GLUCOSINOLATES IN KALE GENOTYPES FROM THE BLACKSEA REGION OF TURKEY

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ABSTRACT

Cruciferous vegetables are important sources of phytochemicals such as phenolics, vitamins, minerals and glucosinolates that are known to possess potential health benefits. When consumed on a regular basis cruciferous vegetables are believed to reduce the risk of several forms of cancer. Kale (Brassica oleracea var. acephala L.) is an important crucifer grown widely around the Blacksea region of Turkey and consumed extensively by the locals. The current study was aimed to determine the glucosinolate profile and content of a collection of 101 kale genotypes at two different plant developmental stages (early and late development). We demonstrated that among the tested genotypes glucobrassicin was the most abundant glucosinolate, followed by glucoraphanin, sinigrin and glucoiberin at much lower levels. The amount of total aliphatics and indols were significantly higher when plants were fully matured compared to the early developmental stage. Potential genotypes with desired glucosinolate profiles were identified for future breeding purposes for the development of new improved cultivars delivering potential health benefits.

Keywords: kale, brassica, glucosinolates

Introduction

Fruits and vegetables are important sources of phytochemicals that may offer potential health benefits when incorporated in human diet. Among these are cruciferous vegetables such as broccoli, cabbage, kale *(Brassica oleracea* var. *acephala* L.*)*, cauliflower, watercress and Brussels sprouts. All of them are members of the *Brassicaceae* family, and are associated with a reduced risk of cancer (6, 8, 16, 21, 22, 25, 28, 29). Cancer protective activity of crucifers is most likely due to the presence of glucosinolates, which are present in plant cells and are hydrolysed to isothiocyanates, thiocyanates, and nitriles or as in the case of indols to indolyl compounds followed by the endogenous β-thioglucosidase activity upon tissue damage. Among these products are the two isothiocyanates, iberin (1-isothiocyanato-3-methylsulphinylpropane) and sulforaphane (1-isothiocyanato-4-methylsulphinylbutane), derived from the corresponding aliphatic glucosinolates respectively. In many cases, these two isothiocyanates may play protective role against several forms of cancer, probably by inhibiting carcinogen activating enzymes, inducing phase 2 detoxifying enzymes followed by the excretion of potential carcinogens from the metabolism, and inducing apoptosis (cell death) as recently described (9, 22). Some of the bioactive compounds derived from indol glucosinolates have also been associated with anticarcinogenic activity (17).

Kale is an important crucifer with an annual production of 79 990 tonnes, grown mainly around the Blacksea region of Turkey (23). The kale consumption by the locals is very high, and fresh leaves are being cooked and ingested mostly as a vegetable soup, as stuffed vegetables or in pickled form. Except as part of the human diet, kale is also used as animal feed in the region.

A project conducted to evaluate the kale genetic resources of the region, identified several kale genotypes obtained from Samsun, Ordu, Giresun, Trabzon and Zonguldak provinces of Turkey, providing almost 74% of the kale production of the country (1). Characterization and evaluation of this collection was performed morphologically according to the guidelines for the conduct of tests for the distinctness, homogenity and stability criteria for new varieties of plants, provided by the International Union for the Protection of New Varieties of Plants (UPOV) (1). However, studies regarding the phytochemical composition of these genotypes have not been performed so far.

The current study aims to determine the glucosinolate profile and content of this collection of kale genotypes that has not been evaluated before. As previously demonstrated by several research groups, genetic background of individual plants, as well as soil properties and environmental factors, influence the glucosinolate content of plants (5). Therefore, variation in terms of glucosinolate content in different cultivars of a single species may occur. Similarly, while commercial cultivars are reported to contain intermediate levels of aliphatic glucosinolates, wild brassicas are reported to contain high levels of aliphatics (14). In addition, with the utilization of this genetic variation in terms of glucosinolate production, high glucosinolate broccoli cultivars are generated as a result of a conventional breeding program by crossing a wild brassica with a commercial broccoli cultivar (15, 20). With the awareness of the effect of genetic background of individuals on glucosinolate production,

profiling of genotypes from this collection of kale genotypes was inevitable, when identifying any existing potential lines with desired levels of glucosinolates.

Mineral nutrient content of the soil, as well as environmental factors during the cultivation period, influence the glucosinolate content of plants (11, 19). In addition, the glucosinolate content of plants at different developmental stages probably varies. Hence it is important to know the amount of glucosinolates produced at different developmental stages, especially when the optimum harvest time is considered for improved health benefits.

In the current paper we demonstrate the glucosinolate production of kale genotypes by profiling and quantifying leaf glucosinolates at two different developmental stages (early and late development). These genotypes may undergo further selection as part of future breeding efforts aiming to generate new cultivars with potential health benefits. Furthermore, we present the changes in glucosinolate content of plants that arise between young and mature ones.

Materials and Methods

Plant Material

A total of 101 kale genotypes belonging to the collection of brassica germplasm from the Blacksea region collected from Samsun, Ordu, Giresun, Trabzon and Zonguldak provinces of Turkey were used for the analysis of glucosinolates. The plants were grown at the same site at two consequent years. Seeds were sown in multipot trays at the beginning of May and seedlings were transplanted to the field conditions at the five-six leaf stage. Seedlings were planted in rows by leaving 60 cm between plants and 90 cm between rows. Leaf samples were taken each year at two different developmental stages. First sampling was from young plants (taken 1 month after transplantation to the field conditions) and second sampling was when the plants were fully mature (taken 3 months after transplantation to the field conditions).

Analysis of Glucosinolates

Leaf samples were freeze-dried prior to the analysis. Extraction of glucosinolates, conversion to desulfoglucosinolates and analysis by HPLC was as described previously (12). Samples were analysed and separated by HPLC-UV (Shimadzu®) detection in the HPLC laboratory at Ankara University, Department of Horticulture. A volume of 80 µl from the extract was injected onto a Waters Spherisorb® 5µM ODS 2, 4.6x250mm analytical cartridge. Analysis was carried out on a gradient of 99% water and 1% acetonitrile (Merck) as presented below, at a flow rate of 1ml/min for 24 min. The detection was carried out at a wavelength of 229 nm.

Benzyl glucosinolate 16mM (glucotropaeolin) was used as the internal standard for the quantification. The quantification of individual glucosinolates was carried out according to Heaney et al. (7) and expressed as $\mu molg^{-1}$ dry weight. Correction factors for glucoiberin, glucoraphanin, sinigrin, glucobrassicin, methoxy-indolylmethyl and hydroxyindolylmethyl glucosinolates were used for calculation.

Statistical Analysis

Multifactorial variance analysis (ANOVA) was performed to evaluate tha data obtained, using MINITAB® version 14. Plant developmental stage and genotype were taken into consideration as variables. Significant differences were evaluated at $P<0.001$ error level. Data were presented as mean values of all genotypes ± standart error (SE) of mean. The relationships among 30 genotypes selected on the basis of their glucosinolate content were determined by Multidimensional Scaling (MDS) analysis using SPSS program, version 15.

Results and Discussion

In the current study, individual and total glucosinolate contents of 101 kale genotypes were determined at two developmental stages. Glucosinolate profiling of individuals revealed that aliphatic glucosinolates glucoiberin, glucoraphanin and sinigrin were the glucosinolates synthesized together with

TABLE 1

Mean ($\mu mol\ g^{-1}$ dw) glucosinolate content ± standard errors of the genotypes at two plant developmental stages: (1) leaf samples taken 1 month after transplantation; (2) leaf samples taken 3 months after transplantation to the field conditions (1st year).

Developmental Stage	Glucoiberin	Glucoraphanin	Sinigrin	Total aliphatics	Glucobrassicin	neoglucobrassicin	4-methoxy-glucobrassicin	4-hydroxy-glucobrassicin	Total Indoles
1	1.011±0.099	0.544±0.06	0.363±0.035	1.918±0.13	28.641±0.902	1.324±0.115	1.935±0.113	0.705±0.083	32.60±1.03
2	2.854±0.254	0.856±0.097	0.654±0.057	4.364±0.26	48.81±2.00	1.3812±0.096	5.258±0.295	1.135±0.149	56.58±2.18

TABLE 2

Mean ($\mu mol\ g^{-1}$ dw) glucosinolate content ± standard errors of the genotypes at two plant developmental stages: (1) leaf samples taken 1 month after transplantation; (2) leaf samples taken 3 months after transplantation to the field conditions (2nd year).

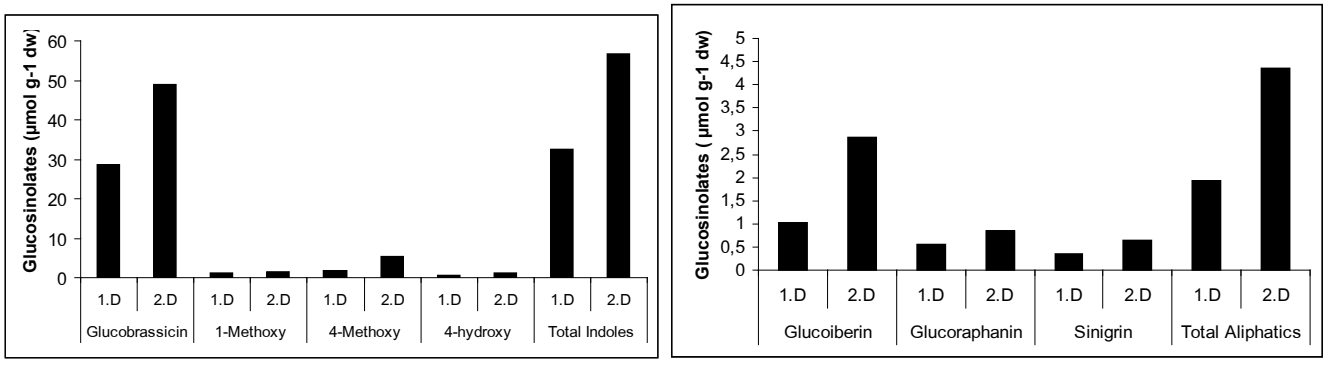
Developmental Stage	Glucoiberin	Glucoraphanin	Sinigrin	Total aliphatics	Glucobrassicin	neoglucobrassicin	4-methoxy-glucobrassicin	4-hydroxy-glucobrassicin	Total Indoles
1	0.102±0.02	0.41±0.037	0.888±0.065	1.41±0.1	34.06±1.58	1.451±0.132	2.503±0.221	1.093±0.088	39.10±1.67
2	0.21±0.04	0.741±0.071	1.364±0.112	2.341±0.16	46.81±1.48	3.17±0.337	5.864±0.379	1.103±0.081	56.96±1.64

indolyl glucosinolates glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin. Among aliphatics glucoraphanin, sinigrin and glucoiberin were detected in almost all genotypes however at very low levels.

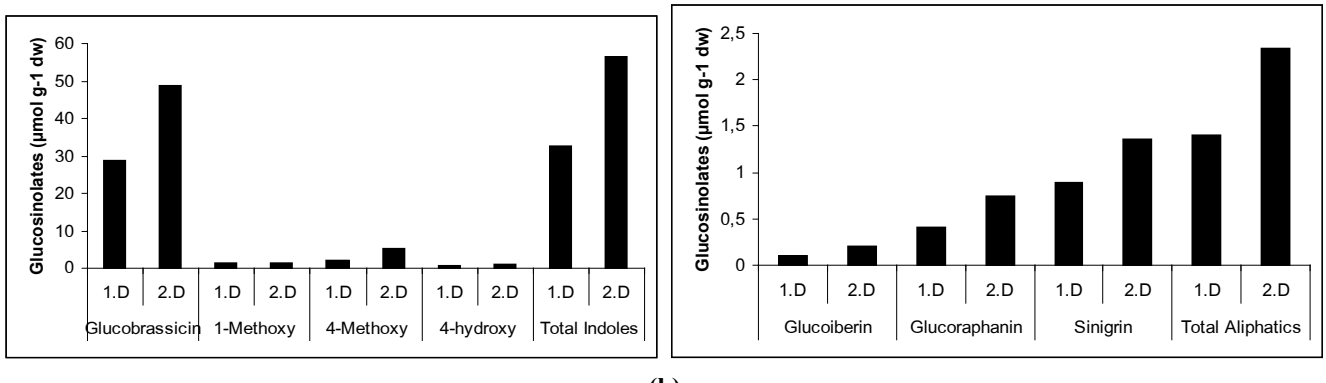
Among indoles, glucobrassicin was the predominant glucosinolate present in all genotypes at very high levels which was followed by 4-methoxyglucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin at much lower levels (**Table 1** and **2**). Depending on the characteristics of the variety studied, the predominant glucosinolates in kales are reported to be sinigrin, and to a lesser extent glucoraphanin and glucobrassicin (10) or sinigrin, glucobrassicin and glucoiberin (24) by different research groups. The analysis of glucosinolates from a collection of kale varieties from Northwestern Spain also revealed that while sinigrin, glucoiberin, glucobrassicin and neoglucobrassicin were present in all varieties studied, glucoraphanin and 4-hydroxyglucobrassicin were detected in 60-70% of the varieties (2). This variation can mainly be attributed to the genetic background of individuals (15, 20).

High indol glucosinolate content is usually associated with stress factors during the cultivation period or as a result of insect herbivory (13). During field experiments, standard cultural practices were performed and plants were kept insect and pest free by spraying twice during the cultivation period, hence the high levels of indolyl compounds could not be due to insect and pest attacks. However, in the conditions of Ankara, summer temperatures arise up to 40˚C which may have probably boosted up the amount of indol glucosinolates as recently demonstrated in cabbages (2).

The amount of aliphatic and indol glucosinolates at two different developmental stages (when plants were young and mature) were also significantly differing from each other. The first year experiment results revealed that while total aliphatic glucosinolate content of genotypes ranged from 0.00-6.93 μmolg^{-1} dw with a mean value of 1.918±0.13 μmolg^{-1} dw, indolyls ranged from 6.73-49.06 μmolg^{-1} dw with a mean value of 32.60±1.03 μmolg^{-1}dw and glucobrassicin being the predominant among all had an amount varying between 5.65-46.46 μmolg^{-1} dw in younger plants. Similarly, the amount of total aliphatics varied between 0.00-14.62 μmolg^{-1} dw, with a mean of 4.364±0.26 μmolg^{-1} dw and total indols ranged from 3.18-71 μmolg^{-1} dw, with a mean value of 56.58±2.18 μmolg^{-1} dw when plants were fully mature (**Table 1**). The experiment was repeated the following year in order to evaluate the effect of the different developmental stage on the glucosinolate content of the plants. The findings from the second year experiment also showed a similar profile. While total aliphatic glucosinolate content of individuals ranged from 0.21-5.47 μmolg^{-1} dw with a mean value of 1.41±0.1 μmolg^{-1} dw, indolyls ranged from 5.88-79.64 μmolg^{-1} dw with a mean value of 39.10±1.67μmolg^{-1} dw, glucobrassicin being the predominant indol, had an amount varying between 5.11-65 μmolg^{-1} with a mean value of 34.06±1.58 μmolg^{-1} dw when plants were young. Similarly, the amount of total aliphatics



(a)



(b)

Fig 1. a and **b** Graph demonstrating the changes in indolyl and aliphatic glucosinolate content of genotypes at two developmental stages (1.D: young; 2. D: mature) of plants in Year 1 (a) and Year 2 (b).

varied between 0.42-9.23 µmolg^{-1} dw, with a mean value of 2.341±0.16 µmolg^{-1} dw and total indols ranged from 19.54-85 µmolg^{-1} dw, with a mean value 56.96±1.64 µmolg^{-1} dw when plants were fully mature (**Table 2**). These results suggested that there is a significant increase in the glucosinolate content of the genotypes studied, occuring at two different plant stages of development in terms of both the amount of total aliphatics and total indols in both experimental years ($P<0.001$) as demonstrated in **Fig. 1 a,b**. These results were in agreement with other research teams (3, 18, 24) who reported changes in sinigrin and total glucosinolate content of plants during later stages of plant development. Environmental conditions also influence the amount of glucosinolates produced (4, 11, 19, 27). High temperatures and long day length in spring/summer season are also reported to positively affect the amount of total glucosinolates and indoles in particular, contrary to aliphatics which are produced at much higher levels in fall season (2, 24).

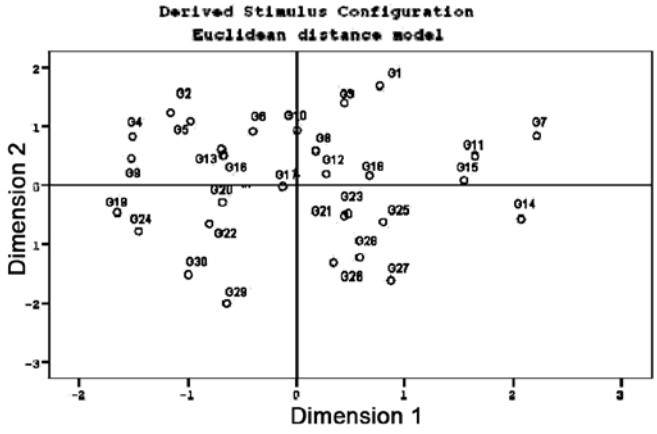


Fig 2. MDS (Multidimensional Scaling) graph showing 30 selected genotypes and the relationships among them.

Despite low aliphatic content of the collection of 101 kale genotypes, 30 potential genotypes consistently synthesizing glucoiberin and glucoraphanin in particular, were selected as potential candidates for further breeding purposes and the relationship between them in terms of individual and total glucosinolates were evaluated using Multidimensional Scaling (MDS) analysis. The genotypes producing glucoiberin (the precursor of iberin) and glucoraphanin (the precursor of sulforaphane) at relatively higher levels were selected for further breeding purposes (**Fig. 2**). According to the graph, genotypes were divided into four major groups: first group including genotypes G2, G4, G5, G6, G9, G13, G16; second group including genotypes G1, G3, G7, G8, G11, G12, G15, G18; third group including genotypes G19, G20, G22, G24, G29, G30 and finally fourth group including genotypes G21, G23, G25, G26, G27, G28, G14. Genotypes G10 and G17 were located in the middle of two groups and genotype G10 being more closely related to genotypes G6 and G8.

Within each group, genotypes G7, G11 and G15 were more related to each other in terms of glucosinolate content compared to the rest of the genotypes in the same group. Genotype G1 was distantly related to the rest of the genotypes within the group but closer to genotype G3. While genotypes G21, G23, G25, G26, G27, and G28 were clustered together, the G14 genotype was distantly related with the rest of the group.

When closely related genotypes were taken into consideration, it has been determined that genotypes G11 and G15 revealed a similar profile to each other except that genotype G11 contained very low levels of neoglucobrassicin contrary to genotype G15. Genotype G7 was different from G11 and G15 in terms of the amount of indoles produced. Genotypes G13 and G16 were very closely related to each other in terms of aliphatic and indol glucosinolates as the only difference was the neoglucobrassicin being present in genotype G13 but not in genotype G16. The two related genotypes G21 and G23 showed a similar glucosinolate profile, only differing in the amount of glucoiberin produced.

Conclusions

Glucosinolates, glucoiberin and glucoraphanin in particular, are considered to contain anticarcinogenic properties. Hence, the initial aim of the current study was to characterize these genotypes as a source of potential genetic material coding high levels of desired glucosinolates and furthermore to utilize this potential germplasm for the development of improved novel cultivars. The results indicated that the genotypes were synthesizing more indoles than aliphatics, which were also reported to possess anticarcinogenic properties (17). The presence of glucoiberin and glucoraphanin in almost all varieties is promising. Individuals synthesizing aliphatic glucosinolates (glucoiberin and glucoraphanin in particular) together with glucobrassicin were selected. These genotypes will further be evaluated together with their agronomic performance for future breeding efforts aiming the development of new kale cultivars with potential health benefits. However, it must be noted that in addition to the improvement of glucosinolate content of crucifers it is also necessary to identify the major glucosinolate breakdown products in order to assess the anti-cancer potential of any crucifer.

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