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Effect of terroir on the glucosinolate content of *Moringa oleifera* grown in three agro-ecological zones of Ghana

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Summary

Environmental factors and cultural practices significantly influence the secondary metabolites in plants, e.g., glucosinolates, depending on the cultivar of each species. The present study analyzed the influence of specific environmental factors (e.g., temperature, rainfall, and relative humidity), elevation, and fertilization (i.e., nitrogen and sulfur) on the glucosinolate content in leaves of wild-grown *Moringa oleifera* from three agro-ecological zones in Ghana and selected *M. oleifera* accessions cultivated under semi-controlled field conditions.

The results showed that climate did not significantly influence total glucosinolate content in leaves of both wild-grown and cultivated accessions of *M. oleifera*, while elevation significantly influenced the total glucosinolate content of wild-grown plants. Fertilization had no significant impact on the total glucosinolate content of the cultivated accessions. Furthermore, wild-collected *M. oleifera* leaves from the three agro-ecological zones did not reveal a significant difference in their total glucosinolate content. For the cultivated accessions of *M. oleifera*, the agro-ecological zone, harvest time, and accession and the interactions among these factors significantly influenced the total glucosinolate content.

The results suggest that selecting suitable accessions, choosing suitable locations, and applying appropriate cultivation practices could contribute to optimizing the production of health-promoting *Moringa* plants with special emphasis on glucosinolate content.

Keywords: agro-ecological zone, climate, fertilization, glucosinolates, *Moringa oleifera* accession

Introduction

Moringa oleifera Lam. has been described as a multipurpose perennial plant with high nutritional and medicinal value. The high nutritional value is attributed to high contents of protein, vitamin A, and iron in the plants' leaves compared to other nutritionally valuable legumes such as soybeans (Makkar and Becker, 1997; Rweyemamu, 2006). Additionally, it has high contents of vitamin C, vitamin B complex, as well as micro-, and macronutrients such as potassium (K), calcium (Ca), magnesium (Mg), selenium (Se), and zinc (Zn) (Fuglie, 2001). Due to these benefits, *M. oleifera* is a highly valuable food source in tropical and sub-tropical areas and has been included in many nutritional programs (Fuglie, 2001; Thurber and Fahey, 2009). Besides its high nutritional value, the different morphological plant parts of *M. oleifera,* especially the leaves, comprise health-promoting compounds with medicinal benefits (Bharali and Azad, 2003; Oluduro et al., 2010; Waterman et al., 2014). These medicinal benefits, including anti-inflammation (Waterman et al., 2014), anti-bacterial (Oluduro et al., 2010), and anti-carcinogenic effects (Bharali and Azad, 2003), are often traced back to the secondary plant metabolites, especially glucosinolates (GS) and their breakdown products (Bharali and Azad, 2003; Oluduro et al., 2010). GS provide plants with unique survival or adaptive strategies (Variyar et al., 2014) and are classified as aliphatic, aromatic, and indolic compounds, depending on the respective amino acid precursor, i.e., methionine, tryptophan, and phenylalanine (Fahey et al., 2001). High contents of aromatic GS have been identified in the leaves of *M. oleifera*, namely, 4-α-rhamnopyranosyloxy-benzyl GS (GS1) and three isomers of acetyl-4-α-rhamnopyranosyloxy-benzyl GS (GS2, GS3, and GS4) (Bennett et al., 2003).

The profile, i.e., the content and composition, of secondary metabolites such as GS in plants is influenced by the environmental and cultural conditions of the plant (Zandalinas et al., 2012). For instance, GS content in Brassicaceae is known to be influenced by temperature, relative humidity, and radiation (Bohinc and Trdan, 2012). The variations in GS profile are also associated with soil type and water supply (Fenwick and Heaney, 1983). Moreover, for the effect of fertilization on GS levels, authors of a previous study have suggested that sulfur (S) supply to broccoli has a strong influence on the nitrogen (N) availability, and hence on GS concentration in the plant (Schonhof et al., 2007a). In contrast, it was found that S supply, regardless of the N content, has no significant influence on GS content in different ecotypes of *M. oleifera* (Förster et al., 2015).

The effects of environmental factors and growing conditions on GS content in plants are species-specific and even cultivar-specific (Charron et al., 2005; Bohinc and Trdan, 2012). The latter has been confirmed by studies on multiple accessions of *M. oleifera* cultivated under field conditions (Doerr et al., 2009) and for ecotypes cultivated under greenhouse conditions (Förster et al., 2015). So far, studies on *Moringa* have not been replicated across different geographical environments (e.g., in tropical areas) that are known to support the optimal growth of the plant. Meanwhile, these different geographical environments have different abiotic and biotic factors, which could modulate the GS content in the plant. Thus, there is limited information on the impact of eco-physiological parameters on the GS dynamics in *M. oleifera*, i.e., the impact of environmental conditions at different agro-ecological zones in Ghana on the GS

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profile of *M. oleifera* plants. Moreover, no study has been conducted so far on the GS profile of wild-grown *M. oleifera* in Ghana. Therefore, the objectives of the present study were to determine the influence of specific environmental factors (e.g., temperature, rainfall, and relative humidity), elevation, and soil minerals (i.e., N and S) on the GS content in leaves of wild-grown *M. oleifera* from three selected agro-ecological zones in Ghana, and in the leaves of selected *M. oleifera* accessions cultivated under semi-controlled field conditions. Moreover, the study was designed to determine the impact of the agro-ecological zone, the selection of accessions, fertilization, and harvest time on the total GS content in leaves of the selected *M. oleifera* accessions.

Materials and methods

Description of the agro-ecological zones

Three agro-ecological zones in Ghana were selected for the study: Guinea savannah, Transitional forest, and Deciduous forest. Whereas the Guinea savannah zone is located within the northern regions of Ghana, the Transitional and Deciduous forest zones span from the East to the West along the middle portions of the country. The Transitional zone spans from the middle portions towards the northern part of the country, while the Deciduous forest zone stretches from the middle portions towards the southern part of the country.

The Guinea savannah zone records a unimodal rainfall pattern, which starts in April and ends in September/October with a mean annual rainfall of 1100 mm and a minimum temperature of 15 °C in January and a maximum of 42 °C in March (Food and Agriculture Organization of the United Nations (FAO, 2005). The rainfall pattern of the Deciduous forest zone is bi-modal, with the major rainy season starting in early March, reaching its peak in June, and tapering off gradually through July. The minor season begins in late August and reaches its peak in September/November. The mean annual rainfall is 1500 mm, and the mean monthly temperature is between 24 °C in August and 30 °C in March (FAO, 2005). The Transitional zone is characterised by a bi-modal rainfall pattern with a mean annual rainfall of 1300 mm (FAO, 2005). Temperatures in the zone range between 17 and 33 °C, with the lowest recorded in August and the highest in December, January, and February (Nketia et al., 2018).

Collection of wild-grown *Moringa oleifera* leaves

Three collection points within each agro-ecological zone were selected for the study (Tab. 1). *M. oleifera* leaflets were collected from five mature plants in each of the three collection points for each of the agro-ecological zones in June 2017. The five plants' leaflets were thoroughly mixed to form a composite for each collection point. Leaflets from the pinnae were harvested as described by Tetteh et al. (2019) from the lower, mid, and upper parts of the tree canopy. Harvested leaf material was transported over a period of 2 h, 4 h, and 8 h from the harvest locations of Deciduous forest, Transitional and Guinea savannah zones, respectively, to the laboratory in the Department of Food Science and Technology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Upon arrival, the leaves were oven-dried at 40 °C for 48 h. All samples were packaged in airtight and waterproof packages and stored in a -20 °C freezer prior to GS analysis.

Moringa oleifera cultivation under semi-controlled conditions

The effects of elevation, temperature, relative humidity, as well as soil minerals and fertilizer application on the GS profile of *M. oleifera* accessions cultivated in the three agro-ecological zones in Ghana were investigated. The point values, representing the experimental fields for the *M. oleifera* cultivation within the agro-ecological zones of Ghana, were recorded (Tab. 1) using a GPS device (Garmin GPS 60, model 010-00322-01, USA).

Tab. 1: GPS coordinates and elevations for the collection points

Agro-ecological zone	GPS coordinates	Elevation [m]
For wild-grown Moringa oleifera		
Transitional zone	N7 16.175 W2 50.950	281
	N7 26.866 W2 34.729	322
	N7 18.955 W2 18.356	300
Deciduous forest zone	N6 43.963 W1 28.436	277
	N6 41.458 W1 37.639	261
	N6 30.460 W1 32.396	252
Guinea savannah zone	N9 29.902 W0 53.971	169
	N9 26.961 W0 51.885	188
	N9 25.333 W0 51.140	191
For the cultivated accessions of Moringa oleifera		
Transitional zone	N7 16.421 W2 48.039	315
Deciduous forest zone	N6 40.650 W1 33.980	264
Guinea savannah zone	N9 35.432 W1 01.667	109

For this experiment, three accessions of *M. oleifera,* including 1) 16016FL-161A PKM 1 (origin: India), 2) 04024FL-041A (origin: Kenya), and 3) 08025FL-081D (origin: Uganda) were used. All accessions were obtained from the Educational Concerns for Haiti Organization (ECHO), Florida, USA.

A total plot size of 15 × 12 m^2 was demarcated, and soil was prepared to a fluffy state in all three experimental fields. Each field was divided into two blocks as fertilized and unfertilized blocks. Further, each block was divided into nine subplots of 1 m^2, each with 0.6 m wide walkways in between the individual plots and borders. A 0.8 m wide border was created around the 18 subplots.

Plants were sown in a Randomised Complete Block Design factorial field layout, and each accession was replicated three times. The seeds were sown directly without any pre-treatment at a 0.2 m × 0.2 m spacing on each of the 1 m^2 subplots at the three experimental fields in February 2017. They were sown at 0.02 m depth with one seed per drill. In total 36 plants per subplot were planted (250,000 plants/ha) and watered soon after sowing in all three locations. *M. oleifera* landrace (local accessions) were sown on the 0.8 m wide borders. The borders were created around the treatment plots (accessions) at each experimental field as a transition zone to prevent predators, pests, and diseases that may directly have a negative impact on the densely populated experimental plants. Germination generally occurred within a maximum of 10 days after sowing at all the experimental fields, and the needed refills on the plots were done with extra seedlings, which were grown specifically for refilling. The seedlings were then left to grow without any treatment for another 14 days before the poultry manure compost was applied. Watering was done every other day on each of the three experimental fields, except on days with rainfall.

Deep litter poultry manure was collected from Aban Farms in Dormaa Ahenkro, Ghana, and analysed for N using the Kjeldahl method (DIN-ISO-13878, 1998), while the other minerals and organic matter composition were determined as described by Motsara and Roy (2008). The mean contents (±standard deviations) were: 1.08 ± 0.01% N, 2.89 ± 0.02% P, 2.04 ± 0.08 cmol/kg K, 1.70 ± 0.01 cmol/kg Na, 4.26 ± 0.03 cmol/kg Ca, 0.42 ± 0.01 cmol/kg Mg, 1.30 ± 0.14 cmol/kg H^+, 0.14 ± 0.01 cmol/kg Al, 0.25 ± 0.01% S, 34.67 ± 0.37% organic carbon, 6.17 ± 0.11 pH, and 59.77 ± 0.78% organic matter.

The poultry manure was then composted for 21 days while turning (aeration) every three days. After 14 days of seed germination, 5 kg

of the decomposed manure were applied per 1 m^2 subplot (50 tons/ha) on the block marked for fertilization. The poultry manure compost was worked into the soil around the seedlings on the subplot and watered afterward.

Harvest and postharvest preparation

At the first harvest of *M. oleifera*, 70 days after sowing, leaf material was manually cut at 0.4 m above ground level (Fig. 1). The leaflets from the plants on each subplot were harvested separately. A composite of leaflets from each subplot was collected into perforated A4-sized envelopes and put on ice packs in ice chests to keep the leaf material at a cold temperature during transportation to the laboratory for drying. Upon arrival in the laboratory, the leaflets were oven-dried at 40 °C for 48 h. The oven-dried samples were kept in a freezer (-18 °C) prior to the GS analysis. Samples were sent to Germany within one month for GS analysis.

After the first harvest, another round of 5 kg per 1 m^2 poultry manure compost was applied to the fertilized plots in all locations. Plants were left to regrow uninterrupted for a period of 35 days until the second harvest. Leaf samples were prepared in the same procedure as described for the first harvest.

Climate data

With respect to the wild-grown *M. oleifera* plants, climatological data for the three agro-ecological zones was obtained from the Ghana Meteorological Agency. Daily climatic data for five months (February to June 2017) of rainfall (mm/year), relative humidity (%), as well as minimum and maximum temperature (°C) recorded before the harvest of *M. oleifera* leaf material, were used. The relative humidity data for the Transitional zone were not available in the climatological dataset from the Ghana Meteorological Agency. The data obtained for the cultivated *M. oleifera* accessions at the same agro-ecological zone were hence also used for the wild-collected variants. Means (± standard deviation) of each climate variables' daily records were calculated (Tab. 2).

For the cultivated accessions of *M. oleifera* at the experimental fields, relative humidity (%) and temperature (°C) were recorded during the experimental period, from February to June 2017 with HOBO data logger (U23 Series Pro v2 Loggers, Onset company, Bourne, MA) within 30 min intervals. The devices were set up at the experimental fields in the Transitional and the Deciduous forest zones, whereas daily records for relative humidity and temperature for the experimental field in the Guinea savannah zone were provided by the Potsdam Institute for Climate Impact Research, Germany. The mean

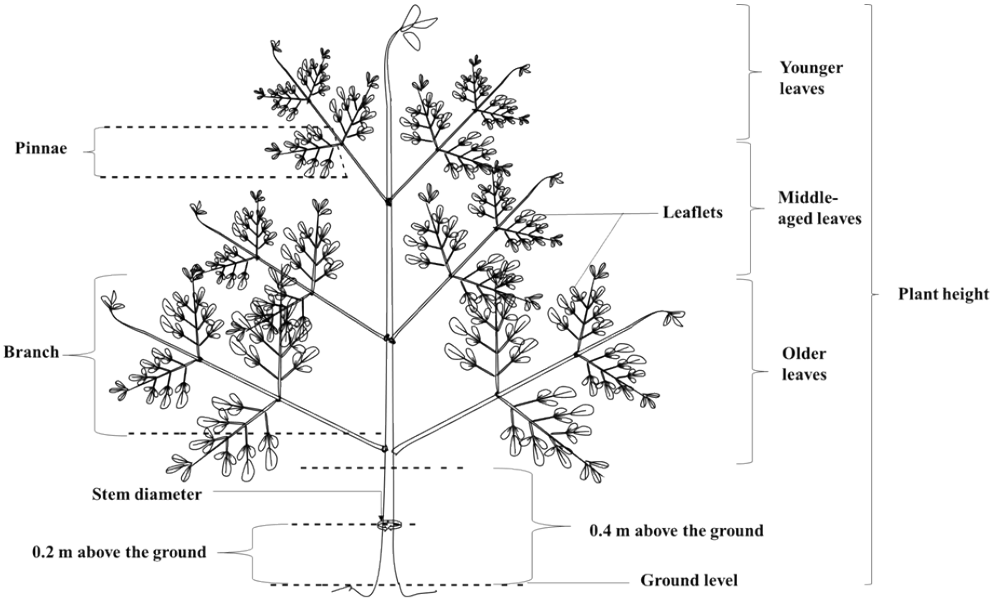

Fig. 1: *Moringa oleifera* morphological sections used for the experiments (source: TETTEH et al., 2019 with modifications)

Tab. 2: Temperature (maximum and minimum), rainfall, and relative humidity recorded daily from the three agro-ecological zones in Ghana.

Agro-ecological zone	Mean daily maximum temperature [ºC]	Mean daily minimum temperature [ºC]	Mean daily relative humidity [%]	Mean daily rainfall [mm]
A				
Transitional zone	34.57 ± 2.60 [a]	23.18 ± 1.28 [c]	74.27 ± 9.60 [a]	4.32 ± 8.99 [a]
Deciduous Forest zone	33.21 ± 1.98 [b]	23.78 ± 1.25 [b]	73.14 ± 14.95 [a]	5.40 ± 10.56 [a]
Guinea savannah zone	35.16 ± 2.88 [a]	25.44 ± 2.10 [a]	63.87 ± 14.87 [b]	3.52 ± 8.34 [a]
B				
Transitional zone	31.96 ± 3.00 [b]	25.71 ± 1.52 [a]	74.27 ± 9.60 [a]	-
Deciduous forest zone	31.25 ± 2.12 [b]	26.33 ± 1.38 [a]	72.60 ± 11.54 [a]	-
Guinea savannah zone	33.87 ± 9.49 [a]	24.88 ± 6.85 [b]	58.10 ± 15.84 [b]	-

The results presented in the table are mean ± standard deviation of daily climate data recorded from February to June 2017. The mean values were compared using one-way ANOVA followed by Tukey's test, $p \leq 0.05$; same lowercase letters in the same column indicate no significant differences in means of climate data among the agro-ecological zones for wild-grown *M. oleifera* **(A)** and cultivated accessions of *M. oleifera* under semi-controlled field conditions **(B)**.

(± standard deviation) of daily climate variables in the three experimental fields within the three agro-ecological zones for *M. oleifera* cultivation were calculated (Tab. 2).

Soil collection

For wild-grown *M. oleifera* plants, topsoil was collected with a hand auger from 0 - 1 m depth around the mature plants. Soils collected around the plants from the three collection points within each agro-ecological zone were mixed as composite soil. The individual composite soils from the three agro-ecological zones were packed in airtight and waterproof containers and transported to the soil science laboratory in the Department of Agriculture, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, for soil mineral analysis.

Prior to the cultivation of *M. oleifera* accessions at the three experimental fields, soil samples were taken with a hand auger from a depth of 0 - 1 m from all the three experimental fields for routine analysis (Tab. 3). Soil samples were also taken at the first and second harvest times of the experiment at five spots on each of the subplots for all the experimental fields in the three agro-ecological zones. The soils from the same accessions from either fertilized or non-fertilized blocks were mixed as composite soil and packaged and transported as described for the soil samples from the wild-collected *M. oleifera* plants.

Tab. 3: Soil composition of the three experimental fields prior to the cultivation of *Moringa oleifera* accessions (mean ± standard deviation).

Mineral/ physicochemical	Agro-eclological zone		
	Transitional zone	Deciduous forest zone	Guinea savannah zone
N [%]	0.13 ± 0.007	0.15 ± 0.014	0.04 ± 0.007
P [%]	0.49 ± 0.014	2.31 ± 0.014	0.15 ± 0.003
S [%]	0.42 ± 0.008	0.25 ± 0.008	0.07 ± 0.004
K [cmol/kg]	0.35 ± 0.007	0.46 ± 0.021	0.08 ± 0.007
Na [cmol/kg]	0.46 ± 0.014	0.53 ± 0.007	0.49 ± 0.014
Ca [cmol/kg]	7.18 ± 0.035	5.86 ± 0.085	3.06 ± 0.085
Mg [cmol/kg]	4.15 ± 0.071	0.85 ± 0.071	1.18 ± 0.035
H^+ [cmol/kg]	0.66 ± 0.014	0.66 ± 0.021	2.35 ± 0.007
Al [cmol/kg]	0.58 ± 0.035	0.52 ± 0.021	0.83 ± 0.021
Cation Exchange Capacity [cmol/kg]	13.37 ± 0.134	8.86 ± 0.141	7.97 ± 0.028
Organic matter [%]	6.51 ± 0.146	3.34 ± 0.079	1.56 ± 0.147
Organic carbon [%]	3.78 ± 0.085	1.94 ± 0.046	0.90 ± 0.086
pH	6.59 ± 0.042	6.10 ± 0.057	5.12 ± 0.014
Sand [%]	50.88	72.80	70.96
Clay [%]	24.00	16.12	7.92
Silt [%]	25.12	11.08	21.12
Textural class	sandy clay loam	sandy loam	sandy loam

Soil analysis

The particle size analysis of the soil was carried out using the hydrometer method (BOUYOUCOS, 1962), and the textural class was determined (MOTSARA and ROY, 2008). Organic matter content was determined by loss of weight on ignition with the aid of Muffle furnace (Naberthem, model L9/11/SKM, Germany), and the soil pH level was determined with an electrode (WTW pH meter, model pH3110, Germany) in 5:1 water to soil extract (MOTSARA and ROY, 2008). Nitrogen (N) was determined by the Kjeldahl method (DIN-ISO-13878, 1998). To determine sulfur (S) in the soil, the Turbidometric method using a spectrophotometer was applied (Buck scientific model 280 G). The determination was done after S extraction with $CaCl_2$ and HNO_3 (MOTSARA and ROY, 2008). The method employed in the analysis of phosphorous (P) was based on the production of a blue complex of molybdate and thiophoshate in an acid solution where P was determined spectrophotometrically (Buck Scientific, model 280 G, USA) at 630 nm (MOTSARA and ROY, 2008). Exchangeable bases determination (potassium (K) and sodium (Na)) was determined using the Volumetric Sodium Tetraphenyl Boron method after dry ashing digestion of the soil, and the composite samples were analysed with a flame photometer (Jenway, model PFP7, UK) (MOTSARA and ROY, 2008). All soil analyses were conducted in duplicate.

For soil samples collected prior to the cultivation of the *M. oleifera* accessions, the means were calculated for the various soil properties analysed (Tab. 3). Due to the strong influence of N and S on the biosynthesis of GS, N and S contents were determined in the soil samples of the wild-grown (Tab. 4) and the cultivated accessions of *M. oleifera* plants (Tab. 5).

Tab. 4: Mineral composition of soils collected from the three agro-ecological zones.

Agro-ecological zone	N [%]	S [%]
Transitional zone	0.13 ± 0.06 [a]	0.40 ± 0.11 [a]
Deciduous forest zone	0.14 ± 0.03 [a]	0.36 ± 0.09 [a]
Guinea savannah zone	0.06 ± 0.03 [a]	0.41 ± 0.26 [a]

Results presented in the table are mean ± standard deviation of soil minerals. The mean values were compared using one-way ANOVA, $p \leq 0.05$; same lowercase letters in the same column indicate no significant difference in the means of the soil minerals from the three agro-ecological zones.

Tab. 5: Mineral composition of soil collected for *Moringa oleifera* accessions cultivated.

Fertilization	N [%]	S [%]
Fertilized	0.14 ± 0.05 [a]	0.2 ± 0.10 [a]
Unfertilized	0.11 ± 0.05 [b]	0.21 ± 0.11 [a]

Results presented in the table are mean ± standard deviation of soil minerals of fertilized and unfertilized soils at first and second harvest. The mean values were compared using the t-test, $p \leq 0.05$; same lowercase letters in the same column indicate no significant difference in the means of the soil minerals of fertilized and unfertilized soils from the experimental fields.

Extraction and analysis of intact glucosinolates

Intact GS in the leaves were extracted according to the method described by FÖRSTER et al. (2015). For the extraction, 20 mg of dried and ground leaf material was used. Leaf material was extracted in 750 µL of 70% methanol (75 °C) and 100 µl of internal standard (1 mM Sinigrin, 2-propenyl GS, isolated from *Brassica juncea* seeds), followed by two additional re-extraction steps with 500 µL of the 70% methanol. The collected supernatants were concentrated in a vacuum concentrator (Thermo Scientific Savent SPD111V Concentrator, vacuum pump: Vacuumbrand PC 3001 series, CVC 3000) to a final volume of 150 µl. A volume of 200 µl of 0.4 M barium acetate was added to each tube and made up to 1 mL with ultrapure water (MilliQ quality). Samples were incubated for 30 min at room temperature and centrifuged at 16,000 g (Thermo Scientific, Heraeus Megafuge 11R Centrifuge) for 10 min. Supernatants were decanted into new Eppendorf tubes and filled to 2 mL with ultra-

pure water. Of each sample, 1 mL was filtered using Costar® SpinX tubes and transferred to HPLC vials. The remaining amount of each sample (about 1 mL) was incubated with 0.05 U myrosinase (thioglucosidase, Sigma Aldrich) for 8 h at 37 °C to hydrolyze GS to their corresponding breakdown products. Hydrolyzed samples of the leaves were filtered through a Costar® SpinX tube and transferred to HPLC sample vials.

Extracts of intact GS were qualitatively and quantitatively analyzed by HPLC (Thermo Scientific) equipped with an autosampler, pump, DAD detector, and column oven. Volumes of 10 µl of the samples were injected on a SB-C18 column (Zorbax 5 µm, 4.6 × 250 mm, Agilent). The following gradient program was used: 0 – 2 min: 0 – 1% B, 2 – 20 min: 1 – 50% B, 20 – 24 min: 50 – 100% B, 24 – 26 min: 100% B, 26 – 27 min: 100 – 1% B, and 27 – 35 min: 1 – 0% B at a flow rate of 1.5 mL/min. Buffered eluents with solvent A: 100% 0.1 M ammonium acetate, B: 40% acetonitrile/0.1 M ammonium acetate were used for the extracts. Detection was at 229 nm, and components were identified by retention time and quantified against internal standard. The peak area remaining after myrosinase treatment of the intact GS extract was subtracted from the peak area of the GS of the intact GS extract without myrosinase treatment (FÖRSTER et al., 2015). GS content was determined on a dry weight (dw) basis.

Statistical analysis

The software, Statistical Package for Social Sciences (SPSS) (IBM SPSS Statistics for Windows, version 25.0), was used for the statistical analysis. The mean values were compared using analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test for pairwise comparison. Spearman's correlation analysis was used to study the relationship between local conditions (climate, soil minerals, elevation in the specific locations) and GS content in the leaves for both experiments.

Results

Glucosinolate profile of wild-grown *Moringa oleifera* leaf material from the three agro-ecological zones

Four different GS, 4-α-rhamnopyranosyloxy-benzyl GS (GS1), and three isomers of acetyl-4-α-rhamnopyranosyloxy-benzyl GS (GS2, GS3, and GS4) were identified in the leaves of *M. oleifera* trees harvested from the three agro-ecological zones. The total GS content analysed in the leaves from all the agro-ecological zones was not significantly different (Fig. 2). Their total GS content varied on average between 41.18 µmol/g dw and 53.42 µmol/g dw. In addition, the contents of single GS were not significantly different comparing the different agro-ecological zones (Fig. 2).

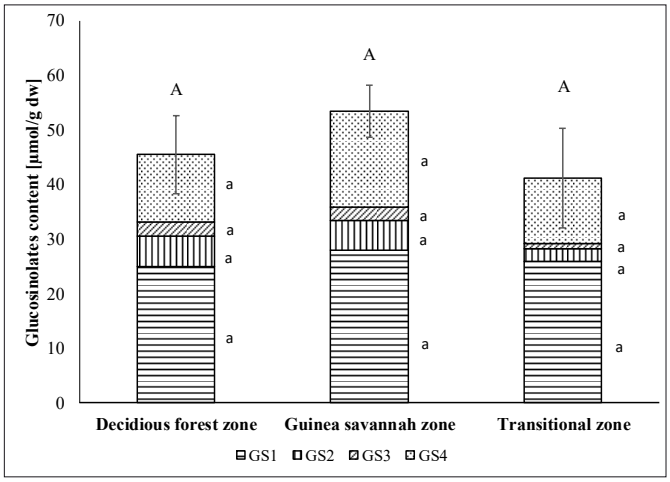

Fig. 2: Glucosinolates (GS) profile of leaves from wild-grown *Moringa oleifera* plants in three agro-ecological zones. One-way ANOVA followed by Tukey's test, $p \leq 0.05$. 4-α-rhamnopyranosyloxy-benzyl GS (GS1), three isomers of acetyl-4-α-rhamnopyranosyloxy-benzyl GS (GS2, GS3, GS4). Same lowercase letters indicate no significant differences for means of single GS among agro-ecological zones; same uppercase letters indicate no significant differences for means of total GS content (comprising of GS1-GS4). Error bars indicate standard deviations of mean total GS content.

Correlation analysis of total glucosinolate content of wild-grown *Moringa oleifera* leaves with soil minerals, elevation, and climate data for the three agro-ecological zones

Results showed no statistically significant correlation between total GS in leaves of *M. oleifera,* and the climate data (Tab. 6). However, a significant negative correlation between total GS and N ($r = -0.85$, $p < 0.05$), as well as elevation ($r = -0.68$, $p < 0.05$) was found (Tab. 6). Elevation correlated negatively with minimum temperature ($r = -0.95$, $p < 0.05$) and positively with relative humidity ($r = 0.95$, $p < 0.05$) (Tab. 6). Maximum temperature had a strong negative correlation with rainfall ($r = -1.00$ $p < 0.05$) (Tab. 6).

Cultivation under semi-controlled conditions

Leaf glucosinolate profiles of three *Moringa oleifera* accessions cultivated with/without fertilizer at two harvest times

The results revealed that the independent factors, i.e., harvest time, accession, and ago-ecological zone, had a significant influence on total GS content in *M. oleifera*, while fertilization had no significant influence on the total GS content (Tab. 7A). Interaction effects

Tab. 6: Spearman's correlation analysis between the total glucosinolate (GS) content in the leaves of *Moringa oleifera* and the local conditions of the three agro-ecological zones.

Variables	(1)	(2)	3)	(4)	(5)	(6)	(7)	(8)
(1) Total GS [µmol/g dw]	1							
(2) N [%]	-0.85**	1						
(3) S [%]	-0.23	0.30	1					
(4) Elevation [m]	-0.68*	0.67*	0.1	1				
(5) Maximum temperature [°C]	0.47	-0.74*	0.05	-0.47	1			
(6) Minimum temperature [°C]	0.63	-0.61	-0.21	-0.95**	0.50	1		
(7) Rainfall [mm]	-0.47	0.74*	-0.05	0.47	-1.00**	-0.50	1	
(8) Relative humidity [%]	-0.63	0.61	0.21	0.95**	-0.50	-1.00**	0.50	1

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

were identified for agro-ecological zone×fertilization, agro-ecological zone×accession, agro-ecological zone×harvest time, and agro-ecological zone×fertilization×harvest time on total GS content (Tab. 7A). Additionally, the comparison of group means revealed that Transitional and Guinea savannah zones showed significantly higher total GS content than those of the Deciduous forest zone (Tab. 7B). Furthermore, group mean comparison showed significantly higher total GS content in the accession 04024FL-041A than that of the accession, 08025FL-081D, which was also significantly higher than that of 16016FL-161A PKM 1 (Tab. 7C). Also, based on group mean comparison, it was found that total GS content at the first harvest was significantly lower than that of the second harvest (Tab. 7D). Results on group mean comparison of the factor fertilization showed that the total GS content in the fertilized variants was not significantly different from the unfertilized variants (Tab. 7E).

Correlation analysis of total GS content of cultivated *Moringa oleifera* accessions with the soil minerals, elevation, and climate data from three agro-ecological zones

The results showed no significant correlation between the total GS content of *M. oleifera* leaves and the soil minerals and climate data (Tab. 8). However, a significant correlation was observed between elevation and soil minerals and between elevation and climate data measured.

Discussion

Impact of temperature on glucosinolate profile of *Moringa oleifera*

The lack of significant correlation between temperature and total GS content in the leaves in both studies (Tab. 6 and Tab. 8), even though significant differences were observed in mean maximum and mini-

Tab. 7: Glucosinolates (GS) profile of *Moringa oleifera* leaves of three accessions cultivated with and without fertilizer and harvested at two harvest times from the three agro-ecological zones.

A	Treatment effects	Total GS (p-value)
	Harvest (H)	p = 0.00
	Agro-ecological zone (AG)	p = 0.00
	Accession (AC)	p = 0.00
	Fertilization (F)	p = 0.18
	H×AG	p = 0.00
	H×AC	p = 0.14
	H×F	p = 0.42
	AG×AC	p = 0.00
	AG×F	p = 0.00
	AC×F	p = 0.91
	H×AG×AC	p = 0.17
	H×AG×F	p = 0.01
	H×AC×F	p = 0.92
	AG×AC×F	p = 0.65
	H×AG×AC×F	p = 0.12

Treatment		GS content [µmol/g dw]				
		GS1	GS2	GS3	GS4	Total GS
B	**Agro-ecological zone**					
	Transitional	19.33 ± 10.27 [a]	6.12 ± 3.01 [a]	2.97 ± 1.83 [a]	13.89 ± 6.37 [a]	42.31 ± 15.42 [a]
	Deciduous forest	19.02 ± 6.56 [a]	3.32 ± 2.73 [b]	1.44 ± 1.42 [b]	10.20 ± 5.64 [b]	33.97 ± 14.98 [b]
	Guinea savannah	23.07 ± 9.17 [a]	4.06 ± 2.29 [b]	.85± 1.22 [b]	10.32 ± 4.45 [b]	39.30 ± 13.58 [a]
C	**Accession**					
	16016FL-161A PKM 1	17.09 ± 6.81 [b]	4.25 ± 3.38 [ab]	2.02 ± 1.96 [a]	8.95 ± 4.37 [b]	32.31 ± 13.12 [c]
	04024FL-041A	22.74 ± 9.86 [a]	5.57 ± 3.05 [a]	2.52 ± 1.72 [a]	14.93 ± 6.53 [a]	45.77 ± 15.68 [a]
	08025FL-081D	21.58 ± 9.04 [a]	3.76 ± 1.96 [b]	1.75 ± 1.06 [a]	10.78 ± 4.69 [b]	37.86 ± 13.32 [b]
D	**Fertilization**					
	Fertilized	22.73 ± 9.50 [a]	4.27 ± 3.00 [a]	1.95 ± 1.67 [a]	11.14 ± 6.41 [a]	40.09 ± 16.12 [a]
	Unfertilized	18.03 ± 7.61 [b]	4.76 ± 2.86 [a]	2.23 ± 1.62 [a]	11.86 ± 5.06 [a]	36.87 ± 13.65 [a]
E	**Harvest**					
	1	18.13 ± 8.27 [b]	3.39 ± 2.37 [b]	1.41 ± 1.26 [b]	10.63 ± 5.76 [a]	33.57 ± 12.52 [b]
	2	22.72 ± 9.00 [a]	5.63 ± 3.03 [a]	2.76 ± 1.72 [a]	12.35 ± 5.71 [a]	43.45 ± 15.69 [a]

A: H, AG, AC, F, H×AG, H×AC, H×F, AG×AC, AG×F, AC×F, H×AG×AC, H×AG×F, H×AC×F, AG×AC×F, and H×AG×AC×F effects on total GS content were compared using ANOVA, $p \leq 0.10$.

B and C: The results presented in the tables are the means ± standard deviations of single GS (GS1: 4-α-rhamnopyranosyloxy-benzyl GS; GS2, GS3, GS4: three isomers of acetyl-4-α-rhamnopyranosyloxy-benzyl GS) and total GS (comprising of GS1 – GS4) content in *M. oleifera* leaves. The mean values were compared using one-way ANOVA followed by Tukey's test, $p \leq 0.10$. Same lowercase letters in the same column indicate no significant difference in means of single GS, and total GS content among the agro-ecological zones (B) and accessions (C)

D and E: The results presented in the tables are the means ± standard deviations of single GS (GS1: 4-α-rhamnopyranosyloxy-benzyl GS; GS2, GS3, GS4: three isomers of acetyl-4-α-rhamnopyranosyloxy-benzyl GS) and total GS (comprising of GS1 – GS4) content in *M. oleifera* leaves. The mean values were compared using the t-test, $p \leq 0.10$. Same lowercase letters in the same column indicate no significant difference in means of single GS, and total GS content between fertilized and unfertilized variants of *Moringa oleifera* (D) and first harvest and second harvest (E).

Tab. 8: Spearman's correlation analysis between the total glucosinolate (GS) content of the leaves of the cultivated *Moringa oleifera* accessions, and the local conditions of the three experimental fields in the three agro-ecological zones.

Variables	(1)	(2)	(3)	4)	(5)	(6)	(7)
(1) Total GS [µmol/g dw]	1						
(2) N [%]	0.16	1					
(3) S [%]	0.19	0.78**	1				
(4) Elevation [m]	0.07	0.87**	0.78**	1			
(5) Maximum temperature [°C]	0.18	-0.40**	-0.16	-0.47**	1		
(6) Minimum temperature [°C]	-0.18	0.40**	0.16	0.47**	-1.00**	1	
(7) Relative humidity [%]	0.07	0.87**	0.78**	1.00**	-0.47**	0.47**	1

* Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

mum temperatures (Tab. 2), was not consistent with findings in the literature, where higher temperatures were reported to significantly increase GS content in different *Brassica* crops (Ciska et al., 2000). However, the transferability of the present results to other studies is difficult, especially when plants are of different species and are grown in an uncontrolled environment combined with high variability of abiotic factors (Ahuja et al., 2010). This assertion holds, particularly for wild-grown *M. oleifera* from the three agro-ecological zones. Here, various parameters such as previous climate events during early plant growth, age, and stage of development of the wild-grown *M. oleifera* plants, which might have had an impact on GS were unknown. Therefore, it is difficult to trace the results back to one particular parameter.

In the wild-grown *M. oleifera*, the lack of significant difference in total GS content for all three agro-ecological zones (Fig. 2) could be due to the perennial nature of *M. oleifera* plants (Duke, 1983). With trends of increasing temperatures in the last several decades (Leary et al., 2013), physiological adaptations might have developed in the plant to deal with the repeated temperature stress and consequently increased the plants' secondary metabolite accumulation (Yang et al., 2018). Therefore, over time, the plants probably retain nearly similar total GS content in the leaves at the three agro-ecological zones irrespective of the climate conditions. On the other hand, *M. oleifera* is a tropical plant (Roloff et al., 2009), and the wild-grown plants could be well adapted to the climate at the three agro-ecological zones. In this context, the plants seem to grow well under nearly optimal temperature and other abiotic conditions such as light, carbon dioxide, water, and nutrients supply. Thus, the plants did not encounter extreme stress at any specific location during the experimental period to cause them to significantly increase their secondary plant metabolites.

Impact of rainfall and relative humidity on glucosinolate profile of *Moringa oleifera*

With respect to wild-grown *M. oleifera*, no significant correlation was found between rainfall and total GS content in the leaves (Tab. 6), which is not in accordance with previous studies (Stamp, 2004; Ramakrishna and Ravishankar, 2011; Förster et al., 2015). These authors reported that water availability affects GS accumulation in such a manner that plants under water stress conditions accumulate higher GS content than well-watered plants. Thus, due to the lack of stress on well-watered plants, GS accumulation is not enhanced (Selmar and Kleinwächter, 2013). Because of the long periods of drought resulting from reduced or no rainfall from September/October to March (Padi, 2017), we expected that the wild-grown plants in their respective environments would respond with a stress-mediated increase in secondary plant metabolites, e.g., GS. In our study, it had been assumed that other edaphic and climate conditions in the three agro-ecological zones might have also impacted the GS content of the wild-collected *M. oleifera* leaves. However, in the present results, rainfall was not significantly different during the experimental period (Tab. 2A). Indeed, at the time of harvest (June 2017), the rainy season in all the three agro-ecological zones was generally at its peak (one-way ANOVA, $p \leq 0.05$, detailed results not shown). Therefore, it is assumed that the increased rainfall might have resulted in a sufficient water supply for the vegetative growth of the plants in all three agro-ecological zones, and consequently, plants did not experience water stress conditions. Furthermore, the increased rainfall at the time of harvest was accompanied by a significant decrease in maximum temperature ($r = -1.00$, Tab. 6). Thus, the absence of high-temperature stress on the plants, in turn, might have led to an inhibition of accelerated GS synthesis. Although not confirmed by the presented results, rainfall might be an important environmental factor for the GS accumulation in *M. oleifera* leaves, as reported. Therefore, a more thorough understanding of the changes in within-season variability of rainfall and its close relation to temperature and their effects on GS content in *M. oleifera* is warranted in future studies.

Further, no significant correlation between relative humidity and total GS content was observed for both the wild-grown and cultivated accessions (Tab. 6 and Tab. 8), even though relative humidity data among the three agro-ecological zones were significantly different (Tab. 2). This contradicts reports suggesting that relative humidity influences GS accumulation in cabbage (Bohinc and Trdan, 2012). Indeed, water requirement in plants is significantly influenced by relative humidity, and consequently, relative humidity influences plant growth, although the effects differ depending on plant species (Ford and Thorne, 1974). Low relative humidity increases transpiration and thus results in water deficiency, which is being caused by stomata closure and consequently reduction of carbon dioxide intake by plants (Talbott et al., 2003). Based on such water stress conditions, there is a significant decrease in NADPH + hydrogen ion (H^+) consumption for carbon dioxide fixation and reduction in the Calvin cycle (Selmar and Kleinwächter, 2013). Therefore, the concentration of the oxidized form of NADPH, $NADP^+$, decreases considerably, which significantly decreases the levels of adequate electron acceptors for the electron transport chain of photosynthesis. As a result, no more electrons from the electron transport chain are transferred to the reduction equivalents, leading to the production of reactive oxygen species. This respective metabolic change could damage the photosynthetic apparatus, referred to as oxidative photodestruction. However, a protective mechanism is induced, which shifts all reactions involving NADPH + H^+ consumption towards an enhanced synthesis of secondary plant metabolites, such as GS (Selmar and Kleinwächter, 2013). Therefore, we expected a correlation between relative humidity and total GS content in our experiments which could, however, not be confirmed in our studies. This is attributable to the absence of water stress of the wild-grown *M. oleifera* plants, as previously explained in the preceding paragraph. Similarly,

the cultivated accessions were well-watered during the experimental period except on days with rainfall. This also indicates that the plants had sufficient water supply for vegetative growth; hence GS synthesis was not enhanced.

Impact of elevation on glucosinolate profile of *Moringa oleifera*

For the wild-grown *M. oleifera*, total GS content in the leaves significantly increased as elevations decreased (Tab. 6). Presumably, high elevations have been associated with stresses, e.g., lower air temperatures, leading to poorer plant growth (BOYER, 1982). However, in the present study, the stresses exist at the zone in the lowest elevation, i.e., the Guinea savannah zone, rather than at the zones in the higher elevations (Tab. 1). This has been reported in studies characterizing the Guinea savannah zone as the zone with very harsh environmental conditions accompanied by lower annual rainfall and higher temperatures than the Transitional and the Deciduous forest zones (LEARY et al., 2013; PADI, 2017). Therefore, due to the longevity of perennial plants such as *M. oleifera* trees, the repeated seasonal stress in this zone probably increased GS accumulation (YANG et al., 2018). In conclusion, this might have tendentiously increased total GS content in the Guinea savannah zone in comparison to the two other zones, although the differences were non-significant (Fig. 2).

Contrarily, for *M. oleifera* accessions cultivated under semi-controlled field conditions, no significant correlation was found between elevation and the total GS content (Tab. 8). We expected that because temperature decreased significantly with increasing elevation and relative humidity increased significantly with increasing elevation (Tab. 8), total GS content would decrease at the higher elevation. Presumably, reduced impact of stress factors on plants prevents GS accumulation (STAMP, 2004; YANG et al., 2018). However, the present study under semi-controlled field conditions contradicted this hypothesis. It is assumed that the differences in temperature and relative humidity values along the elevation gradient at the time of the experiment were not strong enough to trigger a stress condition. Therefore, a trade-off between primary metabolism and secondary metabolism, as depicted by HARTMANN (2007), did not occur to enhance GS accumulation. Although not considered in the present study, other environmental factors, including radiation, could also interact with, e.g., temperature to influence GS accumulation (SCHONHOF et al., 2007b). These authors suggested that temperature and radiation significantly contributed to influence GS content in broccoli in a manner that increasing temperature in combination with increasing radiation significantly increased GS content. Putatively, secondary plant metabolites can be altered in response to changing environmental conditions along the elevational gradient, and this phenomenon can vary across species. It, therefore, becomes more complex to elucidate the specific impacts observed in the present study. Therefore, for *M. oleifera,* further studies, including other environmental factors, are of particular relevance in the area of the present study.

Impact of different accessions on glucosinolate profile of *Moringa oleifera*

Wild-collected leaf material for each agro-ecological zone was a composite of leaf material from different wild-grown *M. oleifera* with diverse genetics as well as origins. As sampling was not carried out based on accession/origin of the *M. oleifera* plants, the impact of accession on total GS content will not be discussed. However, for *M. oleifera* cultivated under semi-controlled conditions, in general, accession significantly differed in their total GS content (Tab. 7A), with higher total GS content found in the accession 04024FL-041A than in the other two accessions (Tab. 7C). This result is in accordance with a study, which found significant varying trends in the GS content of 30 different accessions of *M. oleifera* (DOERR et al., 2009), and another study, where six ecotypes of *M. oleifera* showed high variability in their GS profile (FÖRSTER et al., 2015). The authors attributed these differences to the plant genotypes. Plants of different genotypes have different ways to adapt to their environments, altering the biosynthesis and accumulation of secondary metabolites (YANG et al., 2018). The presented results showed significantly higher total GS content in the accession 04024FL-041A than in the accession, 08025FL-081D (Tab. 7C), although the latter revealed a significantly higher number of leaves than the accession 04024FL-041A (Tetteh, unpublished data). This could be attributed to the fact that GS synthesized in leaves of the accession 08025FL-081D were transported into more leaves than in the accession 04024FL-041A with fewer leaves. Thus, some form of dilution effect occurred to produce a lower yield of GS in the accession 08025FL-081D than in the accession 04024FL-041A. This assumption is confirmed by a study on white cabbage in which leaf expansion was found to be accompanied by dilution of GS (POCOCK et al., 1987).

Impact of fertilization and cultivation practices on glucosinolate profile of *Moringa oleifera*

Results of the wild-collected *M. oleifera* leaf material revealing no influence of S on total GS content (Tab. 6) conforms with a study identifying no influence of S supply on GS content in leaves of *M. oleifera* (FÖRSTER et al., 2015). Further, our results showed that increasing N content decreased total GS (Tab. 6), which corroborates, in part, with a study on broccoli heads indicating a decline in GS content with increasing N content, however only in combination with insufficient S supply (SCHONHOF et al., 2007a). The present study suggests that regardless of S supply, increasing N content decreased total GS content significantly in wild-grown *M. oleifera* (Tab. 6). In contrast, N and S contents had no significant influence on total GS content in the cultivated accessions of *M. oleifera* (Tab. 8). This observation indicates that plants were presumably growing under optimal cultivation conditions, including suitable temperature (ROLOFF et al., 2009), adequate water supply, and rainfall. Plants had also been fertilized with compost manure, a rich source of nutrients, including N and S (AMANULLAH and MUTHUKRISHNAN, 2010), which had a significant positive correlation between N and S (Tab. 8). Consequently, increasing N and S contents increased the leaf numbers of the cultivated accessions significantly (TETTEH, unpublished data). This suggests that plants growing in a resource-rich environment invested their resources into leaf production instead of the enhanced production of secondary plant metabolites (STAMP, 2004).

For *M. oleifera* accessions cultivated under semi-controlled conditions, no influence of fertilizer application on the total GS content in leaves was found (Tab. 7A). The results were supported by the hypothesis of Growth Differentiation Balance. This hypothesis describes the physiological trade-off between growth and differentiation processes regarding nutrient availability, influencing the accumulation of secondary metabolites (HERMS and MATTSON, 1992). Therefore, in plants grown in nutrient-rich soil, biomass production is being promoted to a greater extent than the synthesis of secondary metabolites such as GS (STAMP, 2004; HARTMANN, 2007). Accordingly, fertilization did not significantly influence total GS content (Tab. 7D). However, fertilization significantly influenced the number of leaves of *M. oleifera*, i.e., the fertilized variants had significantly higher leaf numbers than the non-fertilized plants (Tetteh, unpublished data). For further studies, different fertilizer rates and a higher frequency of poultry manure compost application for a longer period than six months might be recommended to better understand the impact of fertilization on GS synthesis in *M. oleifera* growing under semi-controlled field conditions.

An additional trend observed in the cultivated accessions under semi-controlled field conditions was that total GS content in the second harvest was significantly higher than in the first harvest (Tab. 7E), which is consistent with findings of studies on *M. oleifera* (Doerr et al., 2009). The significant interaction effect of agro-ecological zone×fertilization×harvest time for total GS content (Tab. 7A) contributed to this trend observed. Assumingly, seasonal variation at the two harvest times contributed to the differences in total GS content, and thus, *M. oleifera* accessions adapted differently under prevailing environmental conditions at the two harvest times resulting in their different total GS content identified (Charron et al., 2005).

With these findings, it has been shown that under semi-controlled field conditions, the various independent factors, i.e., accession, agro-ecological zone, fertilization, and harvest time, interacted significantly in terms of changes in the total GS content in *M. oleifera*. However, for further studies, the impact of single climate parameters and other abiotic factors on GS content needs to be more specific. To summarize, choosing the appropriate accession and cultivating under appropriate cultural practices at a location with suitable environmental conditions is essential to produce plants with high health-promoting properties in *M. oleifera* in terms of GS content.

Conclusion

This study provides clear evidence that temperature, relative humidity, and rainfall had no significant influence on GS content in the leaves of both the wild-grown and cultivated accessions of *M. oleifera*. Rainfall in the three agro-ecological zones was at its peak at the time of harvest, and their amounts were not significantly different. Therefore, wild-grown plants, assumingly, did not experience water stress conditions, and thus, no significant changes in GS content occurred. This implies that wild-grown *M. oleifera* leaves from the three agro-ecological zones would have nearly similar GS contents when harvested during the rainy season, which is a beneficial outcome for *Moringa* producers and processors in Ghana. This outcome could also be applicable in other tropical areas that have a similar climate as Ghana. Regardless, further studies considering different seasons (i.e., the dry and rainy seasons) in the study area may help to better understand the specific impacts of each of the different climate parameters on the dynamics of GS synthesis in *M. oleifera* leaves.

In determining the influence of elevation on the total GS content for both the wild-grown and cultivated accessions of *M. oleifera*, contradictive results have been identified. Future work is certainly required to further elucidate how the changes of eco-physiological factors along the elevational gradient influence GS accumulation in both wild-grown and cultivated accessions of *M. oleifera*. By determining the influence of soil minerals on the GS profile, we identified that for wild-grown *M. oleifera*, increasing N content significantly increases GS accumulation irrespective of S supply. However, for cultivated accessions, increasing composted poultry manure to increase N and S content during the rainy season did not affect GS accumulation.

Finally, the appropriate selection of *M. oleifera* accessions, which differed in their total GS content, and cultivation under favorable environmental conditions, should be envisaged to produce health-promoting *M. oleifera* plants. Considering that genetics and environmental conditions interact and influence the performance of accessions, genome analyses of accessions and their impact on GS could be of further interest.

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Conflicts of interest

No potential conflict of interest was reported by the authors.

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