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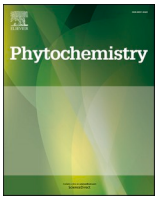

Natural variation in specialised metabolites production in the leafy vegetable spider plant (*Gynandropsis gynandra* L. (Briq.)) in Africa and Asia

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ABSTRACT

The improvement and promotion of leafy vegetables, used for both food and medicine, benefits greatly from detailed knowledge of their health-promoting specialised metabolites. In the present study, we investigated the global metabolite variation in the leaves of 48 accessions of the leafy vegetable *Gynandropsis gynandra* using two complementary analytical platforms: liquid-chromatography mass spectrometry (LC-MS) for an untargeted comparison of non-volatile semi-polar metabolites and gas-chromatography mass spectrometry (GC-MS) for an untargeted comparison of volatile metabolites. Our results revealed large variation in 936 semi-polar compounds including flavonoids, terpene glycosides, glucosinolates and various phenolic compounds. Unsupervised multivariate analysis indicated the variation in levels of the semi-polar metabolites was mainly driven by geography, with accessions from both West Africa and Asia forming a group clearly separated from East African accessions. Detected volatile metabolites included various sesquiterpenes, aldehydes, ketones, and sulphur-containing isothiocyanates. Variation in these compounds was however not geographically specific, but most likely linked to the taste and aroma of the leaves. The relative abundance in glucosinolates and isothiocyanates in the leaves allowed the clustering of accessions into two main groups that could be used for further plant-herbivore interaction studies. This study revealed both the broad spectrum of phytochemicals present in *Gynandropsis gynandra* leaves and the substantial variation in metabolite profiles among accessions from different regions of the world. Our results provide a basis for the development of breeding programs aiming at improving the levels of specialised metabolites in this tropical leafy vegetable for increased resistance against pests and diseases and improved human health.

1. Introduction

Spider plant (*Gynandropsis gynandra*) is a leafy vegetable belonging to the Cleomaceae family, the sister family of the Brassicaceae. Although considered as a major leafy vegetable in several communities in Africa and Asia, *Gynandropsis gynandra* has long been understudied. The recent surge in demand for diversified and nutrient-rich diets in both advanced economies and developing countries has led to an increasing interest for "orphan" or "neglected" crops. Such orphan crops are often both nutritious and an important source of income for local communities. Moreover, such species are adapted to local climates and tolerant to harsh conditions including heat, drought, flood, and pests and diseases. Re-introducing these so-called orphan crops in agri-food systems in developing countries could invert the increasing food insecurity in these regions and contribute to more resilient agricultural ecosystems (Baldermann et al., 2016; Chiurugwi et al., 2019). Hence, *G. gynandra* may be referred to as a "super" leafy vegetable with a tremendous potential to contribute to food and nutrition, especially in regions where it is cultivated (Cernansky, 2015; Sogbohossou et al., 2018a).

Gynandropsis gynandra leaves are rich in nutrients including carotenoids, vitamin C and minerals (Odhav et al., 2007; Omondi et al., 2017; Sogbohossou et al., 2019; Uusiku et al., 2010). Leaves, young shoots and occasionally flowers are eaten boiled in sauces/stews or can be blanched and dried for preservation (Flyman and Afolayan, 2006; van Den Heever and Venter, 2007). *Gynandropsis gynandra* is also used as a medicinal plant in several African and Asian countries. The leaves are used as

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disinfectants on wounds and taken as medicine for body aches and pains, eye infections, malaria, typhoid fever, anaemia and skin conditions (Bala et al., 2010; Chweya and Mnzava, 1997; Shanmugam et al., 2012; Sogbohossou et al., 2018b; Yetein et al., 2013). Pharmacological investigations of the plant species revealed several compounds with confirmed medicinal properties: the leaves contain high concentrations of alkaloids, steroids (Ajaiyeoba, 2000), glucosinolates, flavonoids, tannins, iridoids and other phenolic compounds (Moyo et al., 2013, 2018). Quercetin and kaempferol are the main flavonoids, while traces of isorhamnetin are also present in the leaves of *G. gynandra* (Omondi et al., 2017; Yang et al., 2008). Glucosinolates including glucocapparin, glucobrassicin and 3-hydroxypropylglucosinolate were detected in the plant and have been reported to play a role in plant defence against herbivores (Neugart et al., 2017; Omondi et al., 2017). Upon disruption of the leaf tissues, the hydrolysis of glucosinolates by myrosinases releases volatile sulfuric compounds, mainly isothiocyanates that have repellent properties against pests (Neugart et al., 2017; Omondi et al., 2017; Songsak and Lockwood, 2002).

Consumers' preferences in terms of taste and aroma of spider plant are variable depending on the country and/or region. In the West African countries Benin and Togo, the bitterness of the leaves is considered by some communities as an indicator of healing properties of the species. Bitter and odorous leaves are therefore preferred for both consumption and medicinal uses (Sogbohossou et al., 2018a). East and Southern African communities prefer "sweeter" varieties and use various methods to attenuate the bitterness of the leaves such as by cooking them in milk (Flyman and Afolayan, 2006; Sogbohossou et al., 2018a). Considering consumers' preferences in breeding programs requires an extensive knowledge on secondary metabolites influencing the taste and aroma of the leaves as well as their health-promoting properties. Breeding strategies for the species were broadly discussed in Sogbohossou et al. (2018a). To date, most metabolite surveys conducted were, however, focused on specific compounds or classes of compounds analysed in small sets of accessions mainly collected in Eastern or Southern Africa.

In the present study, we investigated the natural variation in the total metabolite composition of 48 *Gynandropsis gynandra* accessions including 20 from Asia, 23 from East/Southern Africa and 5 from West Africa (Fig. 1; Table 1; Supplementary Table 1), using untargeted metabolomics approaches based on mass spectrometry. Specifically, we focused on: (1) assessing the occurrence and variation in semi-polar and volatile metabolites in the collection; and (2) identifying potential compounds that may be associated with taste, aroma and plant defence in the leaves.

2. Results and discussion

2.1. Natural variation in semi-polar metabolites

The relative intensities of 936 metabolites were extracted in leaf samples using an untargeted LC-MS approach (Supplementary Table 2). A principal component analysis (PCA) on the 48 accessions revealed a separation of the accessions correlating with their geographic origin (Fig. 2; Supplementary Table 2). The first component (PC1) explained 28.8% of the total variation and separated accessions from East and Southern Africa from those from West Africa and Asia. The second principal component (PC2) explaining 10.9% of the variation separated accessions from West Africa from those from Asia (Fig. 2).

Of the 936 metabolites detected, 705 had significantly different abundance levels between East/Southern African, West African, and Asian accessions ($p < 0.05$). Twenty-six of these compounds were putatively identified based on spectral similarity with public spectral libraries or with known compounds of a chemical class. A heatmap (Fig. 3) based on the most contrasting metabolites between accessions, with PCA loadings $> |0.7|$ on both PC1 and PC2, revealed relationships both among accessions and among metabolites. Two main clusters of accessions (A1; A2) and three major clusters of metabolites (B1; B2; B3) were identified (Fig. 3). The cluster A1 included the 20 accessions from Asia and 5 from West Africa while the 23 accessions from East/Southern Africa were all in cluster A2. The same clustering of accessions was obtained with a heatmap including all the 936 metabolites (Supplementary Figure 1). The metabolite cluster B1 comprised of compounds mainly present in East African accessions and compounds present in both West African and East African accessions. Cluster B2 included compounds most abundant in West African accessions and most Asian ones that were also present at intermediate levels in some East/Southern African accessions. Compounds in the cluster B3 were relatively high

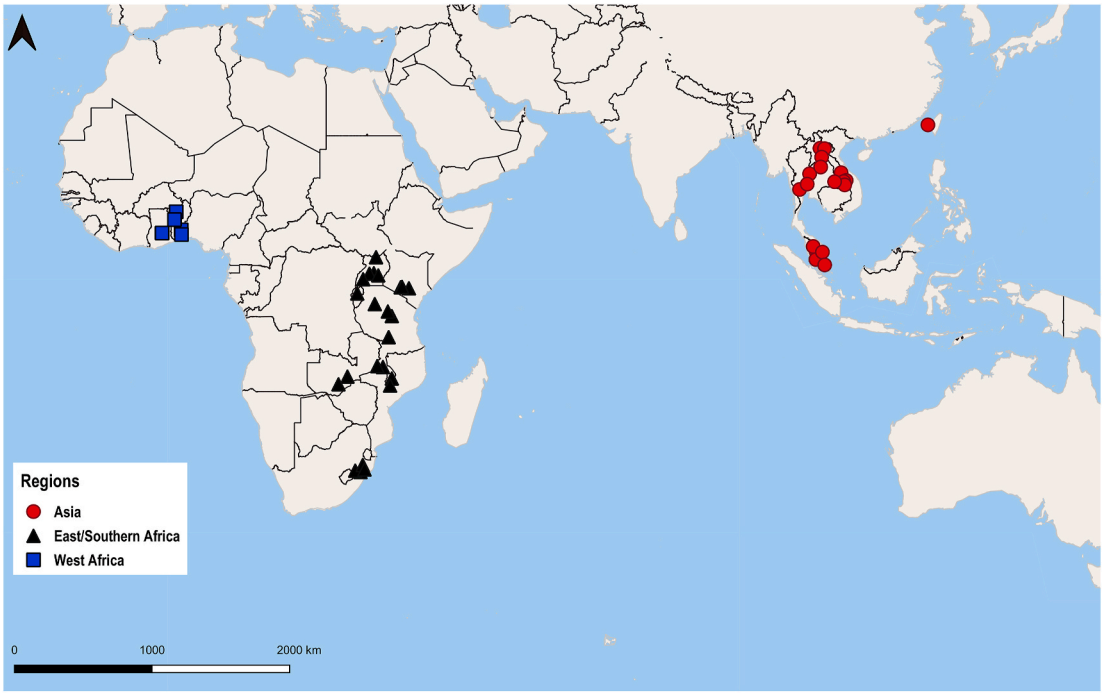

Fig. 1. Collection sites of the 48 accessions of *Gynandropsis gynandra*.

Table 1
List of 48 accessions of *Gynandropsis gynandra* and countries/regions of origin.

#	Accession name	Region	Country
1	Gyn	Asia	Malaysia
2	TOT1048	Asia	Thailand
3	TOT1480	Asia	Thailand
4	TOT3514	Asia	Laos
5	TOT3527	Asia	Laos
6	TOT3534	Asia	Laos
7	TOT3536	Asia	Laos
8	TOT4935	Asia	Thailand
9	TOT4937	Asia	Thailand
10	TOT4976	Asia	Thailand
11	TOT5799	Asia	Thailand
12	TOT7196	Asia	Malaysia
13	TOT7197	Asia	Malaysia
14	TOT7198	Asia	Malaysia
15	TOT7199	Asia	Malaysia
16	TOT7200	Asia	Malaysia
17	TOT7441	Asia	Laos
18	TOT7462	Asia	Laos
19	TOT7486	Asia	Laos
20	TOT8996	Asia	Taiwan
21	RW–SF–10	East/Southern Africa	Rwanda
22	TOT6420	East/Southern Africa	Tanzania
23	TOT6421	East/Southern Africa	Tanzania
24	TOT6422	East/Southern Africa	Tanzania
25	TOT6435	East/Southern Africa	Kenya
26	TOT6439	East/Southern Africa	Zambia
27	TOT6440	East/Southern Africa	South Africa
28	TOT6441	East/Southern Africa	South Africa
29	TOT6442	East/Southern Africa	South Africa
30	TOT8888	East/Southern Africa	Uganda
31	TOT8889	East/Southern Africa	Uganda
32	TOT8890	East/Southern Africa	Uganda
33	TOT8891	East/Southern Africa	Uganda
34	TOT8915	East/Southern Africa	Malawi
35	TOT8916	East/Southern Africa	Malawi
36	TOT8917	East/Southern Africa	Malawi
37	TOT8918	East/Southern Africa	Malawi
38	TOT8925	East/Southern Africa	Kenya
39	TOT8926	East/Southern Africa	Kenya
40	TOT8931	East/Southern Africa	South Africa
41	TOT8933	East/Southern Africa	Zambia
42	TOT8997	East/Southern Africa	Uganda
43	TOT8998	East/Southern Africa	Uganda
44	ODS-15-020	West Africa	Benin
45	ODS-15-044	West Africa	Benin
46	ODS-15-045	West Africa	Benin
47	ODS-15-061	West Africa	Togo
48	ODS-15-117	West Africa	Ghana

abundant in Asian and West African accessions but at relatively low levels in East/Southern African accessions.

The geographic patterns of clustering of semi-polar metabolites suggests that the variation in these metabolites results from the divergence and potential local adaptation of the species. A similar geographic pattern of differentiation for *G. gynandra* was demonstrated using a combination of morphological traits and isoprenoid levels (Sogbohossou et al., 2019) and also based on an analysis of photosynthesis-related traits (Reeves et al., 2018). The congruence between the results of the various studies suggests that the same patterns of geography-specific similarities may also be expected at the genomic nucleotide level. Wahyuni et al. (2013) found comparable patterns of species-specific profiles of semi-polar metabolites in fruits of *Capsicum* spp. The authors stated that differences in the regulation of metabolic pathways, the activity of rate-limiting enzymes, or the substrate specificity of specific modifying enzymes could explain the observed differences in metabolite levels. In our study, differences between accessions different geographic regions could indicate (an on-going) speciation event in *G. gynandra*. Another hypothesis is that these patterns in metabolite profiles were due to active selection of accessions based on in consumers' preferences in different geographic locations. Further genomic analyses, such as the detection of signatures of selection between populations, structural variation and demographic history reconstruction using a broader collection of accessions, could help unravel this question.

The sparse Partial Least Squares Discriminant Analysis (sPLS-DA) with the 936 semi-polar metabolites revealed that the error rate obtained by the prediction models were stabilized after the first two dimensions (Supplementary Table 2). The maximum distance method showed the lowest error rate for the first (12%) and the second dimension (1.4%) (Supplementary Table 2). The first dimension explained 27% of variation and discriminated East/Southern African accessions from West African and Asian ones. The second dimension explained 9% of variation and discriminated Asian from West African accessions. The model selected five metabolites (LC3577, LC2648, LC3081, LC4379 and LC7287) that could help discriminate the accessions (Fig. 4). LC3577 (an unknown C6H6O7-conjugate) was correlated with dimension 1 and relatively abundant in Asian and West African accessions. LC2648 (5-hydroxyferulate; a hydroxycinnamic acid), LC3081 (unknown) and LC4379 (di-pentosylapigenin; a flavonoid) were positively correlated with dimension 2, thus relatively abundant in Asian and East African accessions. LC7287 (hydroxyhexadecanoic acid,-dideoxyhexose; an oxylipid) was negatively correlated with dimension 2 and relatively abundant in West African accessions. Other annotated metabolites that were abundant in Asian and West African accessions but with low levels in East African accessions included for example: hydroxycinnamic acid derivatives (e.g. LC2540: caffeoyl-oxalosuccinate and LC3607: caffeoyl-hydroxycitric acid); terpene glycosides (e.g. LC3341: dihydroxy-eudesmenolide-hexoside and LC2765: icariside B8) and flavonoids (e.g. LC3830: rhamnazin-hexoside-deoxyhexoside) (Fig. 5a–e). East African and West African accessions exhibited high levels of the flavonoid quercetin-3-*O*-rutinoside (LC3890) (Fig. 5f) while glucocapparin (LC880) a glucosinolate was relatively abundant in Asian accessions (Fig. 5g). Classes of metabolites with high levels in East African accessions included other hydroxycinnamic acid derivatives (LC 2021: caffeoyl-citric acid; LC2468: coumaroyl-glucaric acid, and LC2749: feruloyl-glucaric acid) (Fig. 5h–k). In West African accessions, specific compounds with high levels included dihydroxy-eudesmanolide-hexoside (LC5323) a terpene glycoside. (Fig. 5l). The potential benefit of *G. gynandra* leaf consumption on human health, especially in relation to its potential antibiotic and anti-inflammatory activities (Sogbohossou et al., 2018b; Yetein et al., 2013) could be explained by the presence of different flavonoids, terpene glycosides, hydroxycinnamic acid derivatives as well as various other phenolic compounds known for their antioxidant, biocide or anti-inflammatory properties (Baron, 2018; Maurya and Devasagayam, 2010). Flavonoids were previously identified in two accessions of *G. gynandra* (Neugart et al., 2017) and in the present study we identified quercetin-3-*O*-rutinoside, and several hydroxycinnamic acid derivatives. Hydroxycinnamic acids reportedly possess potent antioxidant and anti-inflammatory properties, and may potentially be used for diabetes and hyperlipidaemia prevention (Alam et al., 2016). However, most metabolites detected by the LC-MS analysis were not annotated yet and may include other flavonoids or health-related phenolics. Future experiments towards identification of compounds detected by such untargeted LCMS-based metabolomics approach, e.g. by using dedicated MS/MS or MSn fragmentation experiments (van der Hooft et al., 2012), will help provide more insight into the biodiversity of these non-volatile phytochemicals in *G. gynandra*.

2.2. Natural variation in volatile metabolites in Gynandropsis gynandra

A total of 130 volatile metabolites were detected in the leaves of our accessions (Supplementary Table 3). Accessions TOT6440 and ODS-15-117 were detected as outliers and removed from further analyses. A principal component analysis (PCA) on the remaining 46 accessions and based on the 130 metabolites revealed that there was no clear separation of the accessions along the first two PCs according to their geographic origin (Fig. 6; Supplementary Table 3). A heatmap based on volatile

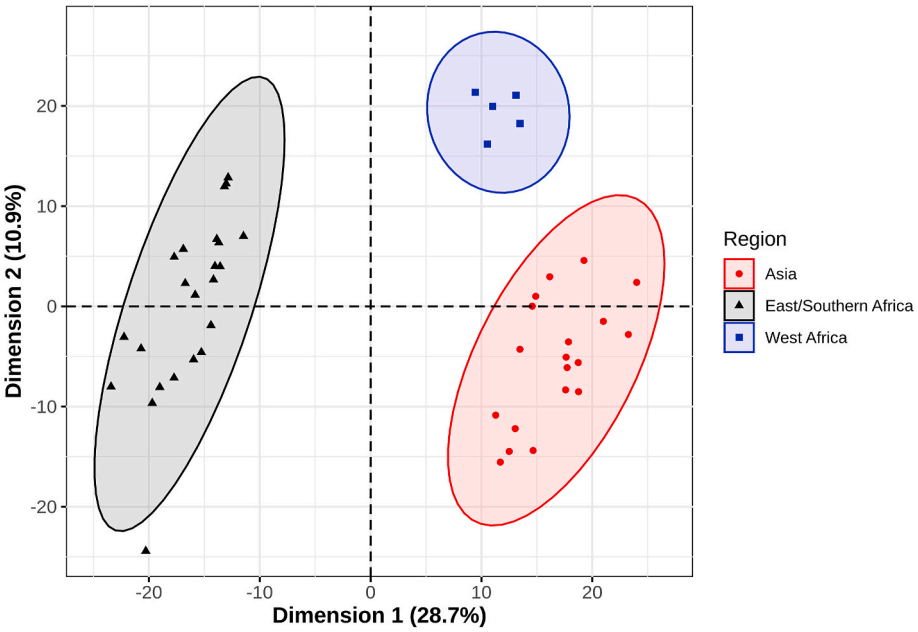

Fig. 2. Principal component analysis score plot of relative levels of 936 semi-polar metabolites detected in the leaves of 48 accessions of *Gynandropsis gynandra* from Asia (red), East/Southern Africa (black) and West Africa (blue). The first two dimensions explaining 39.6% of the total variation are shown. 95% confidence ellipses are displayed for the three regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

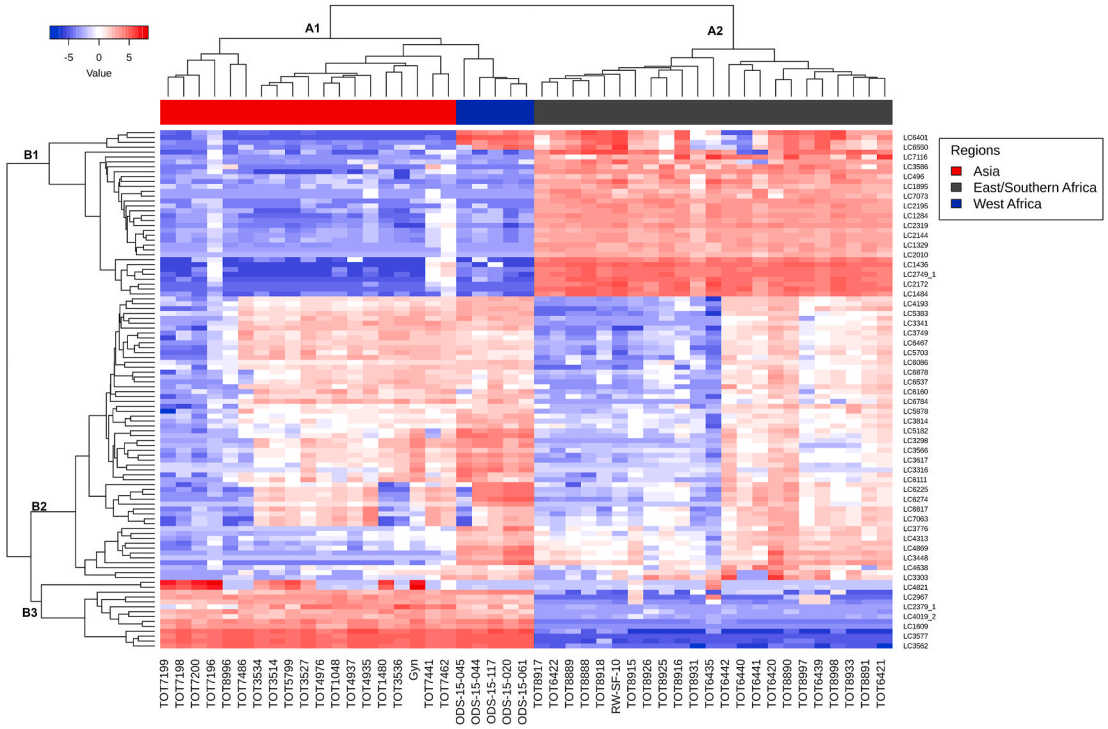

Fig. 3. Heatmap of 107 significant semi-polar metabolites with high PCA loadings (>|0.7|) in 48 accessions of *Gynandropsis gynandra* from Asia (red), East/Southern Africa (black) and West Africa (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metabolite levels revealed three clusters of accessions (C1, C2 and C3) and two main clusters of metabolites (D1 and D2) (Fig. 7). Cluster C1 consisted of 4 accessions (2 from East Africa and 2 from Asia) with overall low levels of volatiles. Cluster C2 included 30 accessions (19 from East Africa, 7 from Asia and 4 from West Africa) with on average high levels of D1 compounds but generally low levels of D2 compounds. Cluster C3 was made up of 12 accessions (11 from Asia and one from East Africa) that had moderate to high levels of volatile compounds. Of the 130 volatiles detected, 54 were putatively identified. Volatile metabolites present in cluster D1 mainly include aldehydes (e.g. GC987: (*E*)-2-pentenal; GC1482: (*E*)-2-hexenal; GC2713: (*E,E*)-2,4,-heptadienal; GC4069: β-cyclocitral), ketones (e.g. GC3119: 3,5-octadien-2-one; GC2492: 6-methyl-5-hepten-2-one), monoterpenes (e.g. GC2858: eucalyptol) and alcohols (e.g. GC1074: (*Z*)-2-penten-1-ol). The metabolite cluster D2 mainly included esters (e.g. GC2148: propyl 2-methylbutanoate; GC2667: isobutyl isovalerate; GC3365: 2-methylbutyl 2-

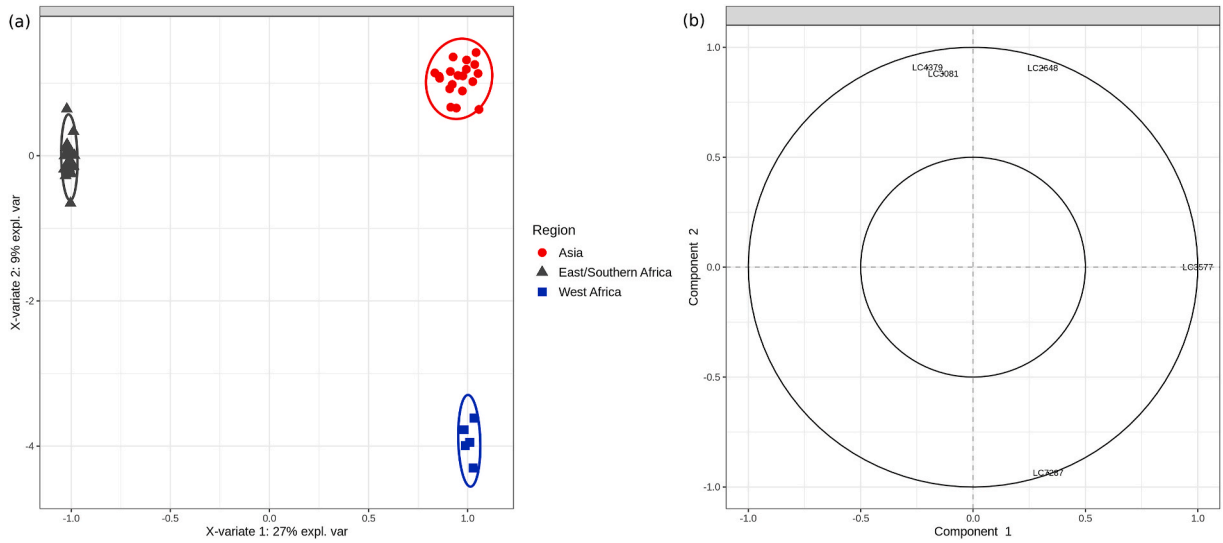

Fig. 4. Sparse partial least square discriminant analysis on the 48 accessions of *Gynandropsis gynandra* based on 936 semi-polar metabolites: (a) Score plot showing the projection of the 48 accessions from Asia (red), East/Southern Africa (black) and West Africa (blue) on the first two dimensions; (b) Selected variables representation on two dimensions on the correlation circles (0.5 and 1 correlation values). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

methylbutanoate), sesquiterpenes (e.g. GC4862: α-humulene; GC4956: bicyclosesquiphellandrene; GC5093: (*E,E*)- α-Farnesene; GC5277: cis-Calamenene), and sulphur compounds (e.g. GC798: methyl-thiocyanate; GC843: methyl isothiocyanate; GC1447: isopropyl isothiocyanate; GC1784: 2-ethyl-thiophene). The profiles of volatile metabolites did not strongly correlate with the geographic origin of the accessions. However, volatile metabolites abundant in cluster C2 were described to have pungent and spicy sensory attributes (The Good Scents Company, 2019). Some of these compounds included 1-Penten-3-ol (GC600; pungent, horseradish-like), *(E)*-2-pentenal, (GC987; pungent, green, fruity apple-like) (*E,E*)-2,4-hexadienal (GC 2033; pungent fatty green), *(Z)*-2-penten-1-ol, (GC1074; mustard horseradish), *(E)*-2-hexenal, (GC1576; sharp, penetrating fresh leafy green, spicy). Compounds abundant in cluster C3 including isobutyl isovalerate (GC2667), butanoic acid, 2-methyl-, 3-methylbutyl ester (GC3301), 2-methylbutyl 2-methylbutanoate (GC3365), isobutyl isobutyrate (GC 1858); 3-methylbutyl 2-methylpropanoate (GC2685), propanoic acid, 2-methyl-, 2-methylpropyl 2-mehtyl propanoate (GC 2011) have been described to have a sweet fruity flavour. Other abundant compounds with specific flavour and taste in the C3 cluster included cubenol (GC5447; spicy), linalool (GC3327; floral, citrus scent), (*E*)-beta-ocimene (GC2893; green, tropical, woody), beta-caryophyllene (GC4609; spicy, woody) (The Good Scents Company, 2019).

Supervised sPLS-DA with the 130 volatiles revealed that the error rate obtained by the prediction models were stabilized after the first five dimensions (Supplementary Table 3). The maximum distance method showed the lower error rate for the five dimensions compared with the centroids and Mahalanobis distances (Supplementary Table 3). The first dimension explained 15% of variation and discriminated Asian accessions from African ones. The second dimension explained 13% of variation and partially discriminated East/Southern African accessions from Asian and West African ones (Fig. 8a). The model selected ten discriminative volatiles, all positively correlated with dimension 2 and thus in relatively low levels in West African accessions. GC1074 ((*Z*)-2-penten-1-ol), GC 1944 (heptanal), GC2164 (unknown), GC2586 (unknown), GC2651 (unknown) and GC3426 (nonanal) were positively correlated with both dimensions and characterised East African accessions. GC515 (unknown), GC 1875 (isopropyl isovalerate), GC2858 (eucalyptol) and GC3900 (unknown) were negatively correlated with dimension 1 and characterised Asian accessions.

From 31 volatiles previously reported in *G. gynandra* (Nyalala et al., 2013), 16 were identified in our study and included mainly isothiocyanates, terpenes and aldehydes. Nyalala et al. (2013) highlighted the inactivity of spider mites exposed to 2,4-heptadienal or *β*-cyclocitral, (*Z*)-2-pentenol, or methyl isothiocyanate, all compounds that were detected in our study.

2.3. Natural variation in glucosinolates and other plant defence related compounds in G. gynandra

Glucosinolates are known to be involved in plant defence against herbivores. Upon disruption of the leaf tissues, the hydrolysis of glucosinolates by myrosinases releases volatile sulphur compounds, mainly isothiocyanates nitriles that have repellent properties against pests (Beekwilder et al., 2008). In our collection, one glucosinolate putatively identified was glucocapparin, also known as methylglucosinolate (LC880). A closer look at the metabolite profiles of the accessions for both non-volatile and volatile glucosinolate-related compounds (Fig. 9) revealed three clusters of accessions. Cluster E1 was comprised of nine East African and one West African accessions which had relatively low levels of glucocapparin and isothiocyanates. Accessions in cluster E2 had overall high levels of glucocapparin and isothiocyanates. Slight correlations were found between glucocapparin and both isopropylisothiocyanate (r^2 = 0.27, ns) and methylisothiocyanate (r^2 = 0.23, ns). A weak correlation between glucocapparin and methylthiocyanate was detected (r^2 = −0.11, ns). In this study we determined these glucosinolates-related compounds as they are in the intact plant cells, by specifically preventing myrosinases activity. Thus, leaves were flash-frozen and ground in liquid nitrogen, and glucosinolates were subsequently extracted in methanol, which denatures proteins, and the thiocyanates were trapped while inhibiting enzyme activities with saturated $CaCl_2$. We therefore expected a low correlation between glucosinolates and isothiocyanates levels. The natural variation in glucosinolates in *G. gynandra* provides a basis for further investigation of the potential of these compounds with herbivore interactions. In our study we identified an aliphatic glucosinolate (glucocapparin). However, Omondi et al. (2017) identified 3-hydroxypropyl glucosinolate as the main glucosinolate present in different plant parts of 30 accessions of *G. gynandra* collected in various East African countries while Neugart et al. (2017) observed mainly glucocapparin and only traces of indole glucosinolates (glucobrassicin and 4-methoxyglucobrassicin) in *G. gynandra* leaves. Glucosinolate levels were strongly influenced by

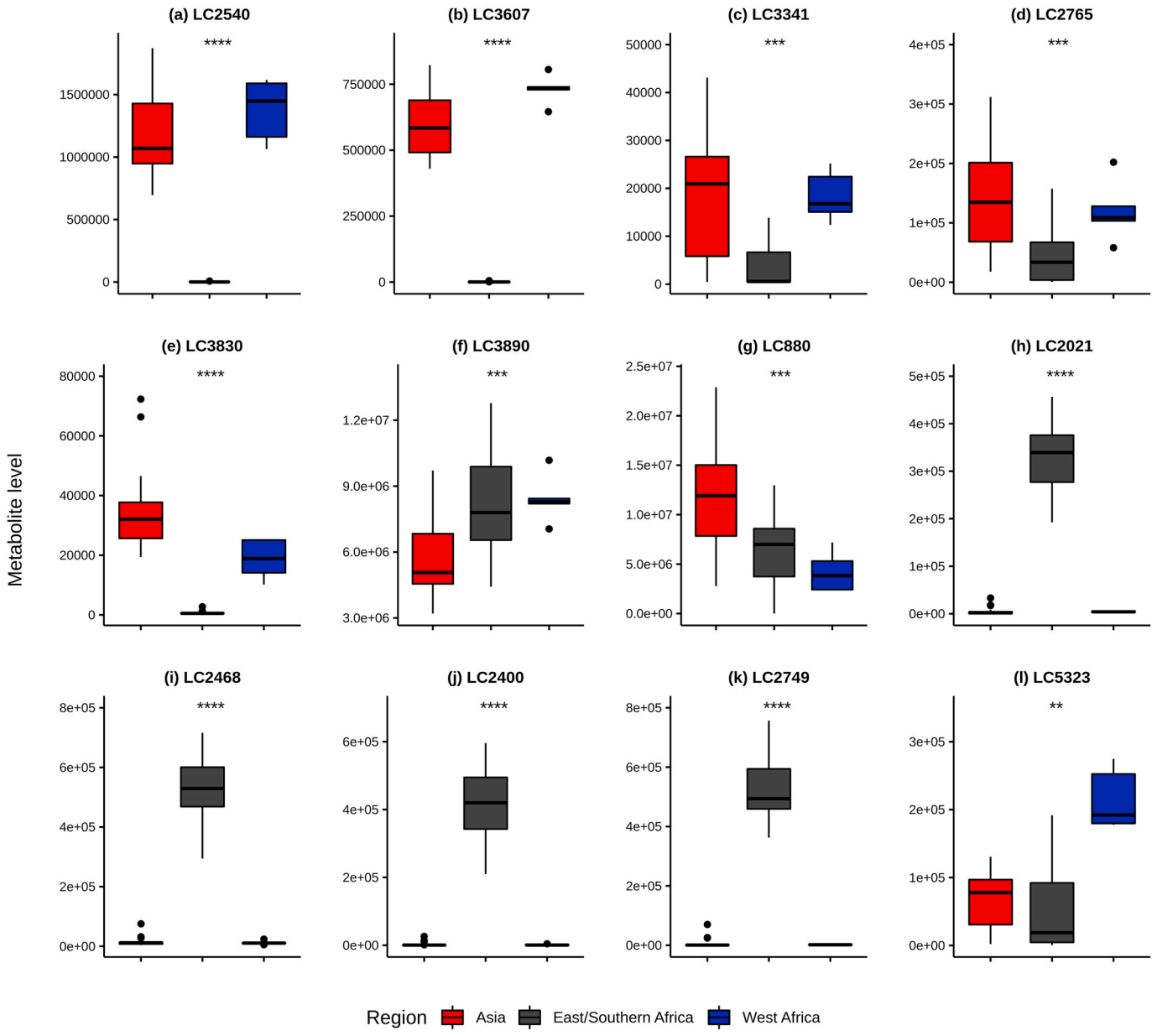

Fig. 5. Box plots showing the variation in relative levels of 14 annotated semi-polar metabolites in the leaves of 48 accessions of *Gynandropsis gynandra*. Lower and upper box boundaries represent 25th and 75th percentiles, respectively, the line inside the box is the median, lower, and upper error lines are 10th and 90th percentiles, respectively. Filled circles represent outliers. Putative identities: (a) LC2540: caffeoyl-oxalosuccinate; (b) LC3607: caffeoyl-hydroxycitric acid; (c) LC3341: dihydroxy-eudesmenolide-hexoside; (d) LC2765: Icariside B8; (e) LC3830: rhamnazin-hexoside-deoxyhexoside; (f) LC3890 quercetin-3-O-rutinoside; (g) LC880: glucocapparin; (h) LC 2021: caffeoyl-citric acid; (i) LC2468: coumaroyl-glucaric acid; (j) LC2400: glucaric acid-C26H26O14 conjugate; (k) LC2749: feruloyl-glucaric acid; (l) LC5323: dihydroxy-eudesmanolide-hexoside.

plant developmental stages in *Aethionema arabicum* and tended to decrease after flowering (Mohammadin et al., 2017). The discrepancies between our results and previous investigations of glucosinolates in *G. gynandra* may therefore be explained not only by differences in the specific accessions used but also by differences in growth conditions and plant developmental stages.

3. Conclusions

Gynandropsis gynandra leaves are rich in specialised metabolites with potential benefits for human health and/or improved plant resistance against pests. Our results provide evidence for geographic-specific metabolite profiles, mainly driven by the variation in semi-polar metabolites. Local adaptation and reproductive isolation might have played a role in shaping the metabolite diversity in the species. Further sampling of accessions in other regions of the world and genomic investigation are required to better understand the observed population structure. Accessions with contrasting metabolite profiles could be selected for further breeding towards lines with relative high levels of compounds of interest to human health, pleasant taste, or odour, but also for compounds key to plant defence against herbivores or pathogens. Additional efforts in annotation of leaf metabolites and investigation of the variation in key metabolites during plant development and in response to different environments and food processing methods could contribute to establishing breeding programs for improved quality in *G. gynandra*.

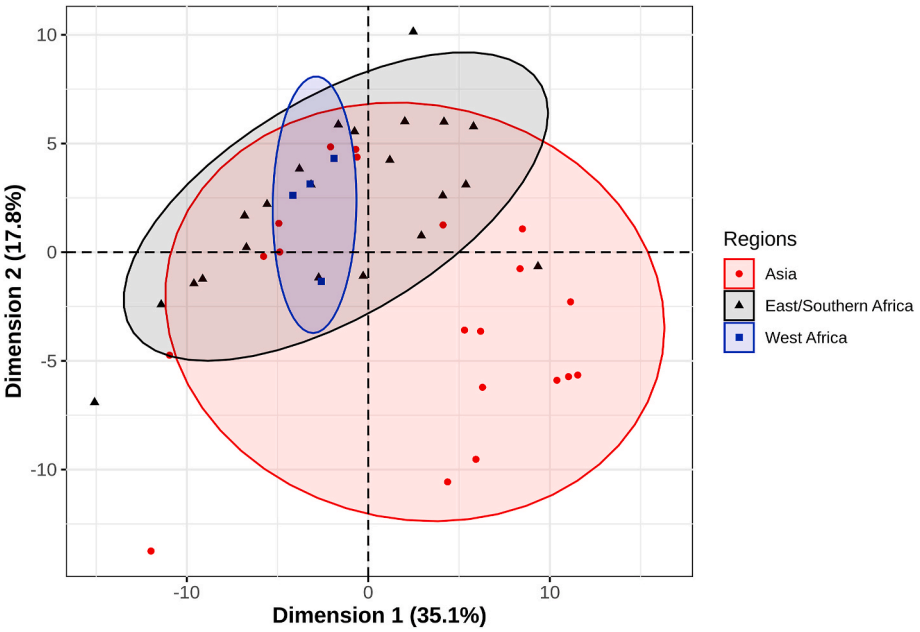

Fig. 6. Principal component analysis score plot of relative levels of 130 volatile metabolites detected in the leaves of 46 accessions of *Gynandropsis gynandra* from Asia (red), East/Southern Africa (black) and West Africa (blue). The first two dimensions explaining 52.9% of the total variation are shown. 95% confidence ellipses are presented for the three regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

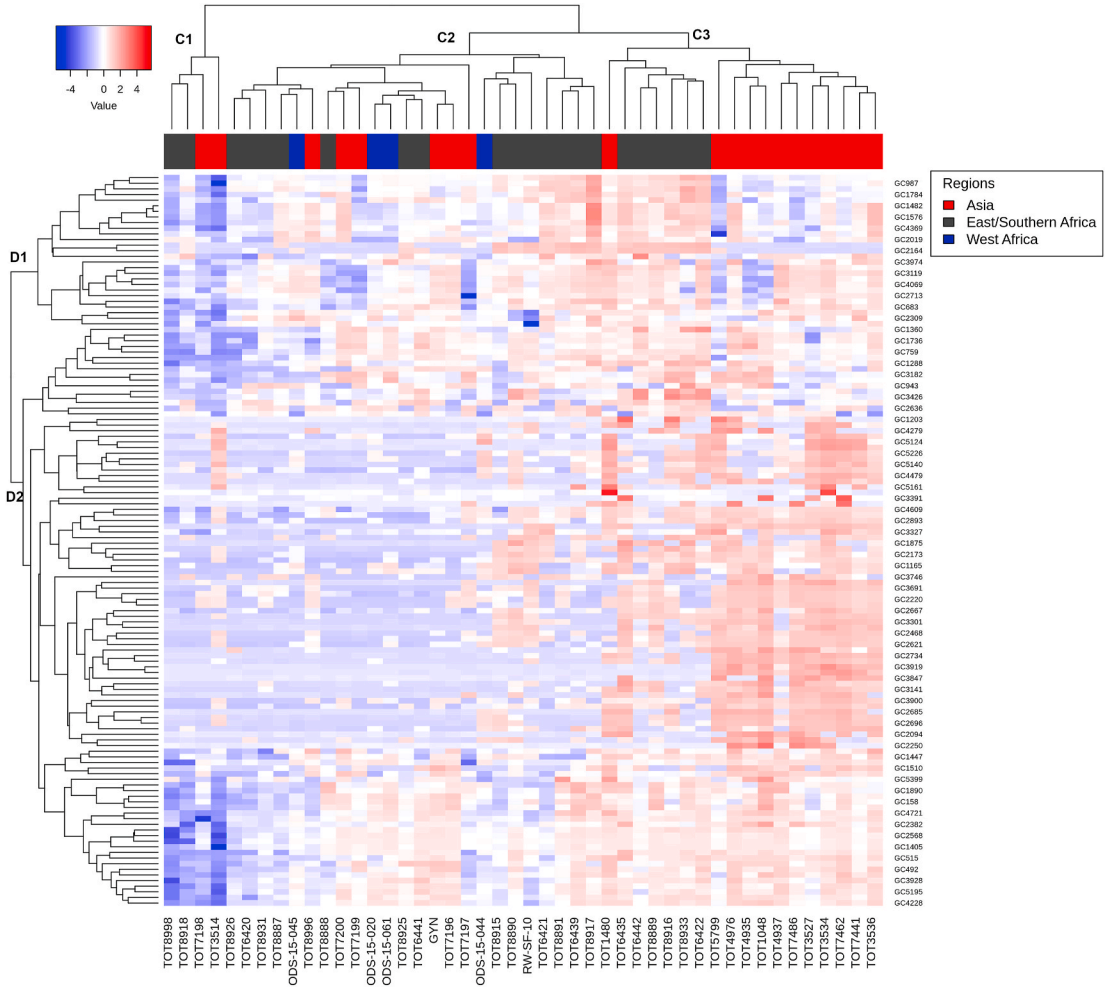

Fig. 7. Heatmap of the 130 volatile metabolites detected in the leaves of 46 accessions of *Gynandropsis gynandra* from Asia (red), East/Southern Africa (black) and West Africa (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

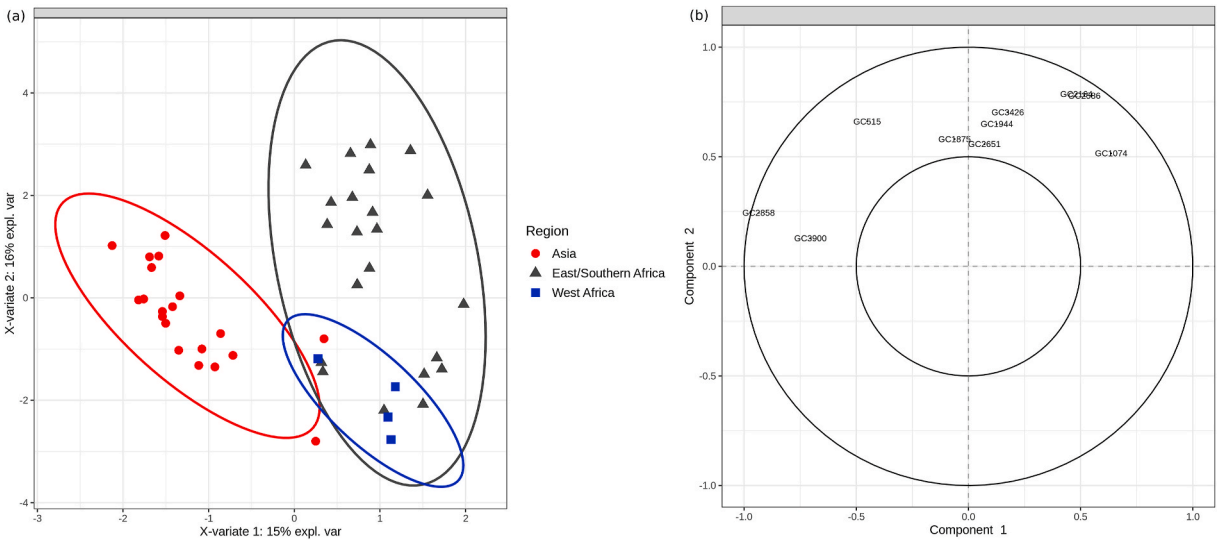

Fig. 8. Sparse partial least square discriminant analysis on the 48 accessions of *Gynandropsis gynandra* based on 130 volatile metabolites: (a) Score plot showing the projection of the 48 accessions Asia (red), East/Southern Africa (black) and West Africa (blue) on the two dimensions; (b) Selected variables representation on two dimensions on the correlation circles (0.5 and 1 correlation values). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

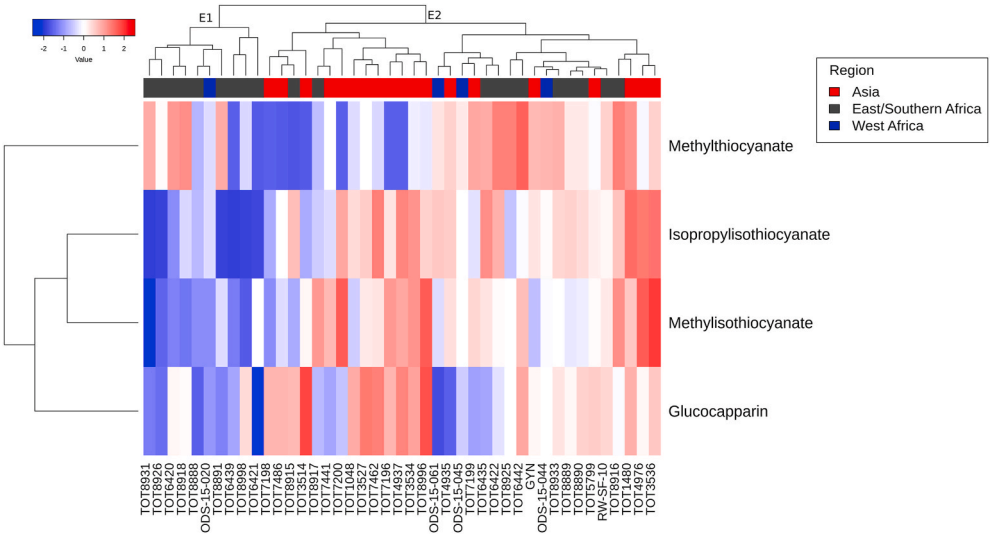

Fig. 9. Relative levels of glucosinolates and isothiocyanates in 43 accessions of *Gynandropsis gynandra* from Asia (red), East/Southern Africa (black) and West Africa (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Experimental

4.1. Plant material

A total of 48 accessions of *Gynandropsis gynandra* (L.) Briq (Cleomaceae) that represented the geographic distribution (Fig. 1.) and morphological diversity of germplasm collections from the World Vegetable Center and the Laboratory of Genetics, Horticulture and Seed Science in Benin were selected (Sogbohossou et al., 2019). The detailed list of accessions and their provenance are presented in Table 1. Three individuals per accession were grown on rockwool under irrigated conditions in a greenhouse at Wageningen University from August to December 2015. Leaf sampling was performed on eight weeks-old plants. Three to five leaves were harvested per individual and leaves were pooled per accession, making a total of ten to fifteen leaves from each accession. The leaves were snap frozen in liquid nitrogen, ground to fine powder and stored at −80 °C until analysis.

4.2. Metabolite data acquisition and processing

4.2.1. Extraction and analysis of semi-polar specialised metabolites

Semi-polar metabolites were extracted using the protocol described previously by van Treuren et al. (2018). A total of 500 mg fresh weight of frozen ground leaf material was extracted with 1.5 ml of 99.87% methanol containing 0.13% formic acid (v/v), which resulted in final concentrations of about 75% methanol and 0.1% formic acid, assuming a leaf water content of about 95%. Frozen samples were vigorously vortexed immediately after adding the extraction material to the frozen material, sonicated for 15 min and centrifuged at maximum speed for 15 min. The resulting extracts were passed through a 0.2 μm polytetrafluoroethylene (PTFE) filter. To check the total technical variation, including extraction, sample analysis and data-processing, five quality control samples were prepared from pooled leaf material of several randomly chosen accessions, extracted using the same procedure and injected after every eight accession extracts.

All the extracts were analysed as originally described by de Vos et al. (2007) and adapted for the currently used LCMS system (van der Hooft et al., 2012). In short a liquid chromatography system (Water Acquity) was coupled to a photodiode array detector (Waters, 240–600 nm) and a high-resolution mass spectrometry (Orbitrap FTMS, Thermo Fisher Scientific), using C18-reversed phase chromatography (Luna C18 column, 2.0 × 150 mm; Phenomenex) and negative electrospray ionization (m/z 95–1350). Five µl of the extract were injected and separated using a binary gradient of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid, with a flow rate of 0.19 ml/min. The initial solvent composition consisted of 95% of A and 5% of B; increased linearly to 35% A and 65% B in 45 min and maintained for 2 min. The column was washed with 25% A and 75% B for 5 min and equilibrated to 95% A and 5% B for 2 min before the next injection. Xcalibur software (Thermo) was used to control all instruments and for data acquisition.

4.2.2. Extraction and analysis of volatile specialised metabolites

Volatile metabolites were extracted using the protocol described by Lopez-Sanchez et al. (2015); Wahyuni et al. (2013). An extraction solution of 5 M $CaCl_2$, 25 mM Tris, 25 mM EDTA (CTE) was prepared. 100 mg of frozen leaf material was weighed in a 10-ml screw-cap glass vials and 1.7 ml of CTE was added. The vials were capped, vortexed and sonicated for 10 min. Collection of volatile compounds was done by dynamic headspace trapping using the Gerstel dynamic headspace (DHS) module mounted onto a multipurpose sampler robot (MPS2) (Gerstel, Mülheim, Germany). Samples were first incubated under agitation at 40 °C for 10 min. After incubation time, volatiles were collected from the headspace by purging the vial with 150 ml of helium on a glass tube filled with Tenax TA (Gerstel, Germany). The temperature of the Tenax was kept at 20 °C. After volatile trapping the glass tube was purged with 30 ml to remove moisture. Volatile compounds were transferred to the gas chromatograph (GC) by thermally desorbing the Tenax tubes at 250 °C for 5 min using a Gerstel thermal desorption unit (TDU) module (Gerstel, Germany) under a constant helium flow in splitless mode. Compounds were re-focussed on a packed sorbent liner filled with Tenax TA at 0 °C that was fitted into a cooled injection system (CIS 4 module, Gerstel, Germany). The CIS was then ballistically heated under a constant helium flow with 12 °C/s to 280 °C with 5 min hold to transfer the compounds to the analytical column with a split ratio of 1:5. The GC (Agilent GC7890A) was equipped with a ZB-5MS column (30 m × 0.25 mm id x 0.25 µm film thickness; Phenomenex). The temperature program of the GC oven started at 45 °C (2- min hold) and rose with 10 °C/min to 280 °C (3-min hold). The column effluent was transferred to a quadrupole mass spectrometer (Agilent 5975C) where volatiles were ionized by electron impact at 70eV. Mass scanning was done from m/z 33 to 350 with a scan time of 2.8 scans/sec.

As for LC-MS, quality control samples consisting of a pool of various random accessions were prepared and injected after eight accession samples to assess the analytical variation.

4.2.3. Mass spectral alignment, filtering and clustering

The MetAlign software package (www.metalign.nl) was used for baseline correction, noise estimation, and ion-wise mass spectral alignment of the GCMS and LCMS raw data. The MetAlign outputs for each GC-MS and LC-MS data sets were subsequently processed with MSClust software (Tikunov et al., 2012), in order to combine mass signals derived from the same molecule, including natural isotopes, adducts and fragment ions, resulting in reduction of data redundancy and reconstruction of mass spectra to be used for annotation purposes.

4.2.4. Putative identification of semi-polar and volatile metabolites

The identification of selected semi-polar metabolites was based on their UV/Vis light absorbance spectra and exact molecular weights. Putative identification of these metabolites was obtained using different databases such as the Dictionary of Natural Products (http://dnp.chemnetbase.com), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and in-house metabolite databases. Volatile organic metabolites were putatively identified by comparing the mass spectra, as compiled by the MSClust program, with those of commercial and in-house mass spectral library entries (e.g., NIST17, using the NIST MS Search v2.3 software; http://chemdata.nist.gov/mass-spc/ms-search/). In addition, linear retention indices (RIs) were calculated based on a series of alkanes (C_8–C_{22}) and calculated RIs were compared with those published in the literature (e.g., NIST17). With respect to the certainty of the identified compounds we followed the guidelines of the Metabolomics Standards Initiative (Fernie et al., 2011; Sumner et al., 2007).

4.3. Multivariate analysis

Metabolites were coded with their unique identifiers (centrotypes), given by MSClust software, depending on whether they resulted from LC-MS or GC-MS analysis. Multivariate statistical analyses were performed on the semi-polar and volatile metabolite data sets containing the relative intensity levels of all detected metabolites for all accessions. Preprocessing of the data was performed by log 2 transformation and mean centering using the R package RFmarkerdetector (Palla and Armano, 2016). The pre-treated data were subjected to analysis of variance followed by a post-hoc Fisher's least significant difference test to identify metabolites that were significantly different among accessions from the three regions. After autoscaling of the pre-treated data, principal component analysis (PCA) was performed with the R package mixOmics (Le Cao et al., 2019). Using the same package, a sparse partial least square discriminant analysis (sPLS-DA) was performed to select discriminative variables among our groups (Lê Cao et al., 2011). The discriminative features were selected on the datasets with all the detected metabolites (48 × 936 for LC-MS and 46 × 130 for GC-MS). The samples were partitioned into K groups (K = 3). Models were validated using the mean classification error rate with 10 cross-validations repeated 10 times for each dimension. Performances of the maximum, centroids and Mahalanobis distances were compared for the class prediction of our samples. Boxplots for significant annotated metabolites not selected by the sPLS-DA were drawn based on the raw levels of these metabolites. A Kruskal-Wallis test was performed to assess the level of significance of the variables. Heatmaps and hierarchical cluster analysis using the UPGMA method were performed using the heatmap.2 function of the R package gplots (Warnes et al., 2016).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112468.

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