DEb}	82.9 ± 4 ^D
Sainfoin	Esky	1	15 ± 2 ^{DE}	15 ± 0.8 ^{DE}	81 ± 4.7 ^D
Sainfoin	Visnovsky	1	15.4 ± 2.8 ^{DE}	15.3 ± 0.5 ^{DE}	82 ± 2.3 ^{Db}
Sulla	Grimaldi	2	24.5 ± 4 ^{ADE}	17.2 ± 2.3 ^{ADE}	93.7 ± 0.4 ^{ADE}
Sulla	Sudda	2	27.7 ± 5.5 ^A	18.2 ± 2.3 ^A	92.3 ± 1 ^A
Birdsfoot trefoil	Bull	2	1.7 ± 0.7 ^B	9 ± 0.9 ^B	30.3 ± 1.9 ^B
Birdsfoot trefoil	Lotar	2	1.8 ± 0.3 ^B	9.7 ± 0.2 ^B	33.8 ± 1.9 ^B
Birdsfoot trefoil	Rocco	2	1.4 ± 0.7 ^{CD}	12.8 ± 1.3 ^{CD}	48.7 ± 4.3 ^C
Big trefoil	Lot 29	2	11.8 ± 5.7 ^{Aa}	18 ± 0.5 ^{Aa}	89.1 ± 0.9 ^A
Big trefoil	Wild type	2	11.6 ± 4.4 ^{AE}	16.4 ± 1.2 ^{AE}	88.3 ± 1.6 ^{AD}
Sainfoin	CPI 63750	2	9.4 ± 3.2 ^{BCa}	11.5 ± 1.1 ^{BCa}	81.9 ± 4.9 ^{CE}
Sainfoin	Esky	2	9.5 ± 2.6 ^{BCD}	12.3 ± 2.4 ^{BCD}	81.1 ± 2.2 ^{CDE}
Sainfoin	Visnovsky	2	10.6 ± 2.2 ^{CDE}	13.9 ± 0.5 ^{CDE}	71.7 ± 3.8 ^{Ea}

PA, proanthocyanidin concentration (g/kg DM); mDP, mean degree of polymerisation; PD%, prodelpinidin percentage in PA. Values identify mean concentration of the measured parameters. Within a column significant differences ($P < 0.05$) between the different cultivars for each harvest is represented by different uppercase letters and the significant differences within the harvests for each cultivar ($P < 0.05$) is represented by different lower-case letters.

Addition of PEG, significantly increased gas and methane production from tannin rich forages

The addition of PEG resulted in increments of GP ($P < 0.0001$) and decrements in CH₄ production ($P < 0.0001$) during the *in vitro* fermentation (Figure 1 and 2, +PEG). These changes were, however, limited to the TRFs. Ribwort plantain and chicory cultivars did not change their fermentation patterns with the addition of PEG. As a result, significant interaction effect of cultivar and PEG treatment existed for GP ($P < 0.0001$) and CH₄ production ($P < 0.0001$). The GP in the +PEG treatment was found to range from 33 to 53 ml/200mg DM, while the CH₄ ranged from 5 to 12 ml/200mg DM. In the first harvest, Svatojansky (ribwort plantain, 5 ml/200mg DM) produced the lowest CH₄ under +PEG treatment, while in the second harvest, Sang 10 (salad burnet, 8.9 ml/200mg DM) had the lowest CH₄ production under the +PEG treatment. The highest change in GP and CH₄ production caused by the PEG addition was found with sulla cultivar, Sudda, where the +PEG treatment increased GP and CH₄ by 46 and 45 percent, respectively compared to the control (-PEG). Conversely, the lowest effect from the PEG addition in TRFs was observed in birdsfoot trefoil cultivars. While the effect of PEG treatment on the MP was significant ($P < 0.001$), there was no significant interaction between treatment and harvest for the MP. There was, however, a

significant effect of three-way interaction between cultivar, treatment, and harvest ($P < 0.0001$) observed for MP. Still, the major variations in MP were observed for birdsfoot trefoil and sainfoin in the first harvest, while in the second harvest, a significant treatment effect was observed on the MP from birdsfoot trefoil, big trefoil, salad burnet and plantain.

Covariates generally explain the variability of fermentation parameters

A model comparison was done by substituting fixed factors, cultivar and harvest, sequentially with measured covariates, to determine whether the measured covariates explain the formation of fermentation end products (CH_4 , GP and MP) equally well, compared to the fixed factors. Yet, replacing the fixed factors with the covariates was not able to improve the quality of the model in terms of the AIC value for neither CH_4 (AIC=312) nor GP (AIC=1749; Table 3). Similarly, for MP the AIC was -646. The marginal R^2 for CH_4 and GP of the final models were generally high with 0.81 and 0.68, respectively. This was despite them being lower than the respective marginal R^2 values of the reference model, with 0.89 and 0.88 for CH_4 and GP, respectively. With regards to covariates, the covariates which significantly affected the fermentation products differed among CH_4 , GP and MP. Accordingly, the GP was significantly increased by the TF concentration and decreased by HHDP, PD% and mDP, while the CH_4 production was decreased by PA concentration, CP and mDP. The MP was only significantly decreased by the PD%.

Table 3

The relationship between forage quality traits and the *in vitro* fermentation end-products (methane production (ml/200mg DM), gas production (ml/200mg DM), and methane percentage in total gas (%)) was estimated by stepwise regression using forward selection. The table shows the model performance of the covariate-based model using measured forage quality parameters (PA, proanthocyanidins (g/kg DM); CP, crude protein (g/kg DM), mDP, mean degree of polymerisation; PD%, prodelphinidin percentage in PA; TF, total flavonoids (g/kg DM); ET, ellagitannins (g/kg DM)) as covariates and its comparison with the reference model. For the R^2 values of the model, marginal R^2 values (variance explained by fixed effects only) and conditional R^2 values (variance explained by the entire model including both fixed and random terms) are presented.

Response parameters	Covariance parameter estimate	Covariates						Model parameters	Model Comparison	
		PA	CP	mDP	PD%	TF	ET		Reference Model	Covariate Based Model
Methane production	F-values	105.36	49.16	7.57				AIC	312.56	325.37
	P values	<0.0001	<0.0001	<0.01				Marginal R^2	0.89	0.81
	Coefficient	-0.17	-0.15	-0.12				Conditional R^2	0.96	0.96
Total gas production	F-values		0.05	13.32	7.26	4.82	6.61	AIC	1749.47	1781.65
	P values		0.83	<0.001	<0.01	<0.05	<0.05	Marginal R^2	0.88	0.68
	Coefficient		0.18	-5.72	-0.55	5.17	-177.75	Conditional R^2	0.98	0.98
Methane percentage in total gas	F-values	0.93			5.41			AIC	-650.08	-646.86
	P values	0.34			<0.05			Marginal R^2	0.26	0.05
	Coefficient	0.001			-0.001			Conditional R^2	0.75	0.81

Discussion

The inclusion of forages with diverse functional groups (e.g. legumes) in mixtures generally improves the yield stability in the grassland-based livestock production systems^{21,22}. Forages rich in tannins and other polyphenols provide even more benefits as they have been shown to decrease CH_4 and N emissions from ruminant based production systems^{23,24}. Although, several studies have analyzed TRFs for their effect on animal performance and CH_4 reduction potential, the results till date have been contrasting, making it difficult to adapt the forages on the large scale. The reason for these contrasting results is assumed to be at least partially on the environmental conditions during plant growth, as these can affect tannin synthesis, as well as on colorimetric tannin assays that do not identify tannin structural characteristics or other compounds with potential interactions^{18,25}. In the present study, the comparative assessment of 17 cultivars from eight agronomically promising temperate forage species and across two harvests provides a novel insight of how the bioactive properties

of these forages vary across the different species, and how stable these CH₄ reduction properties are across different cultivars of the same species and across different harvests.

PA structural characteristics were found to be more stable than forage chemical composition across harvests

The CP levels in legumes are known to generally be in the range of 180 to 300 g/kg of DM and this mostly exceeds animal requirements (130-170 g/kg of DM)²⁶. As expected, in the present study, the CP in the forages was higher in the second harvest where the plants were harvested at the vegetative stage compared to the first harvest, which can be explained by the much higher leaf shares, as leaves are richer in CP than stems²⁷. Higher CP content increases the digestibility of the forage, as it is easily degraded by microbes²⁸. Conversely, high NDF and ADF content in the forages is known to be an indicator of lower forage digestibility²⁶. A meta-analysis by Maccarana, et al.²⁹ found that NDF content in the feed is negatively correlated to GP in the rumen. The NDF content in the feed can reduce the availability of rapidly fermentable carbohydrates leading to lower microbial activity in the rumen and hence a decrease in GP³⁰. Due to potential matrix effects, as well as because of known anti-inflammatory and antimicrobial properties that are beneficial for ruminant immunological system, flavonoid content of the cultivars was also determined (Olagaray and Bradford 2019). Assessing the effect of tannins on ruminants based only on their concentration has given rise to ambiguous results in the past as structural traits of tannins have been found to play an important role in determining their bioactivity¹². Consequently, in addition to PA concentration, PA structural features were also analyzed. In the present study, these features were found to be stable across the harvests, and within the cultivars of the same species the differences were comparatively lower than the variability across the species. This indicates that the methane reduction properties would be stable, and independent of the harvest stage of the plant material.

Methane production from TRFs was consistently lower than lucerne in both the harvests

Numerous studies have demonstrated that TRFs reduced enteric fermentation in ruminants, resulting in lower GP and CH₄ production³¹. Across all the species with varying forage quality and plant metabolite composition, in the first harvest, the largest reductions in CH₄ were observed in TRFs rich species which were sulla, big trefoil, sainfoin and salad burnet in comparison with lucerne. This could be attributed to the high TT concentration in these forages compared to lucerne. A study by Jayanegara, et al.³² found that HTs are more potent compared to PAs. In the present study, however, this was not confirmed, as both the GP and CH₄ production of PI 308861 (salad burnet) were found to be lower than those of Sudda (sulla) and Lot 29 (big trefoil). This was despite the higher TT and TP concentrations in salad burnet, which reaffirms the results from studies indicating that the extent of CH₄ reduction from TRFs does not only depend on tannin concentration but also on the tannin source and structural features^{33,34}. The differences between PAs and HTs bioactivity is likely to be dependent on their fate in biological systems. Proanthocyanidins exist in the form of large polymers, enabling them to bind to the feed components strongly and they do not get easily degraded in the rumen. On the other hand, HTs have been shown to undergo hydrolysis, hence degrading to form low molecular weight phenolics in the rumen, and can thereby, lose part of their bioactivity^{32,35}. A general trend of higher GP and CH₄ production, when plants were harvested at the vegetative stage (second harvest), was recorded compared to when they were harvested at the flowering stage (first harvest) which could be a result of lower tannin content in the plants in the second harvest. Furthermore, a lower content of instantly fermentable fractions, such as water soluble carbohydrates in the leaves at the flowering stage can result into lower GP³⁶. Interestingly, the species with lower TP and TT content, such as ribwort plantain and chicory, were also found to reduce CH₄, but it was not accompanied by reduction in GP. Additionally, it is important to look at the CH₄ percentage (MP) in the total gas, as this can be used as an indicator of the potential CH₄ emissions per unit of organic matter (OM) degraded. Due to the concomitant reductions in GP and CH₄ from TRFs, their MP ranged between 21% and 26% and hence was comparable to that of lucerne (24%) (Supplementary, Table S3). Chicory and ribwort plantain (17-21%) had the lowest MP compared to the other tested species in this study. The reduction in the CH₄ from these species was not accompanied by the reduction in GP indicating their higher potential for CH₄ abatement in ruminants without negatively affecting ruminal fermentation

PEG treatment affirmed the antimethanogenic activity of TRFs, however no influence on methane percentage in total gas was observed

The inclusion of PEG as a tannin inactivating agent, enabled the quantification of the specific tannin-effect from different species on the ruminal fermentation *in vitro*. Polyethylene glycol can inactivate tannins by displacing proteins from tannin-protein complexes, and binding with tannins to form tannin-PEG complexes. Increased GP and CH₄ production from TRFs under +PEG treatment could be a result of high availability of protein after being dissociated from tannin-protein complexes. The increased availability of N compounds that can

no longer bind to tannins with the addition of PEG leads to more substrate for microbial degradation and higher microbial growth. This in turn results into increased ruminal fermentation and higher GP^{34,37}. However, the enzymatic or auto-oxidation of the initial tannins or their hydrolysis products could also produce large bio-oligomers or polymers with tannin-like functions in the rumen or small intestine³⁸. Consequently, making it difficult to assess the fate of tannins in biological systems. As expected, no differences in GP and CH₄ production were observed in samples of ribwort plantain, lucerne and chicory, due to the absence of tannins in these species. Generally, the variation in the extent of GP and CH₄ reduction from across the different TRFs under -PEG and +PEG treatments was in line with the variation in tannin concentration and composition. Accordingly, stronger PEG-treatment effect were observed in cultivars with higher tannin concentrations, which is in line with the studies by Basha, et al.³⁷ and Jayanegara, et al.³⁹.

Although, it is evident that tannins reduce CH₄ emissions, the mode of action by which they reduce CH₄ in ruminants is still not well understood. A study by Hassanat and Benchaar⁴⁰ found that the PA (acacia and quebracho) and HT (chestnut and valonea) extracts had a more pronounced effect on rumen methanogenesis compared to the substrate degradation⁴⁰. Tannins are found to modify the rumen microflora by either directly inhibiting methanogenic population or indirectly affecting methanogen-protozoa symbiosis by inhibiting protozoal population in rumen^{8,18}. In a study by O'Donovan and Brooker⁴¹, PAs from acacia were able to change the morphology of *Streptococcus bovis*, *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*, and, hence, affected their activity. Additionally, HTs and PAs from different plant sources were also found to decrease CH₄ production by suppressing the methanogenic archaea and protozoal populations *in vitro*⁴².

PA structural features were able to explain the variability in methane production from PA containing species but model was not improved

As mentioned above, not only the differences between HT and PA are relevant, but also their structural characteristics affect their potential bioactivity. Both mDP and PD% are among the most relevant features of PAs, determining their protein precipitation activity, which in turn is linked to anthelmintic and antimethanogenic bioactivity of PAs^{12,43}. Hence, in the present study, a stepwise regression was performed to analyze which forage parameters were best suited to explain the total gas and CH₄ production. By identifying covariates as potential predictors, fermentation patterns and CH₄ reduction potential could be estimated from plant traits alone. This would enable extrapolations to any other species, enabling the prediction of their antimethanogenic potential and digestibility from their chemical composition alone. However, the updated model (covariate-based model) with forage composition parameters as covariates did not improve the base model in terms of AIC value. Nevertheless, the conditional R² for CH₄ and GP remained the same and even increased for MP. This indicates that the overall updated model, including both fixed and random effects, was able to explain the variance of CH₄ and GP in the present study for PA containing species. However, in order to predict the fermentation parameters, cultivar remains the better estimate compared to the sum of all covariables, indicating that due to the large number of factors with interconnected effects, the tannin source remains more relevant compared to the traits alone. This could also arise from the fact that in addition to PAs, plants may produce other bioactive PSMs such as terpenes, organic acids, saponins and others which can also influence the CH₄ production from the substrate^{7,44}, yet were not quantified in this study. Furthermore, the influence of matrix effects between tannins and other PSMs on tannin bioactivity, remains an unexplored arena. Still, the model quality was sufficient to identify parameters which had a strong influence on the *in vitro* fermentation end-products. Particularly in case of CH₄, the variables CP, PA as well as mDP were found to exhibit a negative relationship with CH₄ production. This is in accordance with the study by Hatew, et al.⁴⁵, which observed that the polymer size (mDP) was an important determinant of *in vitro* CH₄ and GP production from the substrate. With regards to MP, however, both PA and PD% were found to have limited influence as indicated by their near-zero coefficient and low marginal R² (0.05). One solution to improve the predictive capabilities of CH₄ reductions from tannin traits compared to forage quality traits might be the inclusion of tannin extracts in the *in vitro* incubation instead of the whole plant sample.

The tested forage species are promising alternatives as sustainable feedstock in livestock production systems

The results in the present study underpin that TRFs have high potential for their use in livestock production as their CP content across both harvests was comparable to lucerne. This was further demonstrated by the GP production in the presence of PEG, which did not vary significantly from lucerne. The highest treatment effect was observed when using sulla and big trefoil as a substrate. This indicates their higher antimethanogenic activity owing to high PA content and potent PA structural characteristics. Sulla is a highly palatable forage and its supplementation can reportedly reduce the dependence on proprietary anthelmintics⁴⁶. In the previous studies, supplementation of fresh fed big trefoil was found to reduce CH₄ emissions by 26% (g CH₄ kg⁻¹ DM intake) compared to lucerne in sheep⁴⁷. However, the nutritional value of sulla and big trefoil need to be evaluated before providing them as a substantial feed component. Reportedly, owing to

the high tannin content, sulla and big trefoil can compromise N digestibility and microbial activity in rumen⁴⁸. The concomitant reduction in GP and CH₄ from TRFs in the present study, makes it essential to research on how these forages can be incorporated in animal feed and in which proportion, to exploit their beneficial effects. A study by Orlandi, et al.⁴⁹ found that even though total-tract N digestibility by PA inclusion decreased linearly with the increased concentration, there was a linear increase in N retention and the efficiency of N utilization was improved with the inclusion of tannin extracts of *Acacia mearnsii* until the concentration of 18 g/kg DM, implying that the positive impact of duodenal flux of amino acid supply can outweigh the reduction in protein digestibility when tannins are supplied at optimum levels⁴⁹. Although more research is required to determine the relationship between PAs and post ruminal amino acid availability, it is well established that low to moderate PA concentrations leads to increased rumen undegraded protein and post ruminal amino acid flux which improves the N utilization efficiency in ruminants⁷. Tannin content in the plants is known to be plastic in nature, however, the stability of PA structural features across the different harvests is promising ground to include PA structural features for future structure-activity relationship studies of tannins.

Furthermore, this study also showed that underutilized forbs such as chicory and ribwort plantain have a high CH₄ reduction potential despite being void of tannins. The CH₄ reduction from these species did not result in a negative impact on GP when compared to the other tested species in this study. This is in line with an *in vivo* study by Niderkorn, et al.⁵⁰, which found that sheep produced 23% lower CH₄ per kg DM intake with pure chicory compared to when fed with pure perennial ryegrass (*Lolium perenne* L., cv. AberAvon) which no significant differences in the DM digestibility. Additionally, a study by Navarrete, et al.⁵¹, found that chicory is rich in inulin and sesquiterpene lactones and, ribwort plantain leaves are known to contain acteoside, aucubin, and catapol. These compounds are known to reduce net NH₃ production during ruminal fermentation and acteoside was found to increase potential GP⁵¹. Both chicory and ribwort plantain are promising alternatives to produce high quality feed to overcome feed-deficits during dry seasons due to their drought tolerance, and their inclusion in grass-clover mixtures can enhance above- and below-ground primary production in temporary grasslands^{52,53}. Furthermore, ribwort plantain and chicory are known to benefit animal health due to their antimicrobial and anthelmintic properties which could be attributed to the presence of the PSMs other than polyphenols which were not analyzed in this study^{24,54}.

Conclusions

In summary, low intraspecies variation compared to interspecies variation in antimethanogenic potential of these species is a promising basis for breeding and selection opportunities. The reproducibility of the bioactive effects of TRFs across both the harvests provides a promising outlook of their potential to reduce CH₄ in the livestock production systems. However, as the measurements were conducted under controlled conditions, it is crucial to assess their potential under field conditions. Additionally, inclusion level of these species in ruminant diet should also be assessed to prevent their negative effects on digestibility and animal performance. The current study illustrates that the antimethanogenic property of the PA containing forages is at least in part a consequence of PA structural characteristics as the highest CH₄ reduction was observed from sulla containing highest PD% and mDP value. Furthermore, the stability of PA structural characteristics across the harvests and less variation within the cultivars of the same species than across the species provides an opportunity for breeding and selection programmes based on PA structural traits. Hence, follow up studies to screen the species with high antimethanogenic property for their variation in PA structural features and bioactivity are suggested, which can in turn improve the predictability of structure-activity relationship of tannins, and it is crucial also to validate these relationships under field conditions. Assessing the effect of PA structural features in addition to PA concentration on the bioactivity of these forages could be a useful criterion to understand the diverse effects of different plant sources containing similar PA concentration as these differences have been shown to produce quantifiable effects.

Methods

Experimental design

A greenhouse experiment was conducted with 17 different forage cultivars from 8 species (Table 4). The seeds for the plants were obtained from the seedbank of IPK Leibniz Plant Genetics and Crop Plant Research, Gatersleben. The plants were harvested twice at different developmental stages. In the first harvest, plants were harvested at the flowering stage and in the second harvest, which was done after a 6-week regrowth interval, plants were harvested at the vegetative stage. The plants were grown in flower pots of 24x24x40 cm³ (LxWxH) each and 5 plants were sown in each pot. Each cultivar had 4 pots as replicates. The pots were arranged in a complete randomized design and reflective foils were installed to ensure even lighting. For details see Verma, et al.²⁰. The plants were consequently separated into leaves, stems and flowers. The samples were freeze dried and stored at -80 °C for further analysis. The leaf samples were

subsequently ball milled, and stored at -80 °C to preserve the structural integrity of the polyphenols in the sample. The average monthly temperature in the greenhouse during the plant growth ranged from 24-27°C (March to August). The experimental research on plants complies with the relevant institutional, national, and international guidelines and legislation.

Table 4
The plant species and their cultivars used in the greenhouse experiment

Species	Cultivar	Common name	Plant family
<i>Onobrychis viciifolia</i>	Visnovsky	Sainfoin	Fabaceae
<i>Onobrychis viciifolia</i>	CPI 63750	Sainfoin	Fabaceae
<i>Onobrychis viciifolia</i>	Premier	Sainfoin	Fabaceae
<i>Lotus corniculatus</i>	Bull	Birdsfoot trefoil	Fabaceae
<i>Lotus corniculatus</i>	Lotar	Birdsfoot trefoil	Fabaceae
<i>Lotus corniculatus</i>	Rocco	Birdsfoot trefoil	Fabaceae
<i>Lotus pedunculatus</i>	Lot 29	Big trefoil	Fabaceae
<i>Lotus pedunculatus</i>	Wild type	Big trefoil	Fabaceae
<i>Sanguisorba minor</i>	Sang 10	Salad burnet	Rosaceae
<i>Sanguisorba minor</i>	PI 308861	Salad burnet	Rosaceae
<i>Cichorium intybus</i>	Plumato	Chicory	Asteraceae
<i>Cichorium intybus</i>	Spadona	Chicory	Asteraceae
<i>Plantago lanceolata</i>	PLA60	Ribwort plantain	Plantaginaceae
<i>Plantago lanceolata</i>	Svatojansky	Ribwort plantain	Plantaginaceae
<i>Hedysarum coronarium</i>	Sudda	Sulla	Fabaceae
<i>Hedysarum coronarium</i>	Grimaldi	Sulla	Fabaceae
<i>Medicago sativa</i>	Galaxy	Lucerne	Fabaceae

Chemical composition of forages

Nitrogen concentration of the forages was determined by the DUMAS rapid combustion method using a CN elemental analyser (Vario Max Elementar Analysensysteme, Hanau, Germany). Crude protein (CP) was calculated by multiplying the N concentration with 6.25. The concentrations of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were estimated by sequential fibre analysis according to Van Soest detergent procedure⁵⁵ using a fibre analyser Ankom A220 (Ankom Technology, Macedon, NY, USA).

Polyphenol and tannin analysis with UPLC-DAD-MS/MS

The leaf tannin extracts were prepared for the UPLC-DAD-MS/MS as described by Verma, et al.²⁰. The samples were analyzed with an Acquity UPLC (Waters Corp., Milford, MA, USA) coupled with a XEVO triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) as described by Engström, et al.⁵⁶. The system consisted of a sample manager, a binary solvent manager and a column, coupled with diode-array detector. The UV spectra was recorded. For the elution, acetonitrile (A) and 0.1% aqueous formic acid (B) were used with a constant flow rate of 0.5 mL min⁻¹ with the following elution profile: 0–0.5 min, 0.1% A (isocratic); 0.5–5.0 min, 0.1–30% A (linear gradient); 5.0–8.0 min, 30–45% A (linear gradient); 8.0–11.5 min, column wash and stabilization. The data was recorded from 0 to 6 min for UV-vis (190-500 nm) and MS data (m/z 100 to 2000). The specifications for the negative electrospray ionization are as follows; capillary voltage: 2.4 kV, desolvation temperature: 650°C, source temperature: 150°C, flow rate of desolvation and cone gas (N₂): 1000 and 100 L/h, respectively, and collision gas: argon.

Hohenheim gas test

From the 4 pots per cultivar, due to analytical constraints, 3 pots were selected for analysis with the Hohenheim gas test (HGT) in the first harvest. For the same reason, in the second harvest, the biomass from all pots for each cultivar was pooled. The GP and CH₄ production from the samples were analyzed using *in vitro* HGT⁵⁷. The plant samples (200 ± 1 mg) were added to 100 mL calibrated glass syringes

(Haeberle Labortechnik, Lonsee-Ettlenschieß, Germany) and rotated inside the incubator (39°C). The samples were run in triplicates and each run was performed on two different days leading to 6 replicates per sample. Additionally, to standardize each run, four blanks (no plant material) as well as hay and concentrate standards from the Institute of Animal Science, University of Hohenheim, were used as a reference, and were included in triplicates. The ruminal fluid was collected prior to morning feeding from two ruminally-cannulated, non-lactating crossbred heifers (Jersey × German Black Pied). It was filtered with cheese cloth into a prewarmed insulated flask, immediately transferred to the lab, and mixed with a freshly prepared buffer solution at 1:2 ratio (v/v). The mixture was continuously stirred and flushed with CO₂ while maintained in a water bath 39°C. The buffered ruminal fluid (30ml) was added to the syringes containing plant samples and syringes were placed back in the incubator with the rotor set to about one rotation per minute during the period of incubation (24 hours). The GP and CH₄ production from the samples were measured at the time interval of 8 and 24 hours. The GP was recorded from the scale printed onto the syringes and MP was measured by non-dispersive infrared spectroscopy CH₄ analyser (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany). The measured GP volume from the samples after 24 h was corrected with an average of the factors obtained from hay and concentrate standards. These constant values were calculated based on the measured and the targeted GP values of 45.9 ml/200 mg DM and 65.13 ml/200 mg DM after 24 h incubation of hay and concentrate standards, respectively.

Additionally, to evaluate the specificity of tannin effect on the gas production parameters, PEG treatment was included to attenuate the effect of tannin on fermentation. The samples were incubated with 200 mg PEG (MW: 6,000 kDa). The PEG treatments were referred as +PEG (samples with PEG) and -PEG (samples without PEG).

Statistical Analysis

All statistical analysis were done with using the software R⁵⁸. A linear mixed model was used with cultivars, harvests, treatment, and their interactions (two- and threefold) as fixed factors. Analytical replicates from the HGT per experimental pots and per date of experiment were used as random factor. Graphical residual analysis was performed and based on that, the data was assumed to be normally distributed and heteroscedastic. An ANOVA and multiple contrast tests were conducted to compare the significance of the influence factors on different levels. All tests of significance were made at $P < 0.05$.

Second, to assess whether the measured forage quality parameters of PA containing species can explain the fermentation end products, a (forward) model selection was done based on the AIC value (Akaike information criterion) as described by Venables and Ripley⁵⁹. Therefore, the same model as above was used here as a reference model containing a subset of only PA containing species and the “-PEG” values (without the corresponding PEG treatment effects). In the reference model, the fixed factors were cultivar (only PA containing species), harvest and their interaction effect, and the random effect were the analytical replicates from the HGT per experimental pots and per date of experiment. Subsequently, the previous fixed factors were removed and replaced consecutively with forage trait derived covariates (forage quality and tannin traits). The decision for or against a certain covariate was also based on its significance and its variance inflation factor (VIF; <5) until the optimal model with the lowest complexity was achieved. The final, covariate-based model was then compared with the reference model.

Declarations

Data availability statement. Data sharing not applicable. The data generated during the study is included within this article and supplementary information.

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Author contributions

Conceptualization, C.S.M.; data curation, C.K. and S.V.; formal analysis, S.V., M.H., and C.S.M.; funding acquisition, C.S.M.; investigation, S.V.; methodology, S.V., M.H. and R.B.; project administration, C.S.M.; supervision, C.S.M. and F.T.; visualization, S.V.; writing—original draft,

S.V.; and writing—review and editing, S.V., S.W., J-P. S., M.H., A.S., R.B., C.K., F.T., and C.S.M. All authors have read and agreed to the published version of the manuscript

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Gas production from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar.

Figure 2

Methane production from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar.

Figure 3

Methane percentage in total gas from 17 cultivars of 8 species under different treatments for two harvests. -PEG indicates the samples incubated without polyethylene glycol (PEG) and +PEG refers to the samples incubated with PEG. Means followed by different uppercase letters represent significant differences ($P < 0.05$) between the cultivars for each species, and different lowercase letters represent the significant differences within the treatments for each cultivar.

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