

Ergosterol and polyphenols: Potential biochemical indicators of silage safety

Jhonny Edison Alba-Mejía (✉ jhonny.alba.1604@gmail.com)

Mendelova univerzita v Brne <https://orcid.org/0000-0003-3638-0175>

Vlastimil Dohnal

Univerzita Jana Evangelisty Purkyne v Usti nad Labem

Miroslav Klíma

Vyzkumny ustav rostlinne výroby

Tomáš Středa

Mendelova univerzita v Brne Agronomicka fakulta

Gloria Domínguez-Rodríguez

Universidad de Alcalá de Henares

Jiří Skládanka

Mendelova univerzita v Brne

Research

Keywords: Additives, *Dactylis glomerata*, Fungal marker, Livestock, Phenolic compounds, Silage safety

Posted Date: April 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-19631/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Silage, one of the most important feed sources for cattle, is vulnerable to contamination by spoilage moulds and mycotoxin production because ensilage forage crops are excellent substrates for fungal growth. Whereas there are many factors involved in mycotoxins contamination such as biological factors, harvesting, storage, and processing conditions, the climate is the most important factor. Silage additives can improve the safety of silage and inhibit moulds and other detrimental silage microorganisms; however, their efficacy varies with the type and level of toxigenic fungi contamination. Several studies provided evidence showing that the presence of various mycotoxins was correlated with the presence of ergosterol in cereals and feedstuffs. Therefore, ergosterol (ERG) has been suggested as a potential indicator of fungal contamination with polyphenols concentration analysis allowing accurate prediction on silage safety. The main goal of this study was to use the ERG and polyphenols content as potential biochemical indicators to predict the silage safety in ensiled grasses. The study was carried out using ten orchardgrass varieties (untreated and treated with biological and chemical additives). The determination of the samples was performed on high-performance liquid chromatography using UV detection (ERG) and spectrophotometer UV/VIS (polyphenols).

Results: Interestingly, in the silage 'Bepro' was the unique variety that no presented content of ERG (0.0 mg kg⁻¹ dry matter) in comparison with other varieties in the first cut in 2012. The biological additives reduced the content of ERG in both cuts in 2012 compared with untreated silage. In contrast, the chemical additives in silage grass had a negative effect ($P < 0.05$) by increasing the ERG and polyphenols content in comparison with untreated silage.

Conclusions: These results of this study provide indisputable evidence that silage contaminated with fungi increase the concentration of polyphenols, which prove a progressive deterioration of silage quality. In consequence, ERG and polyphenols seem to be relevant as a rapid method for screening silage safety.

Background

Silage constitutes a major component of ruminant foodstuffs rations worldwide [1], representing 50–80% dairy cow's during the winter [2]. Particularly, orchardgrass (*Dactylis glomerata* L.) is a long-lived perennial grass, shade tolerant and the fourth most important forage grass in the world [3]. Previous studies noted that orchardgrass had greater nutritive value than other forage grasses commonly used as feedstuff for cattle, such as bromegrass, tall fescue, and reed canarygrass [4, 5]. Orchardgrass is widely cultivated on a global scale for grazing, hay or silage production [6], due to its high adaptability to various environmental conditions and considerable tolerance to heat and drought [7, 8], being capable to retain productivity even during dry seasons [9]. Considering the orchardgrass plasticity to adverse climate change conditions, this forage grass would be a promising species, at least in Europe [10].

Low water availability in semiarid and temperate climates [11] limits the forage growth and development, reducing their yield [12]. In view of current climate conditions, silage is the best method to preserve fresh

forage material with minimal losses of nutritive value by fermentation of soluble carbohydrates into organic acids (mainly lactic acid) under anaerobic conditions [10]. Therefore, silage quality is fundamental for animal production, health, and food quality [13]; however, they are at risk of infection by different fungi during storage periods [14]. Fungal growth leads to loss of nutrients and dry matter, palatability reduction and reduction in silage consumption. As a result, fungal contamination in silage impacts negatively in animal performance [15]. In addition, secondary metabolites produced by fungi – mycotoxins – are often responsible for ruminant health damage or death, due to their toxigenic, carcinogenic, mutagenic, teratogenic, estrogenic, nephrotoxic, neurotoxic and hepatotoxic effects [16, 17]

In order to avoid silage degradation from undesirable pathogens, additives have often been used to improve the silage process and quality [18]. Additives can have significant effects on the quality of silage by enabling the production of more organic acids and greater reductions in pH, as well as effectively inhibiting the activity of moulds [19]. Despite that, it is necessary to evaluate the risk of mould contamination through reliable biochemical indicators to ensure the silage hygiene quality and animal health, because the additives do not always improve the quality of silage.

ERG is a principal sterol component of fungal membranes characteristic of moulds and yeasts, which represent between 10 to 80% of all sterols present in the mycelium (depending on growth phase) [20]. This sterol is restricted to hyphal fungi and can be permanently present at appreciable concentrations in the absence of living fungi [21]. Moreover, ERG is a primary metabolite that shares a biosynthetic pathway with other harmful mycotoxins such as deoxynivalenol and nivalenol [22]. Hence, ERG was proposed as an indicator of fungal contamination for various matrices such as solid plant substrates [23], foods and raw materials [24]. ERG is better correlated with fungal biomass than traditional fungal colony forming unit count [25]. Besides, ERG is more sensitive than another fungal biomarker 'chitin' for the detection of early stage fungal growth [26]. In addition, Japelt et al. [27] and Golinski et al. [28] reported that ERG contents increase with increasing humidity and decreasing temperatures during fall and winter months, under favourable environmental conditions for the growth of moulds.

On the other hand, phenolic compounds content has also been proposed as indicator of silage hygienic quality. These compounds are secondary plant metabolites, synthesised in the shikimate pathway (which provides phenylpropanoids), or via acetate/malonate pathway, producing simple phenols. Besides, the monomeric and polymeric phenols and polyphenols can be produced in both pathways [29]. Phenolic compounds are the most important natural antioxidants [30]; however, their oxidation leads to browning and production of toxic compounds. In consequence, feed safety is affected because of rancidity and deterioration of nutritional quality, and other sensorial properties as colour, flavour, texture alteration [31, 32], compromising the animal health.

The aim of this study was to use the content of ERG and phenolic compounds as potential biochemical indicators on silage safety in ten orchardgrass varieties treated with biological and chemical additives.

Material And Methods

Plant materials

Ten orchardgrass varieties from breeding programmes in different European countries ('Greenly' and 'Starly' from France; 'Otello' from Italy; 'Sw-Luxor' from Sweden; 'Amera', 'Dika', and 'Bepro' from Poland; 'Husar' from Germany; and 'Dana' and 'Vega' from the Czech Republic) were used. The plots were established during the second week of April 2011, and the laboratory assessment was conducted in 2012 and 2013 in the Czech Republic, at the Research Station of Fodder Crops in Vatin, (49°31'N, 15°58'E, 560 masl). The research site presents temperate climate, where the precipitation and the average annual temperature were 632, 658 and 705 mm and 7.4, 6.8 and 7.3 °C in 2011, 2012, and 2013, respectively (Fig. 1). The soil type was a Cambisol with a sandy-loam texture. The experimental design consisted of a split-plot with 1.5 × 10 m plots (three replicates). For harvesting, a self-propelled mowing machine (HEGE 212 harvester, Wintersteiger, Ried im Innkreis, Austria) with a mowing width of 1.25 m, was used. The harvested area of individual plots was 12.5 m², with a remaining stubble height of 7 cm. A 20 kg ha⁻¹ dose of seeds was utilised during sowing. Fertilisation consisted of a 60 kg ha⁻¹ N dose per year in urea form. The first and second growth were harvested at the heading phase (vegetation stage when inflorescence is emerging, but before shedding pollen). The biomass was wilted on the plot for 14 h to reduce the water content after mowing. Afterwards, the forage samples (10 kg per treatment) were taken to the laboratory and chopped with a conventional forage harvester to a particle length of 40–60 mm.

Treatment of grass materials

Representative forage samples (6 kg) were placed in mini-silos of polyvinyl chloride (PVC) and compacted to a pressure of 600 kg m⁻³. The mini-silos (three repetitions per treatment) were sealed with a lid and stored at room temperature of 28 °C for 90 days. Treatments consisted of the following silage and inoculant combinations: 1) silage without inoculants, used as control; 2) silage containing biological inoculants [i.e., *Lactobacillus plantarum* (DSMZ 16568) 5 × 10¹⁰ colony-forming unit (CFU) g⁻¹, *Lactobacillus buchneri* (DSMZ 22501/CCM 1819, DSZM: German collection of microorganisms and cell cultures; CCM: Czech collection of microorganisms) 1.25 × 10¹⁰ CFU g⁻¹, and *Enterococcus faecium* (DSMZ 22502/NCIMB, NCIMB: Scottish national collection of industrial and marine bacteria) 6.25 × 10¹⁰ CFU g⁻¹]; and 3) silage containing chemical additives (i.e., formic acid, propionic acid, benzoic acid, ammonium formate, E150d-sulfite ammonia, caramel and water). Biological inoculants were applied at a rate of 2 g ton⁻¹, and chemical additives at 4 L ton⁻¹, according to the manufacturer's recommendations. At the end of the ensiling period (90 days), the silos were opened, and samples were taken for chemical analysis. The pre-drying of subsamples (fresh forage and silage) was performed in a specific drying oven at 60 °C for 48 hours to determine the silage quality. Subsequently, the forage samples for analysis were ground in a mill and then filtered through a 1 mm sieve. The fresh forage and silage samples were analysed to determine the concentration of ERG and phenolic compounds.

Ergosterol determination

In order to determine ERG in fresh grass and silage, a total of 250 mg of the sample and 2 mL of the 10% solution of potassium hydroxide in methanol were weighed using analytical scales into 4 mL glass vials with a screw closure. The vials were then closed with lids with a teflon antiseptic sealing. The content was intensively mixed for 30 s in a mixer (MS2 Minishaker IKA, USA) and the vial was kept in a thermostat (Evaterm, Labicom, CZ) for a period of 90 min at 80 °C. Volume of 0.5 mL of distilled water and 1 mL of hexane were added after cooling to laboratory temperature and the vial content was mixed for 30 s. After thorough separation of aqueous and organic phases, the content was centrifuged (Universal 32R, Hettich, Germany) for 5 min at 4 000 rpm. The upper organic layer was poured into a 1.8 mL vial and evaporated under nitrogen flow. The remaining aqueous phase was added to 1 mL of hexane and the whole extraction process was repeated twice to achieve a quantitative ergosterol yield. The joint extracts were evaporated to the dry phase. The evaporation residue was finally dissolved in 400 µL mixture of methanol/toluene (75:25, v/v) and analysed with the use of liquid chromatography.

The actual determination of ergosterol took place in a HPLC reverse phase using a Zorbax SB-C18 column of size 4.6 × 30 mm at a particle size of 1.8 µm (Agilent Technologies, USA). The separation was carried out at laboratory temperature using isocratic elution – mobile phase with the composition of methanol/water of 97.5:2.5 (v/v) at a volumetric velocity of 0.6 mL min⁻¹. Ergosterol was detected in the ultraviolet zone at 282 nm. The dosed extract volume was 2 µL. To measure the calibration curve, a method of standard addition was used. Various amounts of a standard ergosterol solution were poured into seven vials, corresponding after conversion to a range of 0.1 to 1.000 µg g⁻¹ forage. The vial content was then evaporated by nitrogen flow. A forage amount of 250 mg was inserted into each vial. The eighth vial was filled with forage without the added standard. The following procedure was the same as in the common sample. Each point of the calibration set had three replications.

Phenolic compounds determination

Individual ground samples (0.5 g) were mixed with 10 mL of 75% solution of HPLC grade methanol (Sigma-Aldrich, St. Louis, MO, USA) in Falcon 15 mL Conical Centrifuge Tubes (Corning, Tewksbury, MA, USA). Extraction of samples was performed by Multi Reax shaker (Heidolph, Schwabach, Germany) for 24 h. Samples were centrifuged by MPW 223a centrifuge (MPW, Warsaw, Poland) for 10 min in the frequency of 1024 rpm. Supernatant from centrifuged samples was removed by Hypodermic syringe CHIRANA Luer (CHIRANA T. Injecta, Stará Turá, Slovak Republic) and filtered by Nalgene 25 mm Syringe Filters 0.45 µm (Thermo Fisher Scientific, Waltham, MA, USA). One mL of each filtrated sample was transferred to a 50 mL volumetric flask and diluted with 10 mL of distilled water. The volume of 1 mL of 2N Folin and Ciocalteu's phenol reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to samples, followed by 10 mL of 7% sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA) after 5 min. The volumetric flasks were then filled up with distilled water, mixed and left for 90 min.

Equally, calibration solutions were prepared with a gallic acid calibration curve (1, 0.5, 0.25, 0.1, 0.05 and 0.025 mg mL⁻¹). Distilled water was prepared by Ultrapure Simplicity Water Purification System type 1 (Merck Millipore, Billerica, MA, USA).

Samples were then placed in a quartz cell SM/Q/10 (Exacta + Optech, San Prospero, Italy). Measurement of visible absorbance in the wavelength of 765 nm was carried out by UV/VIS Spectrophotometer Lambda 25 (Perkin Elmer, Waltham, MA, USA). The concentrations of samples were calculated from the calibration line.

The concentration of original solid plant samples was calculated using the formula:

$$m = c_g \times V_c \times m_r \times V_{ex} / m_s \times V_p,$$

where m = weight of phenolic compounds in 100 g of original dry plant sample (g); c_g = concentration of phenolic acid in measured sample received from calculation from calibration line (g mL^{-1}); V_c = volume of calibration solutions used for reaction (1 mL); m_r = weight of reference sample used for calculation (100 g); V_{ex} = volume of extraction solution (10 mL); m_s = weight of sample used for extraction; V_p = volume of extracted sample used for reaction (1 mL). The total content of phenolic compounds was expressed as g gallic acid (GAE) kg^{-1} DM.

Statistical analyses

The data were processed using the statistical software STATISTICA ver. 12 (StatSoft, Inc.). The results were expressed as a mean \pm standard error of the mean (SEM). Differences with $P < 0.05$ were considered significant and determined multifactorial ANOVA test (in particular, Scheffé's test), which was applied for mean comparison.

Results

Silage Additives

The amounts of ERG and phenolic compounds in silages, within the same cut for each orchardgrass variety treated silages with biological and chemical additives compared to their respective controls, showed no differences. Therefore, the ERG and phenolic compounds data with additives and controls were presented in a single average, as will be described below (Tables 2 and 4).

Table 1
 Content of ergosterol (mg kg⁻¹ dry matter) in
 samples of fresh forage

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	6.6	23.8	29.9	39.5
Starly	5.6	5.3	28.1	32.5
Sw Luxor	0.0	14.7	26.7	29.4
Otello	0.0	5.6	20.1	28.5
Husar	0.0	8.1	30.9	22.8
Amera	5.7	16.2	15.4	30.1
Dika	0.0	13.0	22.0	23.9
Bepro	0.0	10.4	18.7	30.7
Dana	0.0	11.4	18.4	29.7
Vega	0.0	6.5	21.8	31.1

Table 2
Content of ergosterol (mg kg^{-1} dry matter) in orchardgrass silage

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	19.7 ± 3.5 ^{ab}	18.5 ± 2.7 ^{ab}	49.9 ± 2.5	77.8 ± 9.9
Starly	11.5 ± 4.0 ^{bc}	19.9 ± 1.1 ^{ab}	44.1 ± 2.2	67.3 ± 3.9
Sw Luxor	7.2 ± 2.4 ^{cd}	22.9 ± 1.6 ^{ab}	54.6 ± 5.7	62.7 ± 4.1
Otello	8.1 ± 2.0 ^{cd}	25.4 ± 1.6 ^a	48.1 ± 3.0	72.0 ± 7.1
Husar	3.4 ± 1.4 ^{cd}	23.3 ± 1.8 ^{ab}	53.9 ± 8.3	83.2 ± 4.4
Amera	23.5 ± 3.8 ^a	21.5 ± 1.9 ^{ab}	34.1 ± 3.6	68.2 ± 3.9
Dika	4.7 ± 0.7 ^{cd}	13.1 ± 3.1 ^b	44.0 ± 3.3	76.2 ± 3.2
Bepro	0.0 ± 0.0 ^d	14.4 ± 2.2 ^{ab}	53.7 ± 2.2	75.0 ± 3.5
Dana	19.7 ± 2.3 ^{ab}	23.6 ± 1.9 ^{ab}	44.8 ± 2.1	70.9 ± 4.6
Vega	6.9 ± 1.7 ^{cd}	18.2 ± 2.9 ^{ab}	50.2 ± 5.3	69.9 ± 1.2
p value	0.0001	0.0005	0.0540	0.1377
Silage Additives				
US	10.4 ± 1.5 ^b	19.3 ± 1.5 ^{ab}	42.7 ± 2.3 ^b	70.3 ± 2.6
BSA	6.9 ± 1.8 ^c	18.5 ± 1.5 ^b	49.7 ± 2.5 ^a	70.9 ± 2.7
CSA	14.1 ± 2.7 ^a	22.4 ± 1.1 ^a	50.8 ± 2.5 ^a	75.7 ± 3.2
p value	0.0001	0.0249	0.0088	0.1577
US: untreated silage; BSA: biological silage additives; CSA: chemical silage additives. Indices (^{a,b,c}) indicate significant differences ($P < 0.05$) determined by the Scheffé's test.				

Table 3
 Concentration of phenolic compounds (g gallic acid kg⁻¹ dry matter) in samples of fresh forage

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	33.9	19.6	40.4	26.4
Starly	33.6	27.5	38.1	25.2
Sw Luxor	26.3	19.0	53.0	27.1
Otello	22.4	14.7	59.6	21.2
Husar	26.1	22.3	63.1	23.7
Amera	39.3	19.1	58.0	21.9
Dika	23.3	20.7	54.1	21.6
Bepro	23.9	19.0	51.6	23.8
Dana	23.8	21.1	61.5	23.6
Vega	26.5	22.8	50.4	23.0

Table 4

Concentration of phenolic compounds (g gallic acid kg⁻¹ dry matter) in orchardgrass silage

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	41.0 ± 1.6 ^{ab}	26.7 ± 0.7 ^{ab}	51.2 ± 3.9	34.0 ± 1.1 ^a
Starly	36.6 ± 2.7 ^{abc}	29.4 ± 0.7 ^a	52.0 ± 3.3	33.3 ± 0.4 ^{ab}
Sw Luxor	29.0 ± 1.6 ^{bc}	23.0 ± 0.5 ^b	69.0 ± 6.9	29.6 ± 0.9 ^{abc}
Otello	26.3 ± 0.4 ^c	22.8 ± 0.9 ^b	76.8 ± 4.5	25.2 ± 0.8 ^c
Husar	29.3 ± 2.6 ^{bc}	26.4 ± 1.3 ^{ab}	75.7 ± 3.3	28.8 ± 0.3 ^{abc}
Amera	49.0 ± 1.2 ^a	24.1 ± 0.5 ^{ab}	76.2 ± 10.7	26.4 ± 0.6 ^c
Dika	29.7 ± 1.5 ^{bc}	23.0 ± 1.3 ^b	77.0 ± 1.4	28.8 ± 0.9 ^{abc}
Bepro	27.5 ± 4.5 ^{bc}	23.2 ± 1.1 ^b	66.4 ± 3.7	28.9 ± 1.2 ^{abc}
Dana	31.6 ± 0.7 ^{bc}	23.2 ± 0.7 ^b	82.3 ± 2.1	28.1 ± 0.5 ^{bc}
Vega	32.6 ± 0.5 ^{bc}	25.9 ± 0.3 ^{ab}	76.1 ± 1.7	28.4 ± 0.8 ^{bc}
p value	0.0001	0.0001	0.0017	0.0001
Silage Additives				
US	32.7 ± 2.0	24.0 ± 0.8	63.9 ± 3.6 ^a	30.1 ± 0.9
BSA	31.3 ± 2.6	24.7 ± 0.8	69.4 ± 3.6 ^{ab}	27.8 ± 0.9
CSA	35.7 ± 2.5	25.5 ± 0.7	77.5 ± 3.8 ^b	29.5 ± 0.9
p value	0.4241	0.4086	0.0439	0.1849
US: untreated silage; BSA: biological silage additives; CSA: chemical silage additives. Indices (^{a,b,c}) indicate significant differences (P < 0.05) determined by the Scheffé's test.				

Analysis of Ergosterol by HPLC

The ERG content in fresh forage of orchardgrass varieties gradually increased from the first cut of 2012 [5.6–6.6 mg kg⁻¹ dry matter (DM)] to the second one of 2013 (22.8–39.5 mg kg⁻¹ DM). In the first cut of 2012, ERG was detected only in Greenly, Starly and Amera varieties, being detected from the second cut of 2012 in all orchardgrass varieties. In 2013, Husar from the first cut, and Greenly, Starly, Amera, Bepro and Vega varieties from the second cut had a high concentration of ERG (> 30 mg kg⁻¹ DM) (Table 1).

As it is shown in Table 2, ERG content in silage differed between orchardgrass varieties and cuts, being higher in 2013 than 2012. In 2012, ERG content ranged from 3.4 to 23.5 mg kg⁻¹ DM in the first cut and 13.1 to 25.4 mg kg⁻¹ DM in the second cut. In the same year (2012), the varieties Amera (first cut) and Otello (second cut) returned the highest ERG content, with Husar (first cut) and Dika (second cut) the lowest ERG. Interestingly, concerning varieties, 'Bepro' proved to be a peculiar variety regarding its zero concentration of ERG (0.0 mg kg⁻¹ DM) in the first cut in 2012. In 2013, ERG content varied between 34.1 to 54.6 mg kg⁻¹ DM in the first cut and 62.7 to 83.2 mg kg⁻¹ DM in the second with no statistically significant differences among orchardgrass varieties.

The application of biological and chemical additives influenced the safety of silage. The hygienic quality and safety of silage groups treated with biological additives was improved in both cuts in 2012, decreasing the ERG content in comparison with untreated silage. In contrast, silages treated with chemical additives showed higher ERG content in both cuts in 2012 in comparison with untreated silage. Nevertheless, the silages treated with both types of additives presented higher ERG content in the first cut of 2013 in comparison to control, while in the second cut of 2013 there were no statistically significant differences in ERG content between silage treatments (Table 2).

Analysis of phenolic compounds by Folin-Ciocalteu assay

In the fresh cut the concentration of phenolic compounds was higher in the first cut in both year (2012 and 2013), where the first cut from 2013 showed higher concentration of phenolic compounds range from 38.1 to 63.1 g gallic acid per kg of DM (g GAE kg⁻¹ DM) in contrast to the others cuts (Table 3).

The concentration of phenolic compounds was no different between silage and fresh forage within the same cultivars. Nevertheless, the total phenolic concentrations showed differences ($P < 0.05$) among orchardgrass varieties, except in the first cut of 2013. Similarly, to ERG, a higher concentration of phenolic compounds was recorded in the silage group treated with chemical silage additives in comparison to control silages. However, there were no differences in phenolic compounds concentrations between the silage groups (treated with biological and chemical additives) in both years, except in the first cut of 2013, where the highest concentrations of these secondary metabolites (77.5 g GAE kg⁻¹ DM) was observed in the silages treated with chemical additives (Table 4).

Discussion

The adverse effects of fungal infestation of silage include allergic airway diseases due to spore inhalation and reduced palatability due to a 'mouldy' scent caused by the production of volatile organic compounds [33]. Moreover, some fungi can produce mycotoxins and are then called toxigenic. In the current silage-making practices, it is difficult if not impossible to avoid mycotoxin contamination of forage crops [34, 35]. Hence, ensiled forages may contain a mixture of mycotoxins, originating from pre-harvest contamination [36, 37] and or from postharvest contamination with toxigenic moulds that are

common in silage [35]. In consequence, in the market exist a large number of silage additives that aim to control silage fermentation and spoilage processes [38]. On one hand, biological additives (homo- and hetero-fermentative strains) which inhibited the growth of spoilage moulds and improve the shelf life of silage at feedout. On the another hand, chemical silage additives with stronger antifungal and antibacterial properties [39]. However, the positive effects of silage additives were not observed in this study in the orchardgrass silage within the same cut for each variety. The nature and intensity of the effect of silage additives may differ across plant species [40], suggesting that either biological or chemical additives no reduced the moulds presence in orchardgrass silage, as consequence were not differences in ERG and phenolic compounds content. Therefore, the use of the silage additives should never be regarded as a substitute for good silage-making practices [38].

ERG is the primary sterol present in the cell wall (membrane) of filamentous fungi [41, 42] used to determine the quantity of moulds [43]. As it is can be seen in Table 2, the augmentative tendency of ERG content found in the fresh forage samples of orchardgrass from 2012 to 2013 was related at the higher rainy conditions in the second year, contributing to the occurrence and development of fungi. The ERG increase at high relative humidity and lower temperature was previously corroborated by Kalač [44]. In addition, the high ERG content is related to the delayed harvest date [27] and the higher frequency of rains [45] at the timing of harvest. This explains why the ERG content was higher in the second cuts of each year than in the first ones. In 2012, 47 mm and 0.5 °C less of precipitation and temperature, respectively, were measured than in 2013 (Fig. 1). These parameters contributed to the higher incidence of fungi in the cuts of 2013, resulting in higher ERG content in fresh forage and silage than cuts of 2012.

This study showed that ERG content in silage increased considerably in comparison to the fresh forage of orchardgrass, thus, our findings are in good agreement with Skládanka et al. [46] which evidenced that ensilage process does not decrease the number of moulds, and hence a higher risk of mycotoxins production in the forage. Therefore, a high fungal infestation by moulds explain the significant correlations of ERG concentration in silage [2]. Fungal growth leads to a loss or reduction in nutrients and dry matter, and a lowering of palatability, with consumption generating losses in animal performance [15]. The importance of ensiled forage crops as sources of mycotoxins in the ruminant diet has been confirmed by numerous authors [2]. Hence, fungal spoilage and mycotoxin contamination are one of the greatest risks in silage.

The ergosterol content found in our study in the first cut of 2012 in the silage treated with biological additives was consistent with previous studies, where the ERG degradation was visible by the inoculation with bacterial additives [46]. While the use of chemical silage additives, based on organic acids and salts widely recommended in silage crops to limit the growth of fungi [39] have not a positive effect. Due to that a high ERG content evidenced that growth of fungi by chemical additives was not prevented in our orchardgrass silages. Hence, it can be suggested that the effect of silage additives in preventing growth of moulds and their metabolites is not always efficient, not even in (in vitro) experiments.

ERG concentrations in forage grasses can vary from 20 to 400 mg kg⁻¹ DM depending on the grass species (see review by Kalač [44]), as well as the variety within the species, as observed in this review for *Festulolium* [47]. Besides, Opitz von Boberfeld and Banzhaf proved [47] the lower the production of ERG content, the higher the quality of silage. Until today, it has not been established a safe limit for ERG content in silages, due to all depends on the mould species which are contaminating the silage, the mycotoxins that it produces and on the ensiled forage species. For example, 110 mg kg⁻¹ DM of ERG in *Festulolium* forage was considered low and this grass as being resistant to mildew infestation in comparison to 139.6 mg kg⁻¹ DM of ERG in *Arrhenatherum elatius* forage that was infected with a high content of zearalenone [48]. Furthermore, Skládanka et al. [49] suggested that ERG amounts (ranging from 3.8 to 190.8 mg kg⁻¹ DM) in different harvest dates (summer and winter) indicated high content of mycotoxins [e.g. deoxynivalenol (DON) and zearalenone (ZEN)] in summer period. As a result, this could indicate that the ERG amounts recorded in this study (ranging from 42.7 to 75.7 mg kg⁻¹ DM in 2013) cannot be considered safe for silage. Previous studies claim that in the case of toxin-forming fungi that occur in the environment, the results of analyses of ergosterol content in cereal grain usually demonstrate a significant correlation with mycotoxin concentrations found in grain crops [50, 51]. For example Cegielska-Radziejewska et al. [52] showed a statistically significant correlation between deoxynivalenol/ERG and total trichothecenes/ERG in poultry feeds. Consequently, high fungal infestation in mouldy parts may explain the high ERG concentration obtained in silage sub-samples [2]. In addition, Pietri et al. [53] reported that the quality of maize is acceptable if the level of ERG content is less than 3 mg kg⁻¹. There is a high possibility of fungal invasion and mycotoxin contamination if the level of ERG content exceeds more than 3 mg kg⁻¹. Because there are no specific regulations on mycotoxins in silage (e.g. grass silage, only for maize-based product guidance value is available), currently recommended levels for animal feed could also be considered as guidelines for silage [54]. Regarding silage the presence of DON and ZEN is recommended not to exceed 12 mg kg⁻¹ and 3 mg kg⁻¹, respectively [55]. Thus, our findings suggest that to determine the ERG content as a biochemical indicator is relevant to assess silage safety but does not allow the establishment of safe limits for ruminants.

Regarding silages safety, high phenolic compound concentration gives us a hint of mould presence as many plant tissues accumulate phenolic compounds on their cell walls on interactions with fungal pathogens. Therefore, the accumulation process constitutes a protective mechanism against cell wall degradation, similar to the barrier provided by lignins, limiting the spread of pathogens [56]. Nevertheless, their presence up to a certain threshold is actually considered to be positive, due to their antioxidant activity, ability to chelate metals, inhibit lipoxygenase and scavenge free radicals [57]. Besides, phenolic compounds play an important role in the synthesis of the biological mimic cell wall, where they may also inhibit the diffusion of extracellular enzymes and toxins, protecting it from degradation [56].

The concentration of total phenolic compounds in orchardgrass silage models ranged from 24.0 to 77.5 g GAE kg⁻¹ DM, which proved a safety issue with the silage, as the minimum phenolic compounds [condensed tannins (CT)] concentration needed to make forages bloat-safe has been proposed to be 5 g kg⁻¹ [58]. Consequently, high CT concentrations (> 55 g kg⁻¹ DM) reduce forage intake and digestibility

and depress rates of body and wool growth in ruminants [59]. Moreover, elevated doses of tannins can impair fibre digestion causing toxicosis in sheep [60]. Plants tend to produce complex mixtures of tannins and not all tannins have the same effects on feeding. The study of polyphenols (tannins) in animal production has primarily focused on CT, and little information is available on the effects of hydrolysable tannins (HT) in livestock production. Therefore, further research is required on the concentration of HT in silage grasses to provide a stronger basis and to prevent intoxications in animals ill-adapted to HT consumption [59].

Similar to ERG, the phenolic compounds concentration can vary between genotypes of the different varieties ensiled and to the experienced environmental [61]. In addition, the concentration of phenolic compounds depends on the type of analysed sources [62].

Nowadays there are better tools which can help us to understand which microorganisms and secondary plant metabolism are involved in the ensiling process over beyond of the fermentation. Despite this, the total concentration of phenolic compounds in orchardgrass has not been reported yet, therefore, further studies are required in these issues to determine the safe limits of phenolic compounds for ruminants in silages.

Conclusions

Biological additives used during ensiling in both cuts of 2012 effectively inhibited epiphytic bacteria and enable successful reduction the content of ERG and consequently moulds proliferation associated with silage production. While in the both cuts of 2013 its effect was not observed. In contrast, silage treated with chemical additives did not avoid ERG production in both years. For this reason, silage additives are not always successful at improving silage hygiene quality, because better-adapted epiphytic microflora might outcompete the additives and dominate the succeeding fermentation.

In consequence, the gradual increase of ERG in silages of orchardgrass between 2012 and 2013 evidenced that forage contamination by moulds simultaneously increased the concentration of phenolic compounds. As a result, this effect proved a progressive deterioration in silage safety, which could be harmful and adversely affect production and health of livestock. Therefore, the determination of ERG and phenolic compounds seem to be relevant as a rapid method for screening silage grass safety, since the animal production depends mainly on forage quality and silage safety.

Abbreviations

CCM: Czech collection of microorganisms; CFU: Colony forming units; CT: Condensed tannins; DM: Dry matter; DON: Deoxynivalenol; DSZM: German collection of microorganisms and cell cultures; ERG: Ergosterol; GAE: Gallic acid equivalent; HPLC: High-performance liquid chromatography; HT: Hydrolyzable tannins; N: Nitrogen; NCIMB: Scotland national collection of industrial and marine bacteria; PVC:

Polyvinyl chloride; SA: Silage additives; SEM: Standard error of the mean; US: Untreated silage; UV: Ultraviolet; UV/VIS: Ultraviolet-visible; ZEA: Zearalenone

Declarations

Acknowledgements

This paper is dedicated to the memory of our dear friend and colleague Ing. Eduardo Alfredo von Bennewitz Álvarez, Ph.D., who died, tragically, in 2019 while this manuscript was being drafted.

Authors' contributions

JEAM and JS conceived and designed the experiments. JEAM and JS collected the forage samples. JEAM, VD and JS performed the experiments. JEAM and JS analyzed the data. JEAM and VD contributed reagents/materials/analysis tools. JEAM, MK, TS and GRD drafted and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by the Czech National Agency for Agricultural Research (Grant No. QJ1310100 "Development and optimisation methods for the determination of biogenic amines in response to increasing health security of silage") and the project of the Ministry of Agriculture of the Czech Republic No. RO0416

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This study does not require ethical approval.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Crop Science, Breeding and Plant Medicine, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic.

²Department of Chemistry, Faculty of Science, Jan Evangelista Purkinje University in Ústí nad Labem, České mládeže 8, 400 96 Ústí nad Labem, Czech Republic.

³Division of Crop Genetics and Breeding, Crop Research Institute, Drnovská 507, 161 06 Prague, Czech Republic.

⁴Departamento de Química Analítica, Química Física e Ingeniería Química, Facultad de Ciencias, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, Alcalá de Henares, 28871 Madrid, Spain.

⁵Department of Animal Nutrition and Forage Production, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic.

References

1. Driehuis F, Spanjer MC, Scholten JM, Te Giffel MC (2008) Occurrence of mycotoxins in maize, grass and wheat silage for dairy cattle in the Netherlands. *Food Addit Contam Part B* 1:41–50
2. Tangni EK, Pussemier L, van Hove F (2013) Mycotoxin contaminating maize and grass silages for dairy cattle feeding: current state and challenges. *J Anim Sci Adv* 3:492–511
3. Stewart A V, Ellison NW (2011) *Dactylis*. In: Kole C (ed) *Wild Crop Relat. genomic Breed. Resour. - millets grasses*, 1st ed. Springer, Heidelberg, Germany, pp 73–87
4. Butkute B, Lemežiene N, Kanapeckas J, Navickas K, Dabkevičius Z, Venslauskas K (2014) Cocksfoot, tall fescue and reed canary grass: Dry matter yield, chemical composition and biomass convertibility to methane. *Biomass Bioenergy* 66:1–11
5. Turner LR, Donaghy DJ, Lane PA, Rawnsley RP (2007) A comparison of the establishment, productivity, and feed quality of four cocksfoot (*Dactylis glomerata* L.) and four brome (*Bromus* spp.) cultivars, under leaf stage based defoliation management. *Aust J Agric Res* 58:900–906
6. Sanada Y, Gras MC, van Santen E (2010) Cocksfoot. In: Boller B, Posselt UK, Veronesi F (eds) *Fodd. Crop. amenity grasses*, 1st ed. Springer, New York, USA, pp 317–328
7. Kochanowska-Bukowska Z (2001) Reaction of selected orchard grass (*Dactylis Glomerata* L.) cultivars to soil moisture. *Electron J Polish Agric Univ* 4:1–12
8. Sanada Y, Takai T, Yamada T (2007) Ecotypic variation of water-soluble carbohydrate concentration and winter hardiness in cocksfoot (*Dactylis glomerata* L.). *Euphytica* 153:267–280
9. Hauck B, Gallagher JA, Morris SM, Leemans D, Winters AL (2014) Soluble phenolic compounds in fresh and ensiled orchard grass (*Dactylis glomerata* L.), a common species in permanent pastures with potential as a biomass feedstock. *J Agric Food Chem* 62:468–475
10. Alba-Mejía JE, Skládanka J, Hilgert-Delgado A, Klíma M, Knot P, Doležal P, Horký P (2016) The effect of biological and chemical additives on the chemical composition and fermentation process of *Dactylis glomerata* silage. *Spanish J Agric Res* 14:1–12
11. Trenberth KE (2011) Changes in precipitation with climate change. *Clim Res* 47:123–138

12. Staniak M, Kocoń A (2015) Forage grasses under drought stress in conditions of Poland. *Acta Physiol Plant* 37:116
13. Tyrolová Y, Výborná A (2011) The effects of wilting and biological and chemical additives on the fermentation process in field pea silage. *Czech J Anim Sci* 56:427–432
14. Weinberg ZG, Muck RE (1996) New trends and opportunities in the development and use inoculants for silage. *FEMS Microbiol Rev* 19:53–68
15. O'Brien M, Egan D, O'kiely P, Forristal PD, Doohan FM, Fuller HT (2008) Morphological and molecular characterisation of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. *Mycol Res* 112:921–932
16. Milićević DR, Škrinjar M, Baltić T (2010) Real and perceived risks for mycotoxin contamination in foods and feeds: challenges for food safety control. *Toxins (Basel)* 2:572–592
17. Van Pamel E, Verbeken A, Vlaemynck G, De Boever J, Daeseleire E (2011) Ultrahigh-performance liquid chromatographic-tandem mass spectrometric multimycotoxin method for quantitating 26 mycotoxins in maize silage. *J Agric Food Chem* 59:9747–9755
18. Seppälä A, Heikkilä T, Mäki M, Rinne M (2016) Effects of additives on the fermentation and aerobic stability of grass silages and total mixed rations. *Grass Forage Sci* 71:458–471
19. Muck RE (2013) Recent advances in silage microbiology Recent microbial techniques. *Agric Food Sci* 22:3–15
20. Vella FM, Laratta B (2017) UV-based evaluation of ergosterol for monitoring the fungal exposure in Italian buffalo farms. *FEMS Microbiol Lett* 364:1–6
21. Gourama H, Bullerman LB (1995) Detection of molds in foods and feeds: Potential rapid and selective methods. *J Food Prot* 58:1389–1394
22. Thammawong M, Okadome H, Shiina T, Nakagawa H, Nagashima H, Nakajima T, Kushiro M (2011) Distinct distribution of deoxynivalenol, nivalenol, and ergosterol in fusarium-infected japanese soft red winter wheat milling fractions. *Mycopathologia* 172:323–330
23. Mohd As'wad AW, Sariah M, Paterson RRM, Zainal Abidin MA, Lima N (2011) Ergosterol analyses of oil palm seedlings and plants infected with *Ganoderma*. *Crop Prot* 30:1438–1442
24. Pitt JI, Hocking AD (2009) The ecology of fungal food spoilage. In: Pitt JI, Hocking AD (eds) *Fungi Food Spoilage*, 3rd ed. Springer Science + Business Media, New York, USA, pp 3–9
25. Schnürer J (1993) Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. *Appl Environ Microbiol* 59:552–555
26. Matcham SE, Jordan BR, Wood DA (1985) Estimation of fungal biomass in a solid substrate by three independent methods. *Appl Microbiol Biotechnol* 21:108–112
27. Japelt RB, Didion T, Smedsgaard J, Jakobsen J (2011) Seasonal variation of provitamin D2 and vitamin D2 in perennial ryegrass (*Lolium perenne* L.). *J Agric Food Chem* 59:10907–10912
28. Golinski P, Opitz Von Boberfeld W, Kostecki M, Kaczmarek Z, Golinski PK (2006) Accumulation of secondary metabolites formed by field fungi in autumn-saved herbage. *J Agron Crop Sci* 192:344–

29. Lattanzio V (2013) Phenolic Compounds: introduction. In: Ramawat KG, Mérillon JM (eds) *Nat. Prod. Phytochem. Bot. Metab. alkaloids, phenolics terpenes*, 1st ed. Springer Berlin Heidelberg, Heidelberg, Germany, pp 1543–1580
30. Scalbert A, Johnson IT, Saltmarsh M (2005) Polyphenols: antioxidants and beyond. *Am J Clin Nutr* 81:215–217
31. Antolovich M, Prenzler P, Robards K, Ryan D (2000) Sample preparation in the determination of phenolic compounds in fruits. *Analyst* 125:989–1009
32. Carpenter R, O’Grady MN, O’Callaghan YC, O’Brien NM, Kerry JP (2007) Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Sci* 76:604–610
33. Wambacq E, Vanhoutte I, Audenaert K, De Gelder L, Haesaert G (2016) Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: A review. *J Sci Food Agric* 96:2284–2302
34. Dell’orto V, Baldi G, Cheli F (2015) Mycotoxins in silage: Checkpoints for effective management and control. *World Mycotoxin J* 8:603–617
35. Alonso VA, Pereyra CM, Keller LAM, Dalcerro AM, Rosa CAR, Chiacchiera SM, Cavaglieri LR (2013) Fungi and mycotoxins in silage: An overview. *J Appl Microbiol* 115:637–643
36. Gallo A, Giuberti G, Frisvad JC, Bertuzzi T, Nielsen KF (2015) Review on mycotoxin issues in ruminants: Occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. *Toxins (Basel)* 7:3057–3111
37. Uegaki R, Tsukiboshi T, Tohno M (2013) Changes in the concentrations of fumonisin, deoxynivalenol and zearalenone in corn silage during ensilage. *Anim Sci J* 84:656–662
38. Driehuis F, Oude Elferink SJWH (2000) The impact of the quality of silage on animal health and food safety: A review. *Vet Q* 22:212–216
39. Wilkinson JM (2005) *Silage*, 1st ed. Chalcombe Publications, Lincoln, UK
40. Tyrolová Y, Bartoň L, Loučka R (2017) Effects of biological and chemical additives on fermentation progress in maize silage. *Czech J Anim Sci* 62:306–312
41. Seitz LM, Sauer DB, Burroughs R, Mohr HE, Hubbard JD (1979) Ergosterol as a measure of fungal growth. *Physiol Biochem* 69:1202–1203
42. Stanis E, Zgoła-Grześkowiak A, Waśkiewicz A, Stępień Ł, Beszterda M (2015) Can ergosterol be an indicator of fusarium fungi and mycotoxins in cereal products? *J Braz Chem Soc* 26:705–712
43. Gutarowska B, Skóra J, Pielech-Przybylska K (2015) Evaluation of ergosterol content in the air of various environments. *Aerobiologia (Bologna)* 31:33–44
44. Kalač P (2012) Carotenoids, ergosterol and tocopherols in fresh and preserved herbage and their transfer to bovine milk fat and adipose tissues: A review. *J Agrobiol* 29:1–13
45. Opitz von Boberfeld W, Banzhaf K, Hrabě F, Skládanka J, Kozłowski S, Goliński P, Szemán L, Tasi J (2006) Effect of different agronomical measures on yield and quality of autumn saved herbage

- during winter grazing - 2nd Communication: Crude protein, energy and ergosterol concentration. Czech J Anim Sci 51:271–277
46. Skládanka J, Nedělník J, Doležal P, Lindušková H, Nawrath A (2012) Influence of forage species and preservation additives on quality and mycotoxins safety of silages. *Acta Fytotech Zootech* 15:50–54
 47. Opitz von Boberfeld W, Banzhaf K (2006) Forage quality of different × *Festulolium* cultivars in winter. *J Agron Crop Sci* 192:239–247
 48. Skládanka J, Dohnal V, Doležal P, Ježková A, Zeman L (2009) Factors affecting the content of ergosterol and zearalenone in selected grass species at the end of the growing season. *Acta Vet Brno* 78:353–360
 49. Skládanka J, Nedělník J, Adam V, Doležal P, Moravcová H, Dohnal V (2011) Forage as a primary source of mycotoxins in animal diets. *Int J Environ Res Public Health* 8:37–50
 50. Saxena J, Munimbazi C, Bullerman LB (2001) Relationship of mould count, ergosterol and ochratoxin A production. *Int J Food Microbiol* 71:29–34
 51. Stuper-Szablewska K, Perkowski J (2017) Level of contamination with mycobiota and contents of mycotoxins from the group of trichothecenes in grain of wheat, oats, barley, rye and triticale harvested in Poland in 2006–2008. *Ann Agric Environ Med* 24:49–55
 52. Cegielska-Radziejewska R, Stuper K, Szablewski T (2013) Microflora and mycotoxin contamination in poultry feed mixtures from western Poland. *Ann Agric Environ Med* 20:30–35
 53. Pietri A, Bertuzzi T, Pallaroni L, Piva G (2004) Occurrence of mycotoxins and ergosterol in maize harvested over 5 years in Northern Italy. *Food Addit Contam* 21:479–487
 54. Cheli F, Campagnoli A, Dell’Orto V (2013) Fungal populations and mycotoxins in silages: From occurrence to analysis. *Anim Feed Sci Technol* 183:1–16
 55. Panasiuk L, Jedziniak P, Pietruszka K, Piatkowska M, Bocian L (2018) Frequency and levels of regulated and emerging mycotoxins in silage in Poland. *Mycotoxin Res* 2002:1–9
 56. Baruah JB (2011) Polyphenolic compounds: nature materials. In: Baruah JB (ed) *Chem. Phenolic Compd.*, 1st ed. Nova Science Publishers, New York, USA, pp 1–6
 57. Martínez-Valverde I, Periago MJ, Ros G (2000) Significado nutricional de los compuestos fenólicos de la dieta. *Arch Latinoam Nutr* 50:5–18
 58. Li Y-G, Tanner G, Larkin P (1996) The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J Sci Food Agric* 70:89–101
 59. Piluzza G, Sulas L, Bullitta S (2014) Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. *Grass Forage Sci* 69:32–48
 60. Hervás G, Frutos P, Javier Giráldez F, Mantecón ÁR, Álvarez Del Pino MC (2003) Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. *Anim Feed Sci Technol* 109:65–78
 61. Solar A, Colarič M, Usenik V, Stampar F (2006) Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.). *Plant Sci* 170:453–461

Figures

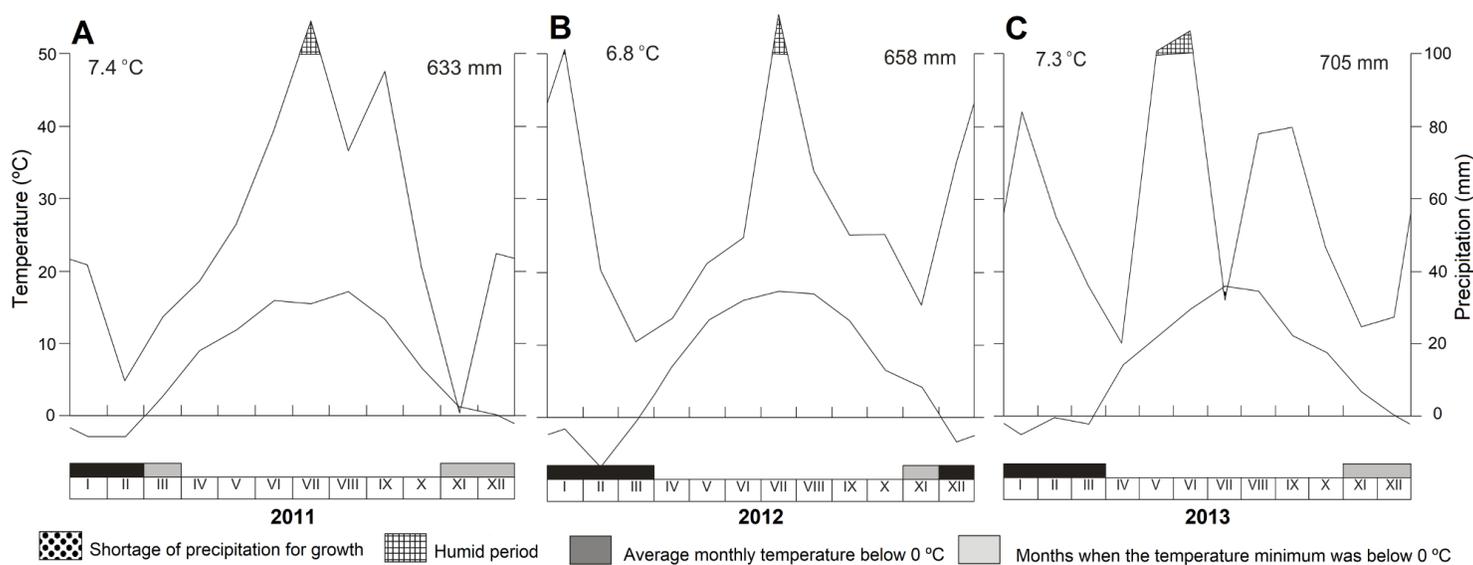


Figure 1

Precipitation and temperatures in years (A) 2011, (B) 2012 and (C) 2013 at Research Station