

Proximate Composition and Spatio-temporal Heterogeneity of Phytochemicals in *Agave sisalana* (sisal) adapted in different agro-ecological zones of Punjab, Pakistan

Sobia Shahzad (✉ sobiafahd@gmail.com)

University of Agriculture Faisalabad

Mumtaz Hussain

University of Agriculture Faisalabad

Hassan Munir

University of Agriculture Faisalabad

Muhammad Arfan

University of Agriculture Faisalabad

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Abstract

Exploring extractable phytochemicals from locally adapted sisal plant vegetation vary seasonally at different locations. This study elaborated proximate composition and phytochemical heterogeneity in sisal due to varying environmental conditions analyzed from five districts, i.e., Chakwal, Khushab, Rawalpindi, Faisalabad, and Layyah in Punjab, Pakistan. Extensive surveying and plant sampling across two years 2017-18 and 2018-19, during mid-spring, summer, autumn, and winter seasons were carried out for understanding the seasonal impact on sisal. The present study was designed in a randomized complete block design (RCBD) and analyzed considering seasonal, yearly, and locational impact. The spatial differences in phytochemicals concentration were strongly associated with environmental conditions prevailing in different seasons. Autumn season reflected saponins, tannins, and flavonoids in higher concentrations during 2018-19 while steroids and terpenoids were higher during spring 2018-19. While Spatio-temporal variations in the proximate analysis were more apparent in different samples collected from different districts. Data recorded for the Khushab district and autumn season reflected the higher composition of a proximate analysis and phytochemical contents as compared to other seasons. Overall, the spatial differences in phytochemicals concentration were strongly associated with soils and environmental conditions prevailing in different seasons in selected districts.

Introduction

Sisal (*Agave sisalana* Perrine) is a xerophytic, perennial, hapaxanthic monocotyledonous, Asparagaceae succulent plant, which yields hard fiber of commercial value, and is the 6th most important fiber crop in the world, and nearly 75% of the global production of hard natural fibers originates only from sisal plants (Davis and Long, 2015; Duarte *et al.*, 2018; Nikam *et al.*, 2019). It is currently cultivated on a large scale in Mexico (native), Central America, Brazil, Asia, and Africa (Coleman-Derr *et al.*, 2016). It is a monocarpic plant that forms an inflorescence after 6 to 9 years then dies (Asfaw, 2011). Sisal reproduction is mainly asexual via suckers originating from the rhizomes and bulbils (Monja-Mio *et al.*, 2019). It has been reported to constitute various biochemical, phytochemical, and antioxidant such as sugars (Arrizon *et al.*, 2010), and a considerable amount of alkaloidal amines, flavonoids, sterols, steroidal alkaloids, and sapogenins, glycosides, terpenoids, tannins, saponins, and flavonoids (Debnath *et al.*, 2010; Ajayi *et al.*, 2011), most of which demonstrated to possess analgesic, anti-inflammatory, anthelmintic, gastroprotective, antioxidant, antiviral, antimycotic, antibacterial, antituberculosis, bactericidal and insecticidal activities (Hamissa *et al.*, 2012; Viel *et al.*, 2017).

Proximate analysis revealed that sisal pulp had a crude protein of 7.3% and a crude fiber of 15.2% (Gebremariam and Machin (2008), and its fiber has high durability, strength, ability to stretch, resistance to deteriorate, interaction with dyestuff (Gonzalez *et al.* 2015), main products derived from this fiber are biodegradable yarns used in handicrafts, production of upholstery and tequilas, ropes of various utilities, pulp for cellulose industry, decorative carpets, animal feed, organic fertilizer, biofertilizers, and bagging (Elanchezhian *et al.*, 2018; de Melo *et al.*, 2019). The aggregations observed in SEM micrographs of sisal

bundles are attributed to $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ crystals responsible for the high calcium content of the sisal ashes (Benitez-Guerrero *et al.*, 2017).

The spatial variability changes in time due to variations in climatic conditions through the years (van Leeuwen, 2010). Su *et al.* (2018) reported that plant growth and nutrient status changed with spatiotemporal variability. Sisal utilizes the Crassulacean acid metabolism (CAM) pathway for fixation of CO_2 (Abraham *et al.*, 2016), and thick leaves have a waxy cuticle layer and are arranged around the stem in a spiral shape which forms a rosette that helps in water retention (Holtum *et al.*, 2014; Davis *et al.*, 2017) and also provides an adaptive advantage in a vast range of geographical zones because of its resistance to abiotic and biotic factors such as extreme temperatures, drought, salinity, and pathogens (Tamayo-Ordóñez *et al.*, 2016; Sarwar *et al.*, 2020). The information regarding the effect of spatiotemporal variability on the proximate analysis and phytochemicals of sisal is still scarce and scanty and provokes new research demands. Based on this, a parallel study was conducted to explore the proximate analysis and phytochemicals of sisal collected from different ecoregions of Punjab, Pakistan, which could provide the precision management of soil nutrients, planting environment suitable for fiber quality, and explore upcoming possibilities for improving phytochemicals in sisal; having prime importance in the medical industry.

Materials And Methods

The study was executed to find Spatio-temporal variations in the phytochemical attributes of sisal (*Agave sisalana* Perrine) collected from three sites of all five selected districts of Punjab, Pakistan, as in Table 1. In each district, three natural vegetation sites were identified and surveyed randomly to collect plant samples during mid of all four seasons (spring, summer, winter, and autumn) repeated in two years study duration. Phytochemical aspects of sisal plant samples were determined using appropriate analytical techniques.

- **Phytochemical Screening**
 - **Saponin determination**

Saponin content was determined by using the prescribed protocol of Nahapetian and Bassiri, (1975). Took 20 g of plant material and put it in 200 ml of 20% ethanol, and the suspension was continuously stirred while heated at 55°C over a hot water bath for 4 h. Approximately at 90°C, the collective extractions were 40 mL after reduction. In this extract, 20 mL of diethyl ether was added and was shaken well after transferring this solution into a 250 mL funnel. Finally, the ether layer was discarded while the aqueous layer remained recovered. Purification was repetitive hence, and 60 mL of n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride. After heating and evaporation, the residual material was dehydrated, and the final calculation was taken in percentage.

- **Determination of total flavonoid content**

Using the method of Chen *et al.* (1993), flavonoid contents were determined. Diluted solution comprising of 1 mL flavonoids, 10 mL of 30% (v/v) ethanol and 0.7 mL of 5% (w/w) NaNO_2 were homogenized, stirred for 5 min, then 0.7 mL of 10% AlCl_3 (w/w) was also added. After that, the mixture was stirred, and 5 mL of 1 mol. L^{-1} NaOH was added. Afterward, the solution was diluted to 25 mL with 30% (v/v) ethanol before the measurement. After 10 min standing, the absorbance of the solution was measured at 500 nm using UV-Vis Spectrophotometer (T60, PG Instruments, UK). The contents of flavonoids were expressed in mg rutin g^{-1} dry weight by comparison with the rutin standard curve, and the yield of flavonoids was calculated using the following formula:

$$Y = (6.404A + 0.2806) BV/W \text{ (mg/g)}$$

where: A – absorbance (500 nm)

B – dilution factor

W – dry weight (g)

V – volume of the extract (ml)

Rutin curve was used for exploiting different mM concentrations of rutin bioflavonoid against its absorbance as described by Payum *et al.* (2013).

- **Estimation of Steroids**

Extracted leaf sample (1 mL) having steroid solution was shifted into volumetric flasks of 10 mL, sulfuric acid (4 N, 2 mL) and iron (III) chloride (0.5% w/v, 2 mL), tracked by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 mL). After 30 min of heating and occasional shaking, the mixture was diluted up to mark and exposed to 780 nm for absorbance in contrast to reagent blank. Steroids were estimated by the method described by (Madhu *et al.*, 2016).

- **Triterpenoid**

Leaf Sample (10 mg) was weighed and independently dissolved in 1 mL of methanol and then added 100 μL vanillin-glacial acetic acid (150 μL , 5% w/v) and perchloric acid solution (500 μL). The sample solution was heated at 60°C for 45 min and then cooled in an ice-water bath, and its absorbance was measured at 548 nm. Ursolic acid (0.025–0.5 mg mL^{-1} in methanol) was used as a standard through a modified method of Chang and Lin (2012).

- **Tannins determination**

Tannins were measured by Folin-Ciocalteu's Phenol Reagent method. About 0.1 mL of the sample extract was added to a volumetric flask (10 mL) containing 7.5 mL of distilled water and 0.5 mL of Folin & Ciocalteu's Phenol Reagent, 1 mL of 35% sodium carbonate solution, and diluted to 10 mL with distilled

water. After 30 min, its absorbance was measured at 700 nm with a UV-Visible spectrophotometer. The tannic acid was used as standard as described by Kavitha and Indira (2016).

2.2. Proximate analysis

The proximate analyses of samples were carried out by the AOAC (2010) procedure and formula.

2.2.1 Determination of moisture content (%)

For moisture content, juice of (10 mL) was evaporated on a water bath in a petri-dish and further heated at 100°C for some time till constant weight was gained. The difference in weight before and after drying gave the moisture content of the sample.

2.2.2. Determination of ash content

The juice (10 mL) obtained from the sample was taken in silica crucible until white ash was formed by heating it in a muffle furnace (600°C). The crucible was made cool by putting it into a desiccator, and then the weight of the ash was obtained.

2.2.3. Determination of crude protein by Kjeldahl's method

A crushed leaf sample (0.5 g) was taken, and 10 mL of concentrated H_2SO_4 along with 10 g of catalyst was added to a digestion tube. After making it cool, it was transferred to a 50 mL volumetric flask. Finally, the volume was made to 50 mL with distilled water. Now 25 mL of boric acid solution was taken in a 250 mL conical flask. The receiving flask was placed in such a way that the outlet of the condenser of the apparatus got dipped into the boric acid solution. 5 mL of acid digestion sample was transferred to the steam chamber along with 5-8 mL of 40% NaOH to the aliquot of digestion sample followed by passing through the steam chamber to distill ammonia till boric acid containing flask changed its color from red to greenish-blue. Now the receiving flask was removed, and the condenser outlet was rinsed into the receiving flask with distilled water. The receiving flask content was titrated against 0.01 N H_2SO_4 till the greenish-blue color changed to electric grey. A blank preparation was run, which was identically prepared except that it did not contain the sample. The amount of nitrogen was calculated using the formula: **see formula 1 in the supplementary files.**

2.2.4. Crude fiber

Under boiling conditions sample was treated initially with 1.25% sodium hydroxide, and then 1.25% H_2SO_4 was used to dissolve alkali and acid-soluble components existing in it. The remainder sample comprising crude fiber was dehydrated to a persistent weight at 500°C. Loss of weight was calculated to determine crude fiber content.

See formula 2 in the supplementary files.

Statistical Analysis:

The experimental design was a randomized complete block design (RCBD) with three factorial arrangements. The data was analyzed statistically by Fisher's Analysis of Variance technique using honestly significant difference (HSD) as mentioned by Steel *et al.* (1997) and with the help of software Statistix version 8.1.

Results And Discussion

Phytochemical analysis

Saponins

Saponins contents determined in sisal leaves elucidated significant ($P < 0.01$), effects of Season, while variations among Locations and the interaction among Season and location was non-significant ($P > 0.05$), during 2017 and 2018. On the other hand, temporal interaction between seasons and years was found statistically significant. For years, saponins were insignificantly different during the year 2017-18, while significant variation in saponin contents of sisal leaves was found during the year 2018-19. Similarly, the analysis revealed a greater accumulation of saponin content during autumn 2018 which was significantly higher than the saponin values recorded during the rest of the seasons and years. The highest value of saponin (2.44%) was recorded in autumn-2018 while the lowest value (0.26%) was recorded during spring-2017 (Fig.2a)

Steroids

Steroids contents determined in sisal leaves showed significant effects of Season during 2018, while non-significant ($P > 0.05$) variations were seen during 2017. Besides, variations among Locations and the interaction among Season and location were non-significant during 2017 and 2018. The highest steroid contents (3.1%) were recorded during spring 2019 followed by the autumn season. Results obtained for steroids were at par during summer season 2018. The lowest steroid contents (0.82%) were recorded during autumn 2017 (Fig. 2b). Faisalabad and Rawalpindi districts revealed a profoundly higher concentration of steroids, while the lowest content was observed in sisal samples collected from district Layyah

Flavonoids

The flavonoids variation among the Seasons x Locations and their interactions were found insignificant ($P > 0.05$) during both years (2017-2018). Fig.2c. depicted a significantly higher concentration of flavonoids recorded during the year 2018-2019 as compared to the 2017-18 experimental year. On the other hand, flavonoids were found significantly higher (7.20 mg g^{-1}) during autumn 2018 followed by (6.52 mg g^{-1}) during summer 2018. Sisal plants accumulated more flavonoids under summer and autumn 2018 conditions, but a non-significant difference was found across the seasons during the year

2017. Samples collected from Chakwal and Rawalpindi districts revealed a profoundly higher concentration of flavonoids (5.37 mg g^{-1}) and (5.34 mg g^{-1}), respectively while the lowest content (5.0 mg g^{-1}) was observed in samples of district Layyah (Fig.2c).

Terpenoids

Non-significant ($P>0.05$), variation was recorded in individual effects of Season and Location during 2017, while significant ($P<0.01$) differences were recorded during 2018. Besides the statistical interaction between Seasons x Locations during 2017 and 2018 was non-significant ($P>0.05$). Besides, terpenoids were found significantly higher during 2019 spring followed by 2018 autumn. For years $\times$ Locations, significantly higher concentrations of terpenoids were recorded during years 2018-2019 as compared to 2017-18 and at locations of Chakwal, Khushab and Rawalpindi. Whereas Faisalabad and Layyah locations were found with lesser terpenoids contents in the sisal plants native to these sites. Samples collected from Khushab district exhibited profoundly higher concentrations of terpenoids (2.539 mg UA/g) during autumn 2018 while the lowest value of terpenoids (1.3075 mg UA/g) was also observed in Khushab site during the year, 2017 (Fig.2d).

Tannins

Significant ($P<0.01$) variation was recorded in individual effects of Season, while non-significant ($P>0.05$) in Season and Locations and their interaction during 2017, while the individual effect of factors and the interaction among them was Significant ($P<0.01$) during 2018. whereas, results obtained from different locations were non-significant for sisal leaf tannins contents, while, it was significantly higher (2.22 mg of tannic acid/g) when assessed during summer, 2018 followed by the value (2.08 mg of tannic acid/g) in samples of sisal leaves obtained and assessed during autumn 2018. Sisal plants accumulated more tannins during summer and autumn 2018, however, their concentrations during the experimental year 2017-18 were comparatively less (Fig.2f).

Proximate analysis

Ash (%)

Statistical results for Ash content exposed Significant ($P<0.01$) variation in individual effects of Season, while non-significant ($P>0.05$) in Season and Locations and their interaction during 2017, while the individual effect of factors and the interaction among them was Significant ($P<0.01$) during 2018. Results elaborated the highest ash contents in sisal leaves from District Rawalpindi (3.03) during spring season followed by Khushab (2.81) in spring, all the districts depicted statistically different values for all seasons and locations. Data exhibited lower values for all sites during the winter season. The least value (0.90) was observed during the winter season at the district Faisalabad site which remained at par for all locations. Results for years interaction with seasons depicted significant variability for interaction as depicted in Fig.3a. The summer season of the year 2018-19 expressed higher ash content (3.48) followed by spring (3.32) which was at par, but autumn and winter seasons followed respectively in descending

order. Fig.2a elaborated the significant variation of the year 2017-18 and 2018-19. The least value of ash content (1.05) was observed during winter season 2017-18 and a similar trend was recorded for all seasons of the year 2018-19.

Moisture content (%):

Interaction across the seasons i.e. (Locations $\times$ Seasons) was found to be significant ($P < 0.01$) during 2017 and 2018. Results depicted the greatest accumulation of water in sisal leaves were from District Khushab (94.10) during spring season followed by Rawalpindi (93.71) in winter, All the districts were statistically similar. Data exhibited lower values for Faisalabad and Layyah sites during the autumn season but statistically, they were at par. Sisal leaf samples elaborated the similar trend in all seasons except autumn for all sites. The least value (82.33) was observed during the autumn season at the district Layyah site which remained at par with the autumn season for Faisalabad location. Data pertaining to years' interaction with seasons exhibited significant variability (Fig.2b). Spring season of year 2018-19 depicted higher moisture content (94.64) followed by winter, summer and autumn seasons respectively. Fig.3b revealed the significant variation of years 2017-18 and 2018-19. The least value of moisture content (87.57) was obtained in autumn 2017-18 and similar was recorded for the year 2018-19 except the autumn seasons, however, other seasons depicted a similar trend during year 2017-18.

Crude protein (or Kjeldahl protein)

Interaction across Locations $\times$ Seasons was found significant ($P < 0.01$) during 2017 and 2018. Crude protein content exposed showed the highest value (16.99) during winter 2018-19 in district Rawalpindi while it remained statistically at par for district Khushab for winter 2018-19. For the year 2017-18 variable trend was observed for all four seasons Spring, summer, winter and autumn, on the other hand, year 2018-19 also expressed significant variation for all seasons. Synthesis of crude protein expressed better results with a descending trend during the year 2017-18 for *Agave sisalana* in winter, summer, spring, and autumn for the year 2017-18, respectively. The lowest value (9.37) was recorded for Layyah site during autumn 2017-18 (Fig.3c).

Crude fiber:

Interaction across the seasons i.e. (Locations $\times$ Seasons) was non-significant ($P > 0.01$) during 2017 and 2018. Results elaborated the highest crude fiber contents in sisal leaves collected from District Layyah (14.07) during summer season followed by a similar trend across all districts. Data exhibited lower values for all sites during autumn season. The least value (9.39) was observed during autumn season at the district Layyah site followed by Faisalabad, Rawalpindi, Chakwal and Khushab sites respectively. The data for years interaction with seasons depicted significant variability, and summer season of the year 2018-19 exposed higher crude fiber content (14.71) followed by spring (13.218) and winter (12.96) which were at par statistically for the year 2018-19, the autumn and winter seasons of both years remained similar. The least value of crude fiber content (10.71) was observed in autumn season of both years (Fig.3d)

Discussion

This study showed that secondary metabolites present in the leaf juice were rich in tannins, saponins, flavonoids, steroids, and terpenoids. Ajayi *et al.* (2011) reported extensive prevalence of a variety of phytochemicals, saponins, tannins, flavonoids, and steroids in sisal juice. Saponins, tannins, and flavonoids were in higher concentration during autumn 2018-19. Szakiel *et al.* (2011) reported that temperature is a very important factor influencing phytochemical synthesis in plants. However, temperature extremes can be the most prominent cause of environmental stress, the elevated temperature was found to reduce photosynthetic rates (by 52% as compared to control plants), stomatal conductance (by 60%) that may indirectly affect the synthesis of phytochemicals in sisal.

The season with the lowest soil and air temperatures, higher relative humidity, and higher soil water availability accumulated fewer flavonoids. Similar findings were reported by Mogren *et al.* (2006) who also found a high correlation between global radiation and levels of quercetin in onion bulbs. Some results indicate that the synthesis of saponins and other phytochemicals is higher in response to stress, suggesting that these compounds could be involved in the adaptation of the plant to survive under adverse soil and climatic conditions (Copaja *et al.*, 2003).

Steroids and terpenoids were high in spring 2018-19, moreover, the terpenoids were found dominant across the years. Conformity of the above results was reported by Onwuliri and Wonany (2005). As per the present study results, summer and autumn seasons were found to affect tannins significantly in sisal leaves. Such a response could be an inference of increased humidity during mid to late summers with a relevant impact on sisal growth in early autumn. Similarly, autumn and summer seasons also triggered the flavonoid concentrations under the arid conditions of Chakwal and Rawalpindi districts whereas, regions understudy with lesser aridity but more soil fertility, i.e., Faisalabad and Khushab followed them. Moderate to high temperatures in autumn with the instance of humidity resulted in more prevalence of flavonoids, however, harshly low temperatures negated flavonoids concentration in the leaves of sisal, i.e., in severe winters.

Tannins and flavonoids were among significant phenolic compounds because of their weighty molecular mass and affinity towards water for solubilization and performing chaperonage to retain moisture. These tannins act as antioxidants by sustaining protein and sugars by bonding with them under adverse conditions that don't let them be available for degradation either by active oxygen species (AOS) or by microbes. Hence, their importance also engulfs anti-microbial significance. High summer and autumn temperatures may cause degradation of protein and lipids in addition to the flaccidity of sisal leaves. However, sisal plants may sustain their growth by accumulating exceptional concentrations of tannins, which may serve extensively to adapt successfully under local conditions, particularly in limited water availability, resulting in an exceptional prevalence of tannins at the plant level (Jouany and Morgavi., 2007).

As far as the ash contents were concerned in sisal leaves, the prevalence was reported ranging between 0.4% to 0.92%. Sisal leaves reportedly increased hydrophilicity under certain drier conditions with the help

of higher ash contents and vice versa. Besides, their incorporation at a certain maturity or in the form of bulbils drop, and the addition of nonviable bulbils in the soil will help chock the soil pore spaces and help sustain water, a sort of mulch (Nair, 2019). Similarly, in the present study, lower values for all sites during the winter season were observed. The least value (0.90) was observed during winter season at district Faisalabad which remained at par with winter season values for all locations. Results for years interaction with seasons depicted significant variability for interaction as in Fig.3a. Plantation of sisal like xeric plants gives impetus to let barren, under-utilized, and marginal lands be productive even to a level of subsistence. Farthest benefits of sisal plantation comprised of dietary, pharmaceutical, and cosmetic uses. Arid to semiarid plantation of sisal was a lucrative character based on its photosynthetic and water use efficient behavior (Fig.3b). Crude protein referred to the quality of leaves of sisal concerning its dietary handling for animal consumption as well as for pharmaceutical uses. Temperature and spatial variations for sisal plantation were reflected in the crude protein assays as proteins were highly degradable under high temperature and dry conditions. Whereas, desiccation had not affected when coupled with lower to moderate temperatures (Verhoeven *et al.*, 2018). Such variation on seasonal basis exposed less emphasis of the sisal plant towards protein biosynthesis under moderate weather conditions which seemed logical and in agreement with findings of this research (Fig.3c). Leaf fiber is the chief remarkable motive after leaf pulp for which it is adapted in a variety of climatic conditions. Sisal fibers were cellulosic and much stronger in nature (Barreto *et al.*, 2011). By applying ANOVA for fiber tensile strength, fiber length, leaf yield and leaf length (data collected on the bases of study sites) show the significant result at $P < 0.0001$ and the data collected regarding the block of the same parameters also statistically significant at the same probability level. The data for years interaction with seasons depicted significant variability, and summer season of the year 2018-19 exposed higher crude fiber content (14.71) followed by spring (13.218) and winter (12.96) which were at par statistically for the year 2018-19, the autumn and winter seasons of both years remained similar. The least value of crude fiber content (10.71) was observed in autumn season of both years (Fig.3d). In conclusion, it has been concluded that various pharmaceutical value components present in *Agave sisalana*, and this potential could be exploited by growing this plant in marginal lands for commercial extraction of these chemicals. In crux, Spatio-temporal variations are a key source of phytochemicals concentration in Punjab, Pakistan.

Declarations

- Ethics approval and consent to participate

All co authors have given consent to corresponding author to submit on behalf of them. No ethical studies are required in this plant study.

- Consent for publication

Not Applicable

- Availability of data and materials

Not Applicable

- Competing interests

"The authors declare that they have no competing interests.

- Funding

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- Authors' contributions

SS- is the main executer of this research, who sampled, analyzed and travelled to different areas in different seasons for the collection of plant samples.

MH-acted as supervisor of study, he planned the thematic layout, assessment of seasonal variation as well as spatio temporal assessments, provided lab facilities and helped with field studies as well.

HM- played important role in funding provision for travelling, sampling, laboratory chemical analysis, statistical analysis and eco-physiological aspect studies along with guidelines to synchronize the project and this study objectives achievements.

MA- acted as co supervisor of the study, helped with data analysis in his lab, data interpretation and analytical work guidance for phytochemical assessment.

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- Authors' information (optional)

Authors belong to University of Agriculture Faisalabad, their professional profiles are also available on university website and can be searched at www.uaf.edu.pk

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Table 1

Due to technical limitations, Table 1 is only available as a download in the supplementary files section.

Figures

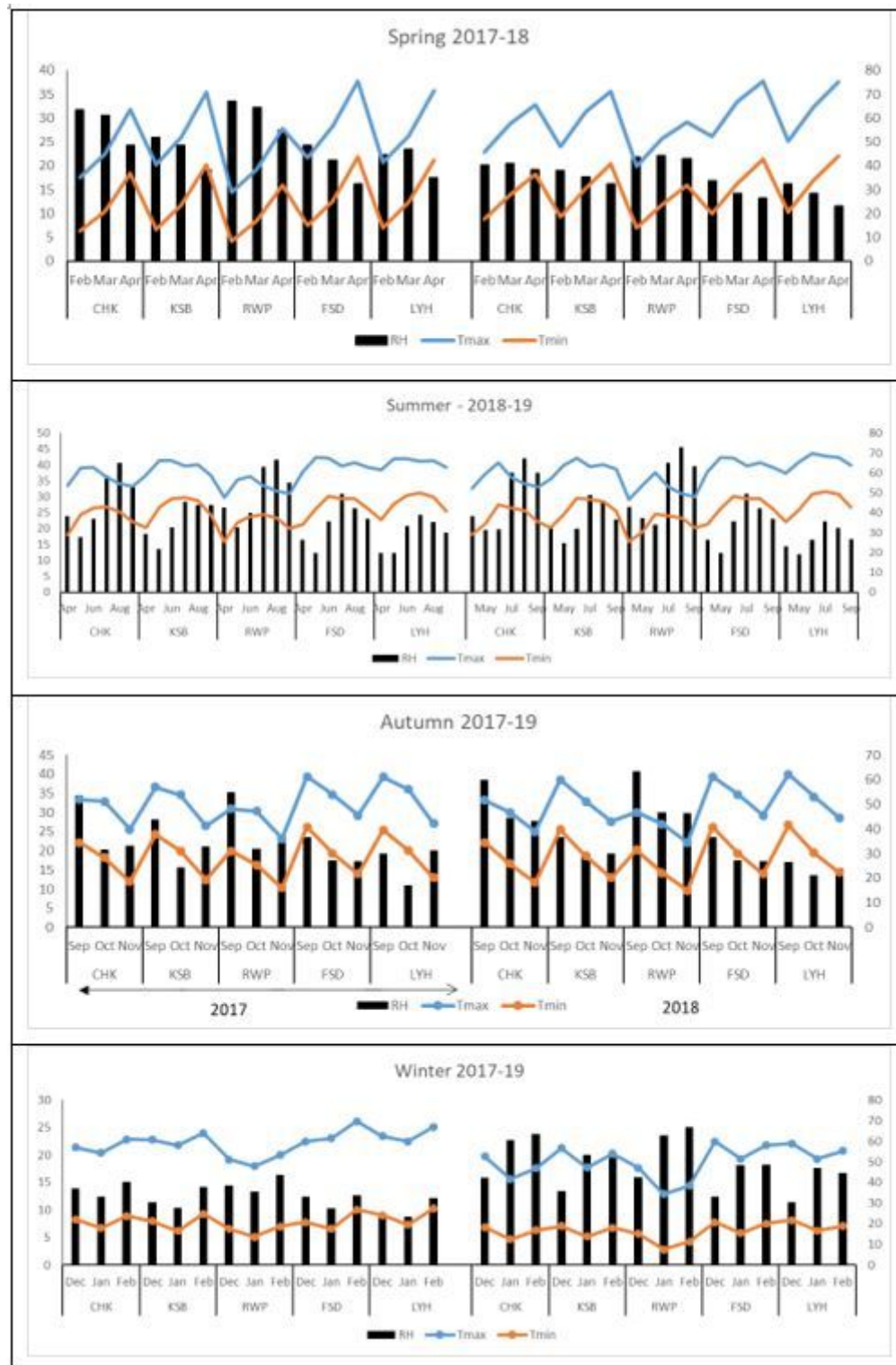


Figure 1

Weather data of two years and four seasons during 2017-19 from study districts

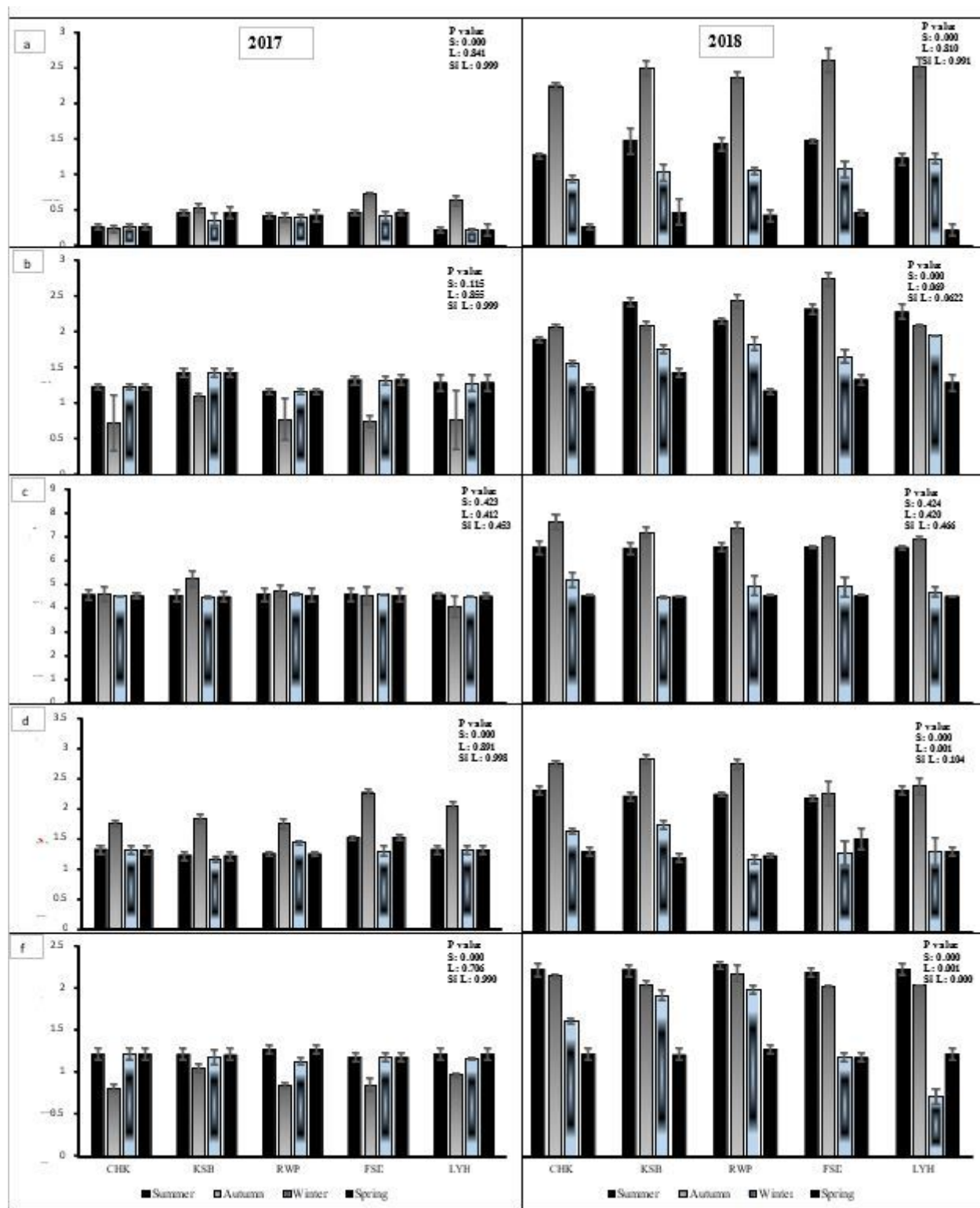


Figure 2

Photochemical analysis of sisal leaves as affected by different seasons of years and location. S (season), L (locations).

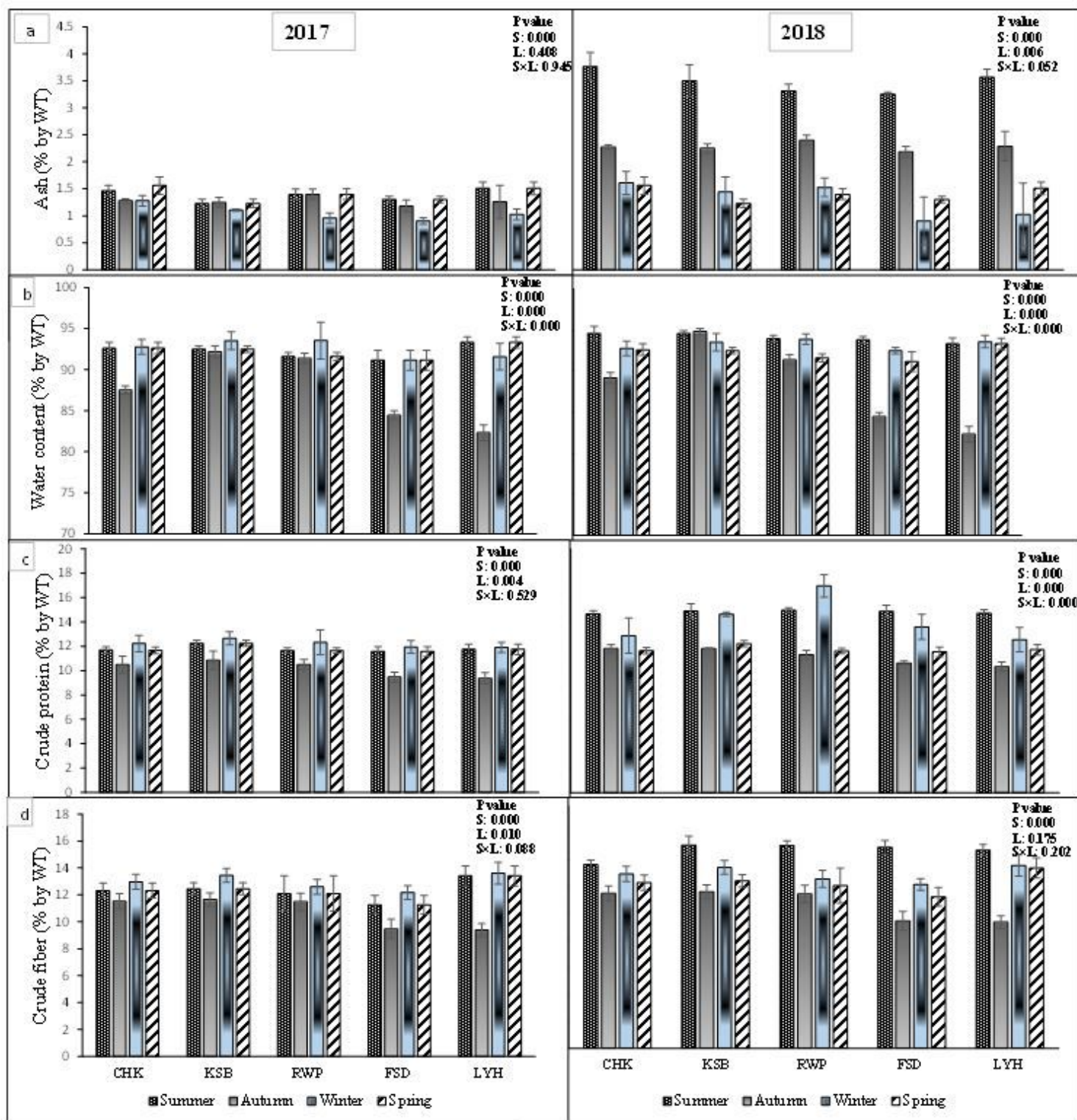


Figure 3

Proximate analysis of sisal leaves as affected by different seasons of years and location. S (season), L (locations).

Supplementary Files

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