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Evaluation of the agronomic performance and metabolome changes in two divergent selections of kale (*Brassica oleracea* var. acephala) for glucosinolate content under different environmental conditions.

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Abstract

Glucosinolates (GLs) are biologically active secondary metabolites of the Brassicaceae family. These compounds contribute to enhancing the resistance to pests and diseases and have allelopathic and anticarcinogenic effects. GLs contents have quantitative inheritance, which is regulated by complex genetic and environmental factors. Kales (*Brassica oleracea* acephala group) are important vegetable crops in traditional farming systems in the world. The glucosinolate content of kales is influenced by environmental factors, plant part examined and phenological stage of plant growth. This study is aimed to the evaluation of the agronomic performance and metabolomics changes occurring in two divergent selections of kale (*B. oleracea* L) for leaf glucosinolates contents (high and low sinigrin and high and low glucobrassicin) at five different environments. The result showed that, the location had a significant effect on agronomic performance for both selections. HGBS and LGBS varieties had a significant effect on agronomic performance except for plant height. The HSIN and LSIN varieties had significant effect on the vegetative growth traits evaluated. Six glucosinolates were detected as SIN, GIB, GBS, OHGBS, NeoGBS, and MeOHGBS in two divergent selections for leaf glucosinolates contents of kale (*B. oleracea* L). The location had a significant effect on all GLs. HGBS and LGBS varieties had a significant effect on GIB, GBS, and NeoGBS. HSIN and LSIN varieties had a significant effect on GBS, GIB, and SIN. The score plot for PLS-DA exhibited an obvious separation between the divergent selections for GLs low and high (SIN) and low and high (GBS). Most of the traits influenced by environmental factors and climatic parameters before harvesting showed the highest importance. These results demonstrated that the plant under different environmental condition gave a good agronomic performance and metabolites profile in the divergent selections for GLs of kale have a clear separation between low and high (SIN) and low and high (GBS)

.**Keywords:** Glucosinolates, Metabolomics, Kale, *Brassica oleracea*, Environmental factors.

Resumen

Los glucosinolatos (GL) son metabolitos secundarios biológicamente activos de la familia Brassicaceae. Estos compuestos contribuyen a mejorar la resistencia a plagas y enfermedades y tienen efectos alelopáticos y anticancerígenos. Los contenidos de GL tienen herencia cuantitativa, que está regulada por factores genéticos y ambientales complejos. Los Kales (grupo Brassica oleracea acephala) son importantes cultivos de hortalizas en los sistemas agrícolas tradicionales del mundo. El contenido de glucosinolato de kales está influenciado por factores ambientales, la parte de la planta examinada y la etapa fenológica del crecimiento de la planta. El objetivo de este estudio es evaluar el rendimiento agronómico y los cambios en la metabolómica en dos selecciones divergentes de col rizada (B. oleracea L) para los contenidos de glucosinolatos de las hojas (sinigrina alta y baja y glucobrassicina alta y baja) en cinco ambientes diferentes. El resultado mostró que la ubicación tuvo un efecto significativo en el rendimiento agronómico de ambas selecciones. Las variedades HGBS y LGBS tuvieron un efecto significativo en el rendimiento agronómico, excepto en la altura de la planta. Las variedades HSIN y LSIN tuvieron un efecto significativo sobre los rasgos de crecimiento vegetativo evaluados. Se detectaron seis glucosinolatos: SIN, GIB, GBS, OHGBS, NeoGBS y MeOHGBS en dos selecciones divergentes para los contenidos de glucosinolatos de la hoja de la col rizada (B. oleracea L). La ubicación tuvo un efecto significativo en todos los GL. Las variedades HGBS y LGBS tuvieron un efecto significativo en GIB, GBS y NeoGBS. Las variedades HSIN y LSIN tuvieron un efecto significativo en GBS, GIB y SIN. El gráfico de puntaje para PLS-DA exhibió una separación obvia entre las selecciones divergentes para GL bajo y alto (SIN) y bajo y alto (GBS). La mayoría de los rasgos influenciados por factores ambientales y parámetros climáticos antes de la cosecha mostraron la mayor importancia. Estos resultados demostraron que la planta bajo diferentes condiciones ambientales dio un buen desempeño agronómico y el perfil de metabolitos en las selecciones divergentes para GL de col rizada tienen una separación clara entre bajo y alto (SIN) y bajo y alto (GBS).

Résumé

Les glucosinolates (GL) sont des métabolites secondaires biologiquement actifs de la famille des Brassicacées. Ces composés contribuent à renforcer la résistance aux ravageurs et aux maladies et ont des effets allélopathiques et anticancérigènes. Le contenu des GL a un héritage quantitatif, qui est régulé par des facteurs génétiques et environnementaux complexes. Le chou frisé (groupe Brassica oleracea acephala) sont d'importantes cultures maraîchères dans les systèmes agricoles traditionnels du monde. La teneur en glucosinolates du kale est influencée par les facteurs environnementaux, la partie végétale examinée et le stade phénologique de la croissance des plantes. Cette étude vise à évaluer les performances agronomiques et les changements métabolomiques intervenant dans deux sélections divergentes de chou frisé (B. oleracea L) pour les teneurs en glucosinolates des feuilles (sinigrin haut et bas et glucobrassicine haute et basse) dans cinq environnements différents. Le résultat a montré que l'emplacement avait un effet significatif sur la performance agronomique pour les deux sélections. Les variétés HGBS et LGBS ont eu un effet significatif sur la performance agronomique sauf pour la hauteur des plantes. Les variétés HSIN et LSIN ont eu un effet significatif sur les caractères de croissance végétative évalués. Six glucosinolates ont été détectés: SIN, GIB, GBS, OHGBS, NeoGBS et MeOHGBS dans deux sélections divergentes pour les teneurs en glucosinolates des feuilles du chou frisé (B. oleracea L). L'emplacement a eu un effet significatif sur tous les GL. Les variétés HGBS et LGBS ont eu un effet significatif sur GIB, GBS et NeoGBS. Les variétés HSIN et LSIN ont eu un effet significatif sur le GBS, le GIB et le NAS. Le diagramme de score pour PLS-DA présentait une séparation évidente entre les sélections divergentes pour les GLs low et high (SIN) et low et high (GBS). La plupart des caractères influencés par les facteurs environnementaux et les paramètres climatiques avant la récolte ont montré la plus haute importance. Ces résultats ont démontré que la plante dans des conditions environnementales différentes donnait une bonne performance agronomique et que le profil des métabolites dans les sélections divergentes pour les GL de chou frisé avait une séparation nette entre faible et élevé (SIN) et faible et élevé (GBS).

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Introduction

1. Introduction

1.1. Origin and Taxonomy of Brassicaceae family.

The Brassicaceae family (Cruciferae) includes around 375 genera and about 3,200 species (Ahuja et al., 2010). The family contains the cruciferous vegetables, including species such as *Brassica oleracea* (e.g. Cabbage, broccoli, tronchuda cabbage, cauliflower, kale, Brussels sprouts, Chinese Kale, Savoy cabbage, and kohlrabi), *Brassica rapa* (e.g. Chinese cabbage, broccoleto, Chinese mustard, bok choy and turnip), *Brassica juncea* (e.g. mustard green, head mustard and cut leaf mustard), *Brassica napus* (e.g., rapeseed and rutabaga), and *Raphanus sativus* (radish) (Ishida et al., 2014). The genus *Brassica* is one of the 51 genera belonging to the crucifer family (Rakow, 2004). Species within the genus would be categorized into oilseed, forage, condiment and vegetable crops by using their buds, inflorescences, leaves, roots, seeds and stems (Francisco et al., 2016).

Table 1. Taxonomy of Brassica genus (Gómez-Campos, 1999, 2003)

Section *Brassica*
B. oleracea L.
B. montana Pourret
B. incana Ten. subsp.*incana*
B. villosa Biv. subsp.*villosa*
B. rupestris Rafin subsp. *rupestris*
B. macrocarpa Guss.
B. insularis Moris
B. cretica Lam. subsp.*cretica*
B. botteri Vis. subsp. *botteri*
B. hilarionis Post.
B. carinata Braun
B. balearica Pers
Section *Rapa* (Miller) Salmeen
B. rapa L. subsp. *Rapa*
B. napus L.
B. juncea (L.)Czen.
Section *Micropodium* DC.
B. fruticulosa Cyr. subsp. *Fruticulosa*
B. nigra (L.) Koch
B. cossoniana Boiss. and Reuter
B. spinescens Pomel
B. maurorum Durieu
B. procumbens (Poiret) O.E.Schulz
B. cadmea O.E.Schulz
B. deserlii Danin and Hedg
Section *Brassicoides* Boiss.
B. deflexa Boiss.
Section *Sinapistrum* Willkomm
B. barrelieri (L.) Janka
B. oxyrrhina Coss.
B. tournefortii Gouan

The genetic relationships among different Brassica species was established in the classical work by U (NU, 1935) (Figure 1). The so-called U-triangle comprises six species (three basic diploids and three amphidiploids). The vertices of the triangle include the three diploid species: *B. oleracea* L. (2n=18; CC), *B. rapa* L. (2n=20; AA) and *B. nigra* L. Koch (2n=16; BB) and the edges of the triangle include the three amphidiploid species: *B. juncea* L. Czern. (2n =36; AABB), *B. napus* L. (2n=38; AACC), and *B. carinata* Braun (2n=34; BBCC) (Gómez-Campo, 2003).

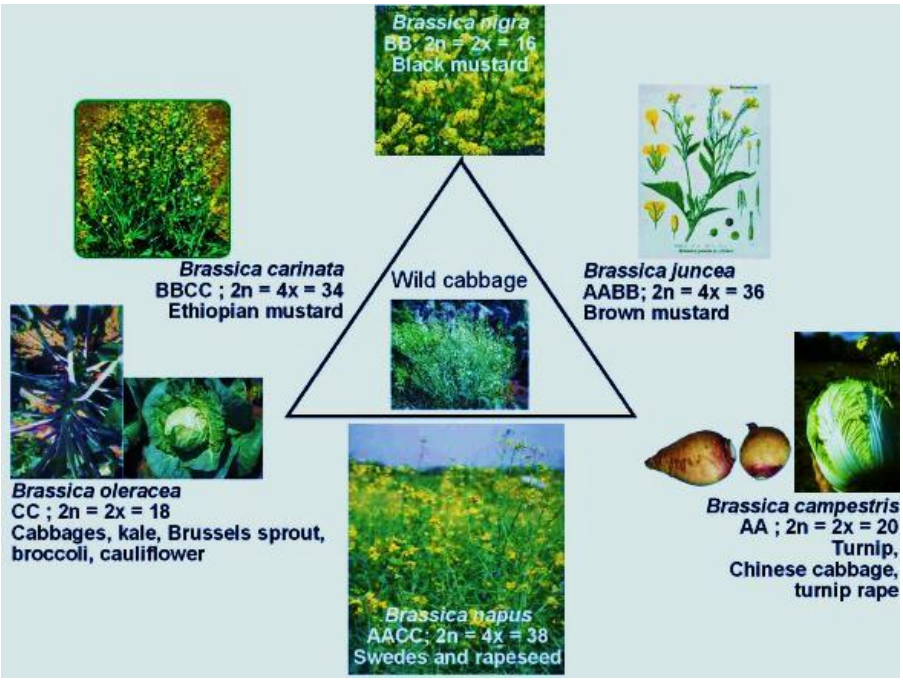

Figure 1. U-triangle showing the different Brassica species(NU, 1935)

The wild species of *B. oleracea* are found in small isolated areas on coasts of northern Spain, western France and southern and southwestern of Great Britain (Snogerup, 1980; Dixon, 2006). The wild species of *B. nigra* grow in the Mediterranean region (such as, near

Tangiers, Morocco, and under semi-cultivated conditions in Rhodes, Crete, Sicily, Turkey and Ethiopia) (Gómez-Campos, 1999). *B. rapa* was found in the highlands near the Mediterranean Sea rather than from the Mediterranean coastal areas (Gómez-Campos, 1999). From here, *B. rapa* spread northward into Scandinavia and westward to Eastern Europe and Germany (Rakow, 2004). *B. carinata* was found in the Ethiopian plateau and south Kenya (Rakow, 2004; Dixon, 2006). The primary center of diversity of *B. juncea* is central Asia (such as, northwest India, including the Punjab and Kashmir)(Dixon, 2006). Wild forms of *B. napus* have been reported to occur on the beaches of Gotland, Sweden, the Netherlands and Great Britain (Rakow, 2004)(Figure 2).

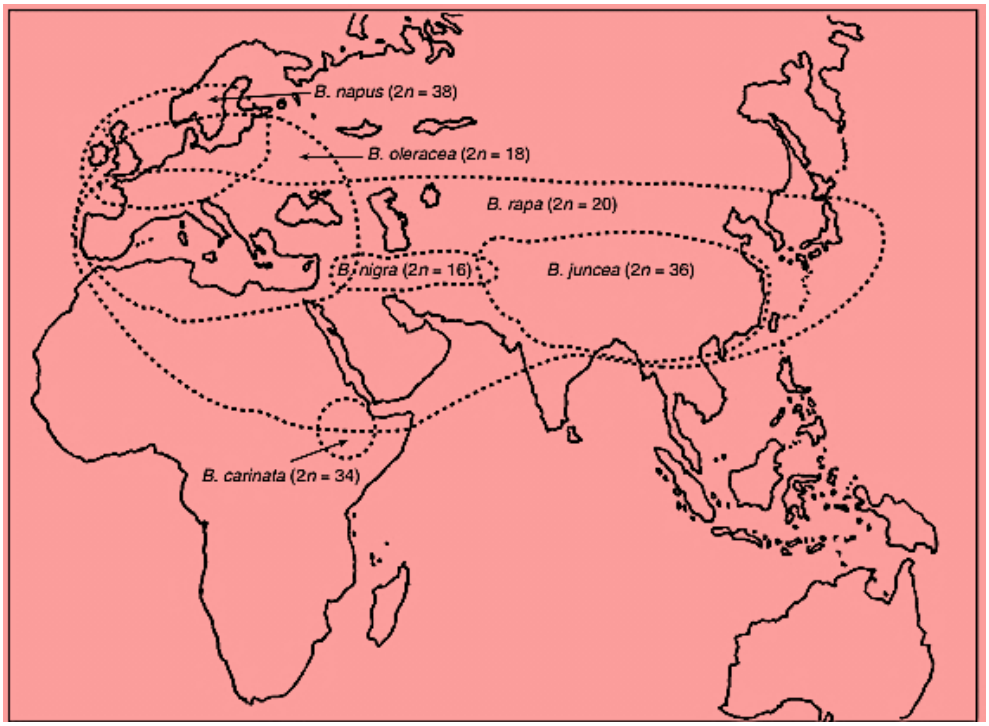

Figure 2. The origin and diversity of the major Brassica species crops according to Dixon(2006).

1.2. Origin and diversity of *B. oleracea* L.

Brassica oleracea L has grown wild in Atlantic coasts of Europe (Britain, France, and Spain) as well as in the Mediterranean basin (Dixon, 2006). The European (Occidental) Brassica vegetables originate from *B. oleracea* and probably some closely related Mediterranean species (Dixon, 2006). The cultivated forms of *B. oleracea* can be subdivided into six groups(Snogerup, 1980):cabbages (var. capitata, var. sabauda, var. bullata) including headed cabbages, Brussel sprouts, Savoy cabbage, and others; kales (var. acephala) including green kale, marrow stem kale, collards; inflorescence kales (var. botrytis, var. italica) including cauliflower, broccoli, sprouting broccoli, and others; kohlrabi (var. gongylodes); branching bush kales (var. fruticosa); and Chinese kale (*B. alboglabra*), used as a leaf vegetable.

Most of the *B. oleracea* crops have a horticultural use and their green parts are consumed as raw (in salads) or cooked as they are part of many dishes around the world. Kale is certain cultivars of cabbage (*B. oleracea*) grown for their edible leaves.

Kale includes leafy kale (var. acephala), thousand head kale (var. ramosa), scotch kale (var. sabellica), marrow stem kale (var. medullosa), palm kale (var. palmifolia), collards (var. viridis) and Chinese kale (var. alboglabra)(Gorka et al., 2018). Different kale types are traditional crops of several European countries such as Italy, Netherlands, Portugal, Scotland, Spain, Turkey (Ferioli et al., 2013). Kale is a highly cross pollinated vegetable crop and is grown under all the agro climatic conditions from subtropical. It is a well-documented fact that variation shown by available genetic resources for

quantitative and quality traits is important for vegetable breeding programme (Gorka et al., 2018).

1.3. Economic importance of Brassica Crops.

Brassica vegetables play an important role in agriculture worldwide. The world statistics of FAO (FAOSTAT, 2016) on horticultural brassicas differentiate two sections: cauliflower and broccoli on the one side and cabbage and other brassicas on the other side, which include red, white and Savoy cabbage, Chinese cabbage, Brussels sprouts and green kale. All these crops belong to *B. oleracea* species, with the exception of Chinese cabbage that belongs to the *B. rapa* species.

Regarding to the production of cabbages and other brassicas, China is the leading country worldwide with more than 33 tons/year followed by India with more than 8 million tons/year, and then other countries such as Russian Federation, the Republic of Korea, Ukraine, etc. (Table 2). Spain produces less than 1% of the world production with almost 158 thousand tons/year, which represents the 38th country in the world production. For cauliflowers and broccoli, the top ten countries by production are China is also the leader worldwide with a production of 10 million tons/year, followed by India with 8 million tons/year and then the United States of America with 1 million tons/year. Spain is the fourth country with a production of 600 thousand tons/year (Table 2).

Table 2. Production of "cabbage and other brassicas" and "cauliflowers and broccoli" in 2016 for the top 10 production countries by tons/year (FAOSTAT, 2016)**.**

Cabbages and other brassicas		Cauliflowers and broccoli	
Area	**Production(tons/year)**	**Area**	**Production(tons/year)**
China	33881515	**China**	10263746
India	8755000	**India**	8199000
Russian Federation	3618771	**United States of America**	1321060
Republic of Korea	2501953	**Spain**	605161
Ukraine	1656440	**Mexico**	583279
Indonesia	1513326	**Italy**	388281
Japan	1446000	**Poland**	314738
Poland	1091653	**France**	308488
Uzbekistan	1030107	**Bangladesh**	268484
United States of America	1027740	**Turkey**	250330

1.4. Phytochemicals in Brassica vegetables.

Brassicaceae vegetables are considered as a rich source of nutrients such as amino acids (e.g., L-alanine, L-aspartic acid, L-glutamic acid and L-glutamine, etc.), carbohydrates (e.g., sucrose and glucose), vitamins (e.g., carotenoids, tocopherol, ascorbic acid, folic acid), minerals (e.g., Cu, Zn, P, Mg, etc.), and another groups of phytochemicals such as indole phytoalexins (brassinin, spirobrassinin, brassilexin, camalexin, 1-methoxyspirobrassinin, 1-methoxyspirobrassinol, and methoxyspirobrassinol methyl ether), phenolics (such as feruloyl and isoferuloylcholine, and hydroxybenzoic, neochlorogenic, chlorogenic, caffeic, p-coumaric, ferulic, and sinapic acids, anthocyanins, quercetin and kaempferol), and glucosinolates mainly including glucoiberin, glucoraphanin, glucoalyssin, gluconapin, glucobrassicanapin, glucobrassicin, gluconasturtiin, and neoglucobrassicin. These phytochemicals play an important role in plant survival, and most of phytochemicals such as glucosinolates are protecting a plant from cell damage by biotic and abiotic stresses or playing their role in plant defense signaling pathway. Some of these phytochemicals are important for human health are considered as anti-carcinogenic and cholesterol-reducing in blood (Podsedek, 2007; Hounsome et al., 2008; Jahangir et al., 2009). When plants have been under abiotic and biotic stresses they produce a diverse array of primary and secondary metabolites (Sudha and Ravishankar, 2002), contributing to defense mechanisms against abiotic and biotic stresses , especially amino acids, phenolics and glucosinolates (Kurilich et al., 1999; Podsedek, 2007; Jahangir et al., 2009).

1.4.1. Primary metabolites.

Primary metabolites are considered as important for plants where their involved in growth and development, respiration and photosynthesis, Primary metabolites such as carbohydrates, amino acids, fatty acids, vitamin B complex, and organic acids(Figure 3).

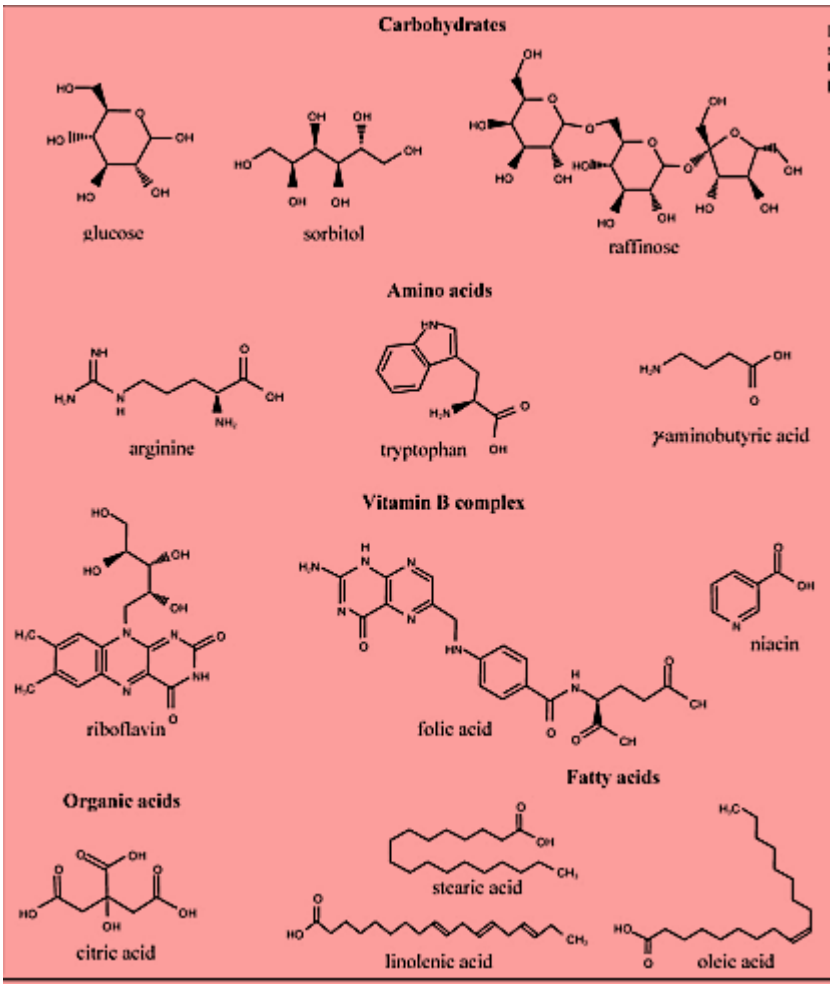

Figure 3. Chemical structure of some plant primary metabolites (Hounsome et al., 2008)**.**

Amino acids is considered one of the important primary metabolites where found in brassica which their play a role as intermediates in plant metabolism and in the production of compounds that directly or indirectly play an important role in plant - environment interactions. Total of 17 amino acids were identified (L-alanine, L-arginine, L-asparagine, L-aspartic acid, glycine, L-glutamic acid, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine) in *B. oleracea* var italic (Gomes and Rosa, 2001; Hounsome et al., 2008; Jahangir et al., 2009; Šamec et al., 2018). Carbohydrates are known have an essential role as vital sources of energy and carbon skeletons for organic compounds and storage components in the plant (Trouvelot et al., 2014). Considered as fructose, glucose, and sucrose are the major soluble sugars found in Brassica (Jahangir et al., 2009). Other carbohydrates found in cabbage, broccoli, cauliflower, and Brussels sprouts such as sorbitol, xylitol, and non-starch polysaccharides are present in (Hounsome et al., 2008). In brassica, fatty acids metabolic pathways play significant roles in pathogen defense. Fatty acids were assigned passive roles in plant defense such as biosynthetic precursors for cuticular components or the phytohormone jasmonic acid (Kachroo and Kachroo, 2009). Vitamin B complex is considered essential not only for human metabolism but also for plants, because of their redox chemistry and role as cofactors, and some of them also have strong antioxidant potential. And Thiamine (vitamin B1) has been shown to alleviate the effects of several environmental stresses on *Arabidopsis* (*Arabidopsis thaliana*), presumably by protecting the plant from oxidative damage (Trouvelot et al., 2014). they are present in broccoli Brussels sprouts, cabbage, and cauliflower (Hounsome et al., 2008).

1.4.2. Secondary metabolites (SMs).

SMs such as flavonoids, carotenoids, sterols, phenolic acids, alkaloids, and glucosinolates (Figure 4) are useful products for plants, as stress response such as play important role in defense against pests and diseases and abiotic stress (Crozier and Clifford, 2006; Jahangir et al., 2009).

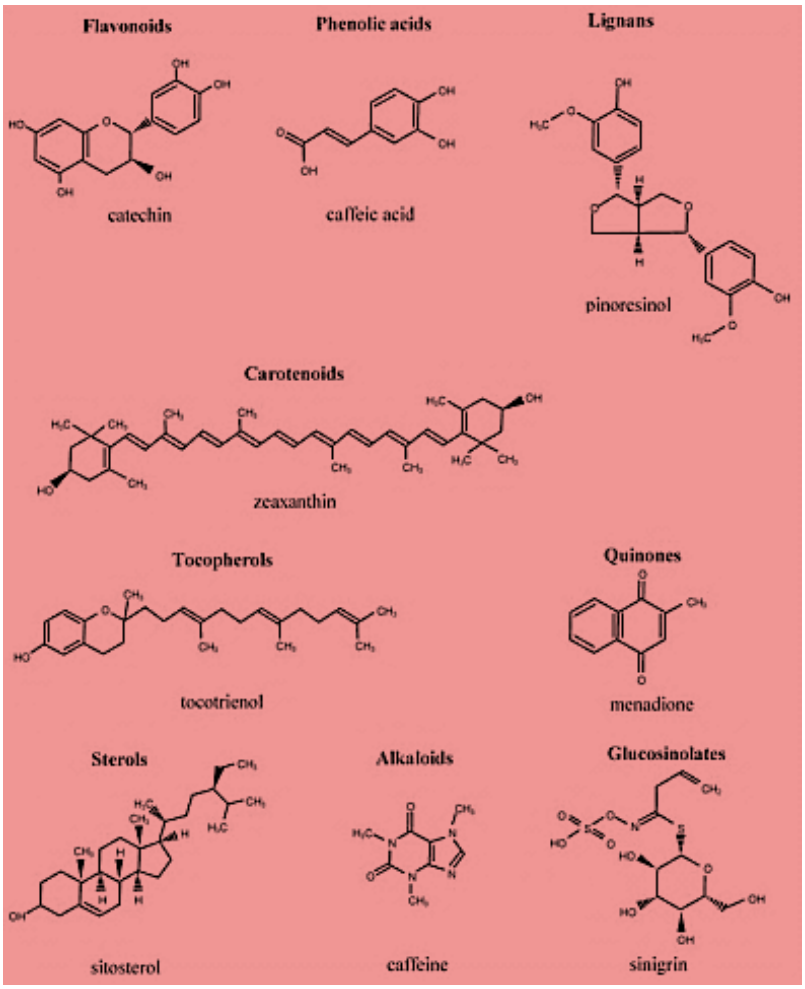

Figure 4. Chemical structure of some plant secondary metabolites (Hounsome et al., 2008).

Phenolic compounds are considered one of the main classes of SMs in brassicas. They perform various physiological functions including roles in growth and reproduction and processes defensive against abiotic and biotic stresses (Duthie and Crozier, 2000; Francisco Candeira, 2010). Phenolic compounds consist of at least one aromatic ring attached with one or more hydroxyl groups (Nicholson and Hammerschmidt, 1992). More than 8000 phenolic compounds have been discovered and they are widely dispersed throughout the plant kingdom (Crozier et al., 2007). Phenolic compounds include flavonoids, phenolic acids, and lignans are present at high concentrations in broccoli, kale, Brussels sprouts, and other brassica vegetables (Hounsome et al., 2008).

Terpenoids are a large family of chemical compounds derived by repetitive fusion of branched 5-carbon isoprene units. Terpenoids have diverse functional roles in plants as structural components of membranes (sterols), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), and hormones (gibberelins, abscisic acid) (Hounsome et al., 2008) . Terpenoids compounds are found in kale, broccoli, brussels sprouts, cabbage, cauliflower, turnip and other brassica vegetables (Piironen et al., 1986; Hounsome et al., 2008).

Glucosinolates (GLs) are a large functional group of sulfur–containing amino acid derivatives, containing a group derived from glucose. GLs are the major class of SMs found in brassica crops (Francisco et al., 2016; Kim et al., 2017). According to Ishida et al(2014) GLs contents have quantitative inheritance, which is regulated by complex genetic factors and which is affected by environmental factors.

GLs are divided into aliphatic, aromatic, and indole glucosinolates, which are derived from methionine, phenylalanine, and tryptophan, respectively (Burow et al., 2006; Velasco et al., 2008; Ishida et al., 2014) (Figure 5).

Common name	Chemical name (side chain R)
Aliphatic glucosinolate	
3 carbon chain length	
Glucoiberverin	3-Methylthiopropyl
Glucoiberin	3-Methylsulfinylpropyl
Sinigrin	2-Propenyl
4 carbon chain length	
Glucoerucin	4-Methylthiobutyl
Dehydroerucin	4-Methylthio-3-butenyl
Glucoraphanin	4-Methylsulfinylbutyl
Glucoraphenin	4-Methylsulfinyl-3-butenyl
Gluconapin	3-Butenyl
Progoitrin	2-Hydroxy-3-butenyl
5 carbon chain length	
Glucoberteroin	5-Methylthiopentyl
Glucoalyssin	5-Methylsulfinylpentyl
Glucobrassicanapin	4-Pentenyl
Gluconapoleiferin	2-Hydroxy-4-pentenyl
Aromatic glucosinolate	
Gluconasturtiin	2-Phenylethyl
Indole glucosinolate	
Glucobrassicin	3-Indolyl methyl
4-Hydroxyglucobrassicin	4-Hydroxy-3-indolymethyl
4-Methoxyglucobrassicin	4-Methoxy-3-indolylmethyl
Neoglucobrassicin	N-methoxy-3-indolymethyl

Figure 5. Common and chemical name of major aliphatic, aromatic, and indole glucosinolates present in Brassicaceae vegetables(Ishida et al., 2014)

GLs with an aliphatic side chain, derived from methionine, are the most prominent in leaves of Brassica vegetable (Giamoustaris and Mithen, 1996). Aliphatic GLs are classified by side-chain size as 3-carbon (3C), 4-carbon (4C) and 5-carbon (5C) GLs. They can also be classified by side-chain structure into methylthioalkyl, methylsulfinylalkyl, alkenyl and hydroxyalkenylglucosinolates (Li and Quiros, 2003). BoGS-Elong controls production of GLs with three carbon or four carbon side chains. BoGS-Alk is responsible for production of alkenyl glucosinolates. BoGS-OH controls production of 2-hydroxy-3-butenyl glucosinolate (Kliebenstein, 2001) (Figure 6).

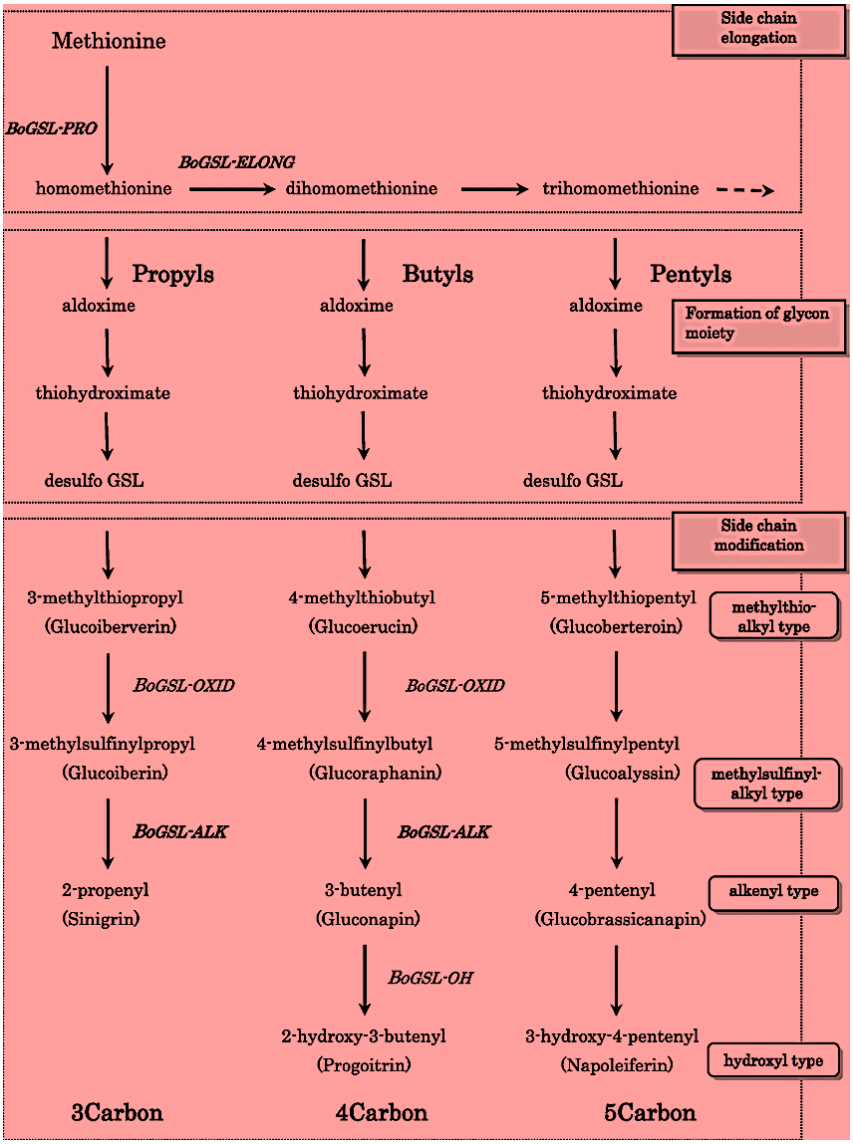

Figure 6. Glucosinolate biosynthesis pathway for 3C, 4C and 5C aliphatic glucosinolates of Brassica. Others have been inferred from inspection of glucosinolate profiles in *B. oleracea*(Ishida et al., 2014).

GLs are well known to be related to the plant defense response mechanisms, being induced after wounding and/or pathogen attack (Doughty et al., 1991; Cole, 1997), exposure to salt and drought stresses (Qasim et al., 2003; López-Berenguer et al., 2009), and diverse environmental factors (Engelen-Eigles et al., 2006;Velasco et al., 2007). When plant tissues and cells are damaged, glucosinolates were hydrolyzed by the enzyme myrosinase, resulting in several degradation products, including isothiocyanates, nitriles, thiocyanates, epithionitriles and oxazolidines (Bones and Rossiter, 2006) (Figure 7).

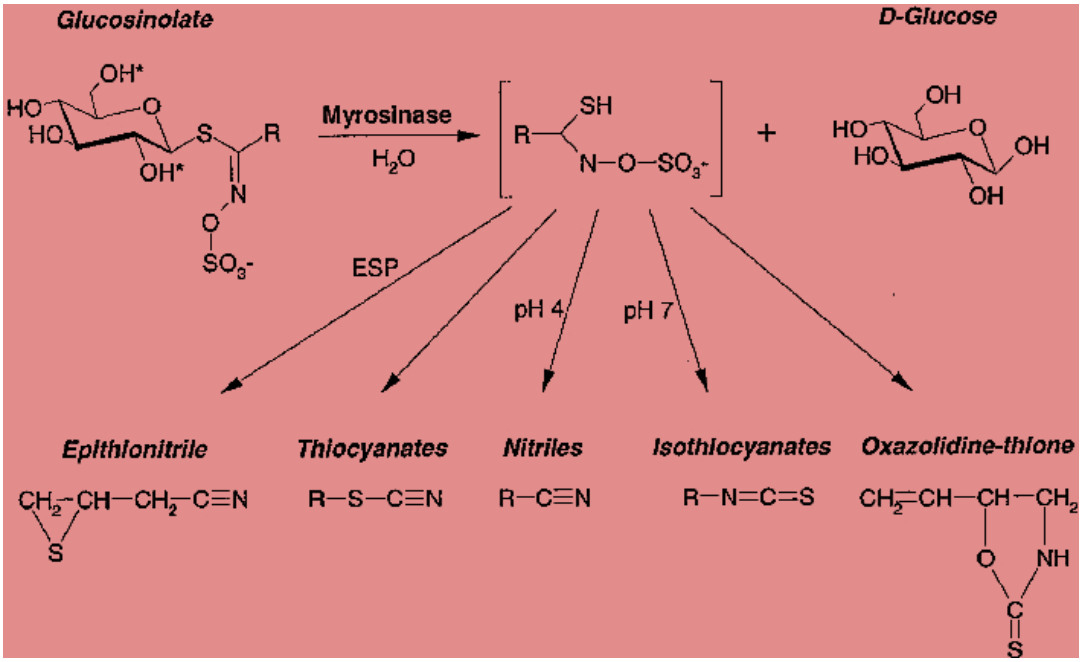

Figure 7. Depiction of the glucosinolate-myrosinase reaction and the various compounds produced under different conditions. ESP: epithiospecifier locus(Rask et al., 2000).

1.4.3. Glucosinolate profiles in *Brassicaceae* vegetables.

Distribution of the glucosinolates varies among plant organs, with both quantitative and qualitative differences between roots, leaves, stems and seeds (Fahey et al., 2001). Glucoraphanin was found in *B. oleracea*. Glucoiberin is contained in cabbage, broccoli, and cauliflower. Gluconapin and progoitrin are found in many Brassica vegetables such as *B. rapa* (Chinese cabbage, mustard spinach, mizuna, and turnip), *B. oleracea* (cabbage, broccoli and cauliflower), *B. juncea* (mustard green), and *B. napus* (rapeseed vegetable). Sinigrin is produced in *B. oleracea* vegetables, mustard green (*B. juncea*). Glucoerucin is found in garden rocket (*Eruca sativa*). De-hydroerucin is specific to radish (*R. sativus*) and is the predominant aliphatic glucosinolate, accounting for over 80% of the all glucosinolates. Glucobrassicanapin is the main glucosinolate constituent of *B. rapa* vegetables (Fahey et al., 2001; Ishida et al., 2014).

Table 3. Main glucosinolates identified in leaves of brassica vegetables (Cartea and Velasco, 2008).

Crop	Aliphatic Glucosinolates										Indole Glucosinolates				Aromatic glucosinolates
	GIB	PRO	SIN	GAL	GRA	GNA	GBN	GIV	GER	GNL	GBS	NGBS	4HGBS	4MGBS	GST
Brassica oleracea															
White cabbage	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Savoy cabbage	+	+	+	-	+	+	+	+	-	-	+	+	-	+	+
Red cabbage	+	+	+	-	+	+	-	+	-	-	+	+	-	-	-
Kale	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+
Collard	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-
Tronchuda cabbage	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
Broccoli	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Brussels sprouts	+	+	+	-	+	+	-	+	-	-	+	+	-	-	-
Cauliflower	+	+	+	-	+	-	-	+	-	-	+	+	-	-	-
Kohlrabi	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-
Brassica rapa															
Turnip	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+
Turnip greens	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+
Turnip tops	+	+	-	-	-	+	+	+	-	-	+	+	+	-	+
Chinese cabbage	+	+	-	-	-	+	+	-	-	+	+	+	-	+	+
Brassica napus															
Swede	-	+	-	-	+	-	+	-	-	+	+	+	+	+	+
Leaf rape	-	+	-	+	-	+	+	+	-	+	+	+	-	+	+

Table 4. Trivial name and chemical name for some of glucosinolates identified in leaves of brassica vegetable

Code	Trivial name	Chemical name
GIB	**Glucoiberin**	**3-methylsulfinylpropyl**
PRO	**Progoitrin**	**2-hydroxy-3-butenyl**
SIN	**Sinigrin**	**2-propenyl**
GAL	**Glucoalysiin**	**5-methylsulphinylpentyl**
GRA	**Glucoraphanin**	**4-methylsulphinylbutyl**
GNA	**Gluconapin**	**3-butenyl**
GBN	**Glucobrassicanapin**	**4-pentenyl**
GIV	**Glucoiberverin**	**3-methylthiopropyl**
GER	**Glucoerucin**	**4-methylthiobutyl**
GNL	**Gluconapoleiferin**	**2-hydroxy-4-pentenyl**
GBS	**Glucobrassicin**	**3-indolylmethyl**
NGBS	**Neoglucobrassicin**	**1-methoxy-3-indolylmethyl**
4HGBS	**4-hydroxyglucobrassicin**	**4-hydroxy-3-indolylmethyl**
4MGBS	**4-methoxyglucobrassicin**	**4-methoxy-3-indolylmethyl**
GST	**Gluconasturtiin**	**2-phenylethyl**

1.4.4. Metabolomics technology

Metabolites are the end products of cellular regulatory processes with low-molecular-weight and their levels can be regarded as the ultimate response to genetic modification and physiological, pathophysiological, and environmental changes (Clarke and Haselden, 2008). As it is impossible to measure all metabolomic changes simultaneously, systems biology as a holistic approach can be used to examine different biological processes, operating as an integrated system and visualize how individual metabolomic pathways are interconnected to each other. Depending on selectivity and sensitivity different analytical approaches can be used. It includes nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS).

NMR spectroscopy is considered as nondestructive, highly reproducible, and easy for sample handling. It allows the analyses of a large group of compounds in a single run, and is the most suited quantitative and qualitative metabolomic technique. It is used for high-throughput screening, metabolite fingerprinting, metabolite profiling and can also be used to investigate the operation of plant metabolomic networks. NMR profile may contain 2 to 3,000 peaks, but only about 10% of these will map to known chemical entities. Thus, unknown peak areas may require further assessment by technologies such as MS which produces a much enhanced resolution profile, and containing possibly 15,000 peaks (Clarke and Haselden, 2008).

Gas-chromatography–mass-spectrometry (GC–MS or GC–TOF–MS) and liquid-chromatography–mass-spectrometry (LC-MS or LC–TOF–MS) are currently the principal mass- spectrometry methods for metabolite analysis (Fiehn et al., 2000; Shulaev, 2006). GC–TOF–MS allows the assessment of the exact mass (ppm range) of a molecule or fragment thereof, which improves the identification of compounds (van Dam and Bouwmeester, 2016). Liquid Chromatography (LC-MS) is being increasingly used in metabolomics applications due to its high sensitivity and a range of analyte polarity and molecular mass wider than GC-MS (Shulaev, 2006). LC–MS has one advantage over GC-MS, in that, there is largely no need for chemical derivatization of metabolites (which is required for analysis of non-volatile compounds by GC-MS) (Fiehn et al., 2000; Shulaev, 2006). Electrospray ionization (ESI) is the most commonly used method of small molecule ionization employed in LC-MS-based metabolomics studies (Roberts et al., 2013).

1.4.5. Metabolomics data analysis.

Metabolomics generates a huge volume of data similar to transcriptomics and proteomics, that necessitate specialized bioinformatics and data mining tools to gain knowledge (Shulaev et al., 2008). Metabolite profiling approaches analyze all features detectable without any pre-selection and prior knowledge (Schmidt and Bancroft, 2011). Untargeted metabolomics aims to gather information on as many metabolites as possible in biological systems by taking into account all information present in the data sets (De Vos et al., 2007).Untargeted metabolomics data processing workflows incorporate several defined steps including noise filtering, peak detection, peak deconvolution, retention time alignment, and finally feature annotation. Analyte identification can be performed by searching the experimental MS or MS/MS data through public available tools to perform metabolomics data analysis and databases (e.g., ChemSpider (http://www.chemspider.com), METLIN (https://metlin.scripps.edu), Human Metabolome DataBase (HMDB) (http://www.hmdb.ca/), MassBank, mzCloud (https://www.mzcloud.org), GNPS (https://gnps.ucsd.edu), metAlign (Lommen, 2009), mzMine (Katajamaa et al., 2006), OpenMS (Sturm et al., 2008), XCMS (https://xcmsonline.scripps.edu) (Smith et al., 2006) and LipidBlast) , or for a nominal fee [e.g., NIST Mass Spectral Library (http://chemdata.nist.gov)].

Objective

As we have seen, glucosinolates are highly recognized by their biological effects, which include resistance to diseases and pest, allelopathic effects and anticarcinogenic effects. For this reason, the Brassica group at the Misión Biológica de Galicia started a program to create phenotypes with different glucosinolates profile. Glucosinolate contents have quantitative inheritance and a divergent selection applied to a base population can be useful in investigating the genetic control of quantitative traits, the correlated response of other characters, and developing genotypes for physiological studies (Tuberosa et al., 1986) , for this, the plant material used in this experiment was a divergent selection for high and low concentration of sinigrin (HSIN and LSIN) and high and low concentration of glucobrassicin (HGBS and LGBS) in kale (*B. oleracea* var. acephala).

The divergent selections were started in 2006 by using seeds of the kale population MBG-BRS0062, kept at the brassica germplasm bank at Misión Biológica de Galicia (MBG-CSIC) (Galicia, NW Spain). This population is a local variety. The population presents variability for GLs concentration and this is a necessary characteristic to realize a mass divergent selection for high and low content.

2. The specific objectives are:

1- Evaluation of the adaptation of a divergent selection of kale (*B. oleracea* var. acephala) for leaf glucosinolates content (HSIN, LSIN and HGBS, LGBS), for agronomic traits in five different environmental conditions.

2- Studying the metabolomics change in the divergent selection of kale under five environmental conditions.

3- Studying the effect of climatic on kale under the five different environmental conditions.

Materials and Methods

3. Material and Methods

3.1. Location and Growth Conditions

This work was carried out during the growing season of 2017 at five locations: **Pontevedra (PO), Spain (**42°26'N, 8°38'W), **Badajoz (BA), Spain** (38°53'N, 6°51'W), **Córdoba (CO), Spain** (37°53'N, 4°42'W), **Gottingen (GO), Germany (51°32'N , 9°54''E), and Tromsø (TR), Norway** (69°40'N, 18°56'E) (Table 5).

Table 5. Planting and harvest dates for the five locations

	PO	BA	CO	TR	GO
Planting date	02-Apr	24-Mar	05-Apr	14-Jun	16-May
Harvest date	26-Sep	26-Sep	29-Sep	31-Aug	05-Sep

Climatic data (e.g., temperatures, radiation and precipitation) were obtained from meteorological stations located close to the experimental fields (Table 6).

Table 6. Average of temperature, precipitation and radiation for the whole season and last 30 days of growth season of kale (*B. oleracea* var. acephala) for the five locations.

	PO		BA		CO		TR		GO	
	Season[1]	**30D[2]**	**Season**	**30D**	**Season**	**30D**	**Season**	**30D**	**Season**	**30D**
Max temp (°C)	24.0	22.4	28.8	30.2	32.7	32.7	13.6	13.7	23.0	22.7
Med temp (°C)	18.0	16.9	20.4	21.2	24.3	24.0	10.8	11.0	16.7	16.3
Min temp (°C)	12.0	11.3	12.1	12.4	15.7	15.1	8.1	8.4	10.5	10.2
Precipitation (mm)	277.0	30.1	81.4	12.9	131.0	0.2	198.9	96.0	427.3	85.9
Max temp absolute (°C)	37.0	28.0	41.3	36.0	44.9	37.0	21.9	19.4	33.7	29.8
Min temp absolute (°C)	5.0	5.0	3.9	6.7	5.7	9.6	4.6	4.6	5.0	5.0
Radiation (MJ/m2)	20.5	15.3	24.5	22.5	25.1	21.1	37.0	31.8	60.2	60.2

1- Data recorded during from planting to harvest in each location. 2- Data recorded during 30 days before harvest in each location

3.2. Plant material

The plant material used in this experiment was a divergent selection for high and low concentration of sinigrin (HSIN and LSIN) and high and low concentration of glucobrassicin (HGBS and LGBS) in kale (*B. oleracea* var. acephala). This selection was made from a Galician local population (MBG-BRS0062) obtained from the brassica germplasm bank of the Mision Biologica de Galicia (MBG-CSIC) (Galicia, NW Spain). The seeds from these four selections were sown in multi pot-trays in a greenhouse. At a stage of 5-6 leaves plants (50 plants/ plot) were transplanted in the field in a randomized block design experiment with two replications. Cultivation operations, fertilization, and weed control were carried out according to local practices and crop requirements.

3.3. Agronomic parameters.

Twenty-five leaves (7^{th}-8^{th} leaf from the upper leaves) were collected randomly from each plot in the last week of August in TR and the first week of September in GO and the last week of September in PO, BA, and CO. Samples were placed in paper-bags and weighed to record the leaves fresh weight (FW) and dried in a heater at 70 ^{o}C until reaching constant weight to record the leaves dry weight (DW). Plant height was measured from the soil to the base of the upper leaf in 10 plants from each plot.

3.4. Biochemical Composition

For metabolomics and glucosinolates analyses, the 4th leave from the upper leaf of 15 plants/plot were collected at the last week of August in TR and the first week of September in GO and the last week of September in PO, BA, and CO, to make three bulks of 5 plants/bulk. Samples were collected on liquid nitrogen and stored at -80 ^{o}C until freeze-dried in a lyophilizer (GAMMA 2-16 LSC plus, Christ, Germany). Samples were mechanically milled to a fine powder in a grinder (Janke and Kunkel A10 mill, IKA-LabortechnikStaufen, Germany) before analysis.

3.4.1. Glucosinolates extraction

Sample extraction and desulfation were performed according to Kliebenstein et al(2001) with minor modifications.

❖ **Sample extraction.**

12 mg of frozen-dried kale powder were weighed into 2 ml Micro tubes. The samples were mixed gently with 400 µl 70 % (v/v) methanol preheated to 70^{o}C, 10 µl of PbAc (0.3M) and 120 µlmiliQ water preheated to 70 ^{o}C. Then, 20 µl of Glucotropaeolin were added as an internal standard. The tubes were mixed gently in a Qiagen TissueLyser II (Hilden, Germany), at 25.0 Hz for 1.30 min. Then, the samples were shaken in a Microplate incubator (Model OVAN Orbital Midi) at 250 rpm in the dark for an hour, at room temperature. After that, the samples were centrifuged (centrifuge model 5804, Eppendorf, Germany) at 3700 rpm for 12 min.

The supernatant was used for anion-exchange chromatography. Ninety-six–well filter plates from Millipore were loaded with 45 µL of DEAE Sephadex A-25 by using the Millipore multiscreen column loader.

300 µL of water was added and allowed to equilibrate for 2 to 4 hr. Water was removed with 2 to 4 sec of vacuum on the Qiagen vacuum manifold.

150 µL of the supernatant was added to the 96-well columns, and the liquid was removed by 2 to 4 sec of vacuum. This step was repeated once to bring the total volume of plant extract to 300 µL.

The columns were washed four times with 100 µL of 60% methanol, and four times with 100 µL of mili Q water.

To desulfate glucosinolates on the column, we added 10 µL of water and 10 µL of sulfatase solution to each column, and the plates were incubated overnight at room temperature.

Desulfoglucosinolates were eluted by placing a deep-well 2-mL 96-well plate in the bottom of the 96-well vacuum manifold and aligning the 96-well column plate. The DEAE Sephadex then was washed twice with 100 µL of 60% methanol and twice with 100 µL of water.

The chromatographic analyses were carried out on an Ultra-High-Performance Liquid Chromatograph (UHPLC Nexera LC-30AD; Shimadzu, Kyoto, Japan) equipped with a Nexera SIL-30AC injector and one SPDM20A UV/VIS photodiode array detector. The UHPLC column was a X Select ®HSS T3 (2.5µm particle size, 2.1 × 100 mm i.d.) from

Waters (Waters Corporation, Waters is from USA) protected with a Van Guard pre-column. The oven temperature was set at 35 ^{o}C. Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile, with a flow of 0.5 mL min^{-1}: 1.5 min at 100% H_2O, an 11 min gradient from 5% to 25% (v/v) acetonitrile, 1.5 min at 25% (v/v) acetonitrile, a minute gradient from 25% to 0% (v/v) acetonitrile, and a final 3 min at 100% H_2O.

Data were recorded on computer with Lab Solutions software (Shimadzu). Specific GLs were identified by comparing retention times and UV spectra with standards. Sinigrin (SIN, sinigrin monohydrate from Phytoplan, Diehm and Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoplan, Diehm and Neuberger GmbH, Heidelberg, Germany) were used as external standards, and expressed in µmol g^{-1} DW. Calibration equations were made with, at least, five data points, from 0.34 to 1.7 nmol for SIN and from 0.28 to 1.4 nmol for GBS. Regression equations for SIN and GBS were y = 148.818 × (R^2= 0.99), y = 263.822 × (R^2 = 0.99), respectively (Sotelo et al., 2016).

3.4.2. Metabolomics extractions

❖ **Sample preparation**

Fifty mg of frozen-dried kale powder were weighed in a microcentrifuge tube, mixed with 50 µl of 80 % aqueous methanol, and immediately vortex for 10 s at room temperature. The samples were sonicated for 15 min at 40Hz continuously in a water bath at room temperature. After that, the samples were centrifuged (centrifuge 5415D, Eppendorf,

GmbH, Hamburg, Germany) at 14.600 rpm for 10 min in order to remove plant debris at room temperature. Supernatants were recovered and were filtered through 0.2 µm syringe PTFE filters (Whatman ™, Germany) and then transferred to UHPLC vials.

❖ **LC–QTOF analysis**

Five µL of each sample were injected into an ultra-high performance liquid Chromatography system (UHPLC) (Thermo Dionex Ultimate 3000 LC) connected to a quadrupole time of flight detector (Bruker Compact™ QTOF-MS) with a heated-electrospray ionization source (ESI). Chromatographic separation was performed in a Bruker UHPLC Intensity Trio C18 2.1 × 150 mm 3 µm pore size column using a binary gradient solvent mode consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was used: 3% B (0–4 min), from 3 to 25% B (4-16 min), from 25 to 80% B (16-25min), from 80 to 100% B (25–30 min), and to hold 100% B until 36 min. The flow rate was established at 0.4 ml/min and column temperature set at 35 °C.

Calibration solution was introduced directly into the ESI source, at an initial flow of 0.06 ml/h. MS data were acquired using an acquisition rate of 2 Hz over the mass range of 50-1200 m/z. Both polarities (+/-) ESI mode were used under the following specific conditions: gas flow 8 l/min; nebulizer pressure 38 psi; dry gas 7 l/min; dry temperature 220 °C. Capillary and end plate offset were set to 4500 and 500 V, respectively. The performance of data acquisition, run sequence was started with 3 blanks (methanol, the solvent used in sample extraction), and a standard compound triphenyl phosphate (TP) for

positive ionization mode and chloramphenicol for negative ionization mode (Tortosa et al., 2018b).

3.4.3. Metabolite selection and identification

Data were analyzed by using interactive XCMS software (https://xcmsonline.scripps.edu/). Parameter settings for XCMS processing data acquired by UHPLC/Bruker Q-TOF were as follows: centWave for feature detection (Δm/z= 10 ppm, minimum peak width = 5 s, and maximum peak width = 20 s); obiwarp settings for retention-time correction (profStep = 1); and parameters for chromatogram alignment, including mzwid = 0.015, min frac = 0.5, and bw = 5. The relative quantification of metabolite features was based on EIC (extracted ion chromatogram) areas. The generated data set was imported into Metaboanalyst (http://www.metaboanalyst.ca) to perform statistical analyses. In order to remove non-informative variables, data was filtered by using interquantile range filter (IQR). Moreover, the Pareto variance-scaling was used to remove the offsets and adjust the importance of high and low abundance ions to an equal level. The resulting three-dimensional matrix (peak indices, samples and variables) was further subjected to multivariate data analysis. Principal components analysis (PCA) and Partial least-squares-discriminant analysis (PLS-DA) were carried out to investigate and visualize the pattern of metabolite changes. These analyses were applied to obtain an overview of the complete data set and discriminate those variables that are responsible for variation between the groups. PLS-DA model was evaluated through a cross validation (R^2 and Q^2 parameters). The quality assessment (Q2) and R-squared (R^2) statistics provides a qualitative measure of consistency between the

predicted and original data, or in other words, estimates the predictive ability of the model (Worley and Powers, 2012).

The ten ions with higher VIP-scores (variable importance) at GBS and SIN selections for ESI- mode were identified using METLIN database (https://metlin.scripps.edu/). For accurate mass filtering, 10 ppm mass tolerance was used.

3.5. Data Analysis

Combined analyses of variance for the total and individual GLs and agronomical traits (e.g., plant height, leaves fresh weight, and leaves dry weight) were computed using the GLM procedure of SAS (SAS Institute Inc., Cary, USA 2008) where genotype was considered a fixed effect and environment was considered as a random effect. Comparisons of means among geographic origins were performed for each GLs by using the Fisher's protected least significant difference (LSD) at $P \leq 0.05$ (Steel et al., 1997). Linear Regression analyses were performed for climate data (e.g., temperatures, precipitation, and radiation), agronomical traits, and 20 important metabolomics concentration using the REG procedure of SAS (SAS Institute Inc., Cary, USA 2008) where the climate data was the independent variable and the agronomical traits and the concentration of 20 important metabolites were dependent variables.

Results

3. Results

3.1. Vegetative Growth

We evaluated the agronomic performance of two divergent selections for leaf glucosinolates content (high and low concentration of GBS (HGBS; LGBS) and high and low concentration of SIN (HSIN; LSIN)) in kale (*B. oleracea* L) in five different environments. Comparisons between genotypes and locations were made within each selection. There were highly significant differences for both selections among locations for all vegetative growth traits evaluated (plant height, fresh weight and dry weight) (Table 7).

For the divergent selection for GBS, location × genotype showed a significant quantitative interaction (non-crossover) for fresh weight and no significant interaction for plant height and dry weight. HGBS and LGBS varieties were significantly different for fresh and dry weight but not significant differences were observed for plant height (Table 7).

For the divergent selection for SIN, the interaction was significant and qualitative (crossover) only for plant height, for that reason, means for the different varieties will be presented by location for this trait (Table 8). LSIN gave the highest plant height in PO, CO, TR, and GO, while, in BA, there were no significant differences. The HSIN and LSIN varieties were significantly different for the rest of vegetative growth traits evaluated (Table 8).

Table 7. ANOVA results of the effect of location, genotype (low and high concentration of GBS and low and high concentration of SIN) and location × genotype interaction on plant height, fresh weight, and dry weight for two divergent selections for glucosinolate content of kale**.**

	GBS			SIN		
	Mean square	F Value	*P*	Mean square	F Value	*P*
Plant height (cm)						
Location	8049.07	91.53	< 0.01	9025.56	104.81	< 0.01
Genotype	2.21	0.03	0.87	950.48	11.04	0.01
Location × genotype	52.66	0.60	0.66	227.62	2.64	0.03
FW (g/25 leaves)						
Location	5872973.12	223.74	< 0.01	6479125.62	108.54	< 0.01
Genotype	244520.03	9.32	0.01	388187.32	6.50	0.03
Location × genotype	100523.52	3.83	0.04	70283.68	1.18	0.4
DW (g/25 leaves)						
Location	23740.93	49.09	< 0.01	27344.98	23.72	< 0.01
Genotype	3597.23	7.44	0.02	7287.61	6.32	0.03
Location × genotype	1082.23	2.24	0.14	1882.94	1.63	0.24

Table 8. Mean for HSIN; LSIN and location for plant height (cm) for divergent selections for glucosinolate content of kale.

		Plant height (cm)
Location	**SIN**	**Mean**
PO	HIGH	54.10[b]
	LOW	64.80[a]
CO	HIGH	52.85[b]
	LOW	54.25[a]
BA	HIGH	72.30[a]
	LOW	71.15[a]
TR	HIGH	29.65[b]
	LOW	32.90[a]
GO	HIGH	42.95[b]
	LOW	50.55[a]

LS-mean with the same letter are not significantly different

For the divergent selection for GBS, the HGBS gave higher fresh and dry weights than LGBS (Table 9). On the contrary, for the divergent selection for SIN, the LSIN gave higher fresh and dry weight than HSIN (Table 9). Among locations, in BA the plants showed the highest fresh and dry weight for both selections (Table 9), and showed the highest plant height for GBS selection (Table 9).

Table 9. Mean for genotypes and locations for fresh weight and dry weight (g/25leaves) and GBS selection for plant height (cm) for two divergent selections for glucosinolate content of kale.

	Plant height	**FW(g/25leaves)**		**DW(g/25leaves)**	
GLs	GBS	GBS	SIN	GBS	SIN
HIGH	51.59[a]	1562.80[a]	1428.40[b]	171.95[a]	158.93[b]
LOW	51.38[a]	1341.70[b]	1707.10[a]	145.13[b]	197.11[a]
Location					
PO	57.85[b]	1149.30[bc]	1186.07[b]	124.30[c]	134.45[c]
CO	49.45[c]	379.18[d]	459.25[c]	58.92[d]	67.64[c]
BA	70.87[a]	3535.00[a]	3770.00[a]	263.75[a]	288.75[a]
TR	32.45[d]	953.75[c]	1186.25[b]	148.75[c]	193.75[b]
GO	46.80[c]	1244.00[b]	1237.25[b]	197.00[b]	205.50[b]

LS-mean with the same letter are not significantly different

3.2. Glucosinolate content.

Six glucosinolates were detected belonging to two chemical classes: aliphatic (sinigrin (SIN) and glucoiberin (GIB)) and indolic (glucobrassicin (GBS), 4-hydroxyglucobrassicin (OHGBS), neoglucobrassicin (NeoGBS) and 4-methoxyglucobrassicin (MeOHGBS)) (Table 10).

For the divergent selection for GBS, location × genotype interaction was significant for GBS, NeoGBS and MeOHGBS concentration (Table 10). This interaction was quantitative (non-crossover) for GBS and NeoGBS concentration, where the HGBS have a higher GBS and NeoGBS concentration at PO, BA and TR locations but for CO and GO there were no significant difference between HGBS and LGBS (Table 11). For MeOHGBS concentration, the interaction was qualitative (crossover), where HGBS gave higher MeOHGBS concentration than LGBS at BA. In GO, PO and TR and CO locations there were no significant difference between genotypes for MeOHGBS concentration (Table 11). The effect of location was significant for all GLs (Table 10). HGBS and LGBS varieties were significantly different for GIB, GBS, and NeoGBS concentration but not significant differences were observed for SIN and OHGBS concentration (Table 10).

For the divergent selection for SIN, location × genotype interaction was significant for GIB, SIN and NeoGBS concentration (Table 10). For GIB the interaction was qualitative (crossover), where LSIN gave higher GIB concentration than HSIN at PO, BA, GO and TR locations. In CO no significant differences were observed between HSIN and LSIN for GIB

concentration (Table 11). For NeoGBS concentration the interaction was also qualitative (crossover) but no significant difference between HSIN and LSIN were observed in any location (Table 11). For SIN concentration, the interaction was quantitative (non-crossover) where the HSIN gave higher SIN concentration than LSIN at all locations (Table 11).

A difference among locations was significant for all GLs content (Table 10). For the divergent selection for GBS, GIB and SIN concentrations showed the highest concentration at TR, and OHGBS concentration showed the highest concentration at BA (Table 12). For the divergent selection for SIN, OHGBS, GBS, and MeOGBS concentrations showed the highest concentration at BA location (Table 12). HSIN and LSIN varieties were significantly different for GBS concentration where HSIN gave higher GBS concentration than LSIN but were no significantly different for OHGBS and MeOHGBS (Table 12).

Table 10. ANOVA results of the effect of location, genotype (low and high concentration of GBS and low and high concentration of SIN) and interaction for six GLs detected in leaves of two divergent selection for glucosinolate content of kale.

		GBS			SIN		
GLs		**mean square**	**F value**	***P***	**mean square**	**F value**	***P***
GIB	Location	71.10	28.49	<0.01	74.30	25.85	<0.01
	Genotype	32.35	12.96	0.01	78.04	27.15	<0.01
	Location × genotype	3.58	1.44	0.24	11.31	3.94	0.01
SIN	Location	33.11	19.48	<0.01	40.79	28.19	<0.01
	Genotype	3.10	1.83	0.18	538.92	372.52	<0.01
	Location × genotype	4.03	2.37	0.06	14.17	9.80	<0.01
OHGBS	Location	0.11	17.78	<0.01	0.26	12.12	<0.01
	Genotype	0.02	3.69	0.06	0.00	0.01	0.92
	Location × genotype	0.01	1.56	0.20	0.04	2.07	0.10
GBS	Location	467.94	23.68	<0.01	620.47	51.85	<0.01
	Genotype	622.93	31.52	<0.01	48.69	4.07	0.04
	Location × genotype	129.60	6.56	0.01	3.32	0.28	0.89
MeOHGBS	Location	0.12	8.68	<0.01	0.11	4.87	0.01
	Genotype	0.04	2.71	0.10	0.02	0.82	0.37
	Location × genotype	0.08	5.74	0.01	0.04	1.54	0.20
NeoGBS	Location	216.63	38.37	<0.01	341.56	40.69	<0.01
	Genotype	214.76	38.03	<0.01	16.37	1.95	0.16
	Location × genotype	77.29	13.69	<0.01	49.87	5.94	0.01

Table 11. Means for GLs Compounds (µmol g^{-1} dw) of HGBS, LGBS, HSIN, LSIN in five location for two divergent selection for glucosinolate content of kale**.**

		GBS			SIN		
		GBS	**MeOHGBS**	**NeoGBS**	**GIB**	**SIN**	**NeoGBS**
Location	**Level**						
PO	HIGH	11.39[a]	0.39[a]	2.24[a]	0.76[b]	5.20[a]	1.18[a]
	LOW	3.95[b]	0.37[a]	0.53[b]	3.74[a]	2.03[b]	1.52[a]
CO	HIGH	16.94[a]	0.30[a]	11.59[a]	7.40[a]	5.79[a]	18.53[a]
	LOW	12.82[a]	0.42[a]	8.76[a]	5.92[a]	1.12[b]	9.78[a]
BA	HIGH	28.34[a]	0.77[a]	15.41[a]	5.08[b]	9.95[a]	7.76[a]
	LOW	10.40[b]	0.45[b]	2.49[b]	7.75[a]	2.15[b]	11.67[a]
TR	HIGH	4.61[a]	0.38[a]	0.56[a]	6.92[b]	11.89[a]	0.68[a]
	LOW	2.79[b]	0.42[a]	0.24[b]	11.23[a]	4.02[b]	0.87[a]
GO	HIGH	8.50[a]	0.51[a]	3.36[a]	3.33[b]	10.50[a]	3.42[a]
	LOW	6.03[a]	0.43[a]	1.31[a]	6.83[a]	2.56[b]	2.26[a]

LS-mean with the same letter are not significantly different

Table 12. Means for GLs compounds (µmol g^{-1} dw) of HGBS, LGBS, HSIN and LSIN at five location for a divergent selection for glucosinolate content of kale.

	GBS			SIN		
	GIB	**SIN**	**OHGBS**	**OHGBS**	**GBS**	**MeOHGBS**
Gls						
HIGH	6.41^{b}	6.27^{a}	0.32^{a}	0.39^{a}	11.63^{a}	0.39^{a}
LOW	7.95^{a}	5.79^{a}	0.28^{a}	0.40^{a}	9.74^{b}	0.43^{a}
Location						
PO	3.35^{d}	5.33^{c}	0.22^{b}	0.26^{b}	6.79^{c}	0.36^{bc}
CO	7.84^{b}	3.37^{d}	0.39^{a}	0.50^{a}	12.89^{b}	0.28^{c}
BA	8.95^{ab}	6.72^{b}	0.42^{a}	0.61^{a}	22.23^{a}	0.56^{a}
TR	9.40^{a}	8.35^{a}	0.23^{b}	0.29^{b}	3.94^{d}	0.39^{bc}
GO	6.37^{c}	6.39^{bc}	0.22^{b}	0.33^{b}	7.56^{c}	0.45^{ab}

LS-mean with the same letter are not significantly different

3.3. Metabolomics.

The metabolic changes were analyzed using an UHPLC-QTOF under both ionization modes with an electrospray ionization source (ESI+ and ESI-). From the diverse multivariate handling techniques, principal component analysis (PCA) was first used to identify metabolic changes. PCA showed no discrimination between HGBS and LGBS or between HSIN and SIN at different locations (Figure 8).

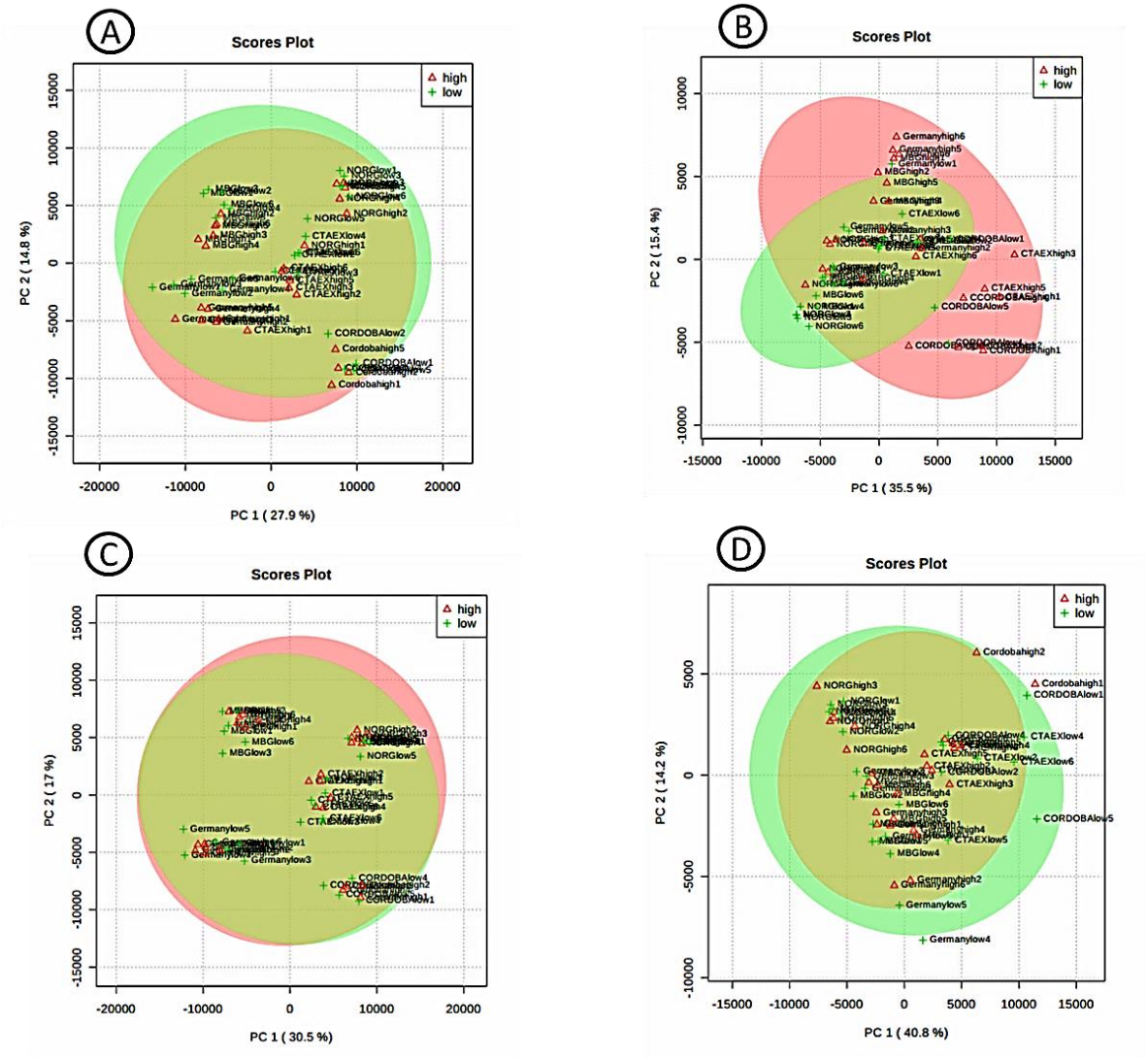

Figure 8. Score plot from a PCA for a divergent selection for GBS and SIN selections in kale (*B. oleracea* L.) at five different locations for both ionization modes (ESI+ and ESI-).A: ESI+ mode for GBS; B: ESI- mode for GBS; C: ESI+ mode for SIN; D: ESI- mode for SIN.

Partial least squares discriminant analysis (PLS-DA) is a frequently used classification method and is based on the PLS approach (Barker and Rayens, 2003), it can find the components or latent variables which discriminate as much as possible between two or more different groups of samples and PLS-DA is a better discrimination model in comparison to PCA, for that, we used PLS-DA, as, it was most effective in separating sample into groups. The PLS-DA can be verified about model validity based on cross-validation and permutation tests. When one of this test is not correct then we cannot be used the model. Within the cross-validation analysis the model is believed to be reliable when $Q^2 > 0.5$ and $R^2 > Q^2$ and can be thought to correspond to good models with a high discriminating power (Tortosa et al., 2018). For the divergent selection for GBS, the PLS-DA score plot showed discrimination between low and high GBS for ESI- mode and ESI+ mode (Figure 9). In this analysis, a $Q^2 > 0.8$ was obtained. The total variance explained was 28.5% from component 1 and 17.6 % from component 2 for ESI- mode and was 9.8% from component 1 and 14.6 % from component 2 for ESI + mode (Figure 9). Permutation tests show that the model is significant ($p<0.001$) based on 1000 permutations for ESI- mode, and for ESI+ mode the model is no significant ($p = 0.301$) based on 1000 permutations. For this reason, we only have the analysis for negative mode and not for positive mode.

For the divergent selection for SIN, also the PLS-DA score plot (Figure 9) showed discrimination between low and high GBS at different locations for ESI- mode and ESI+ mode. A $Q^2 > 0.8$ was observed. The total variance explained was 9.7 % from component 1 and 37.2 % from component 2 for ESI- mode and was 7.9 % from component 1 and 20.4

% from component 2 for ESI +mode (Figure 9). Permutation tests show that the model is significant (p = 0.041) based on 1000 permutations for ESI- mode and the model is no significant (p = 0.16) based on 1000 permutations for ESI+ mode. For this reason, we only have the analysis for negative mode and not for positive mode.

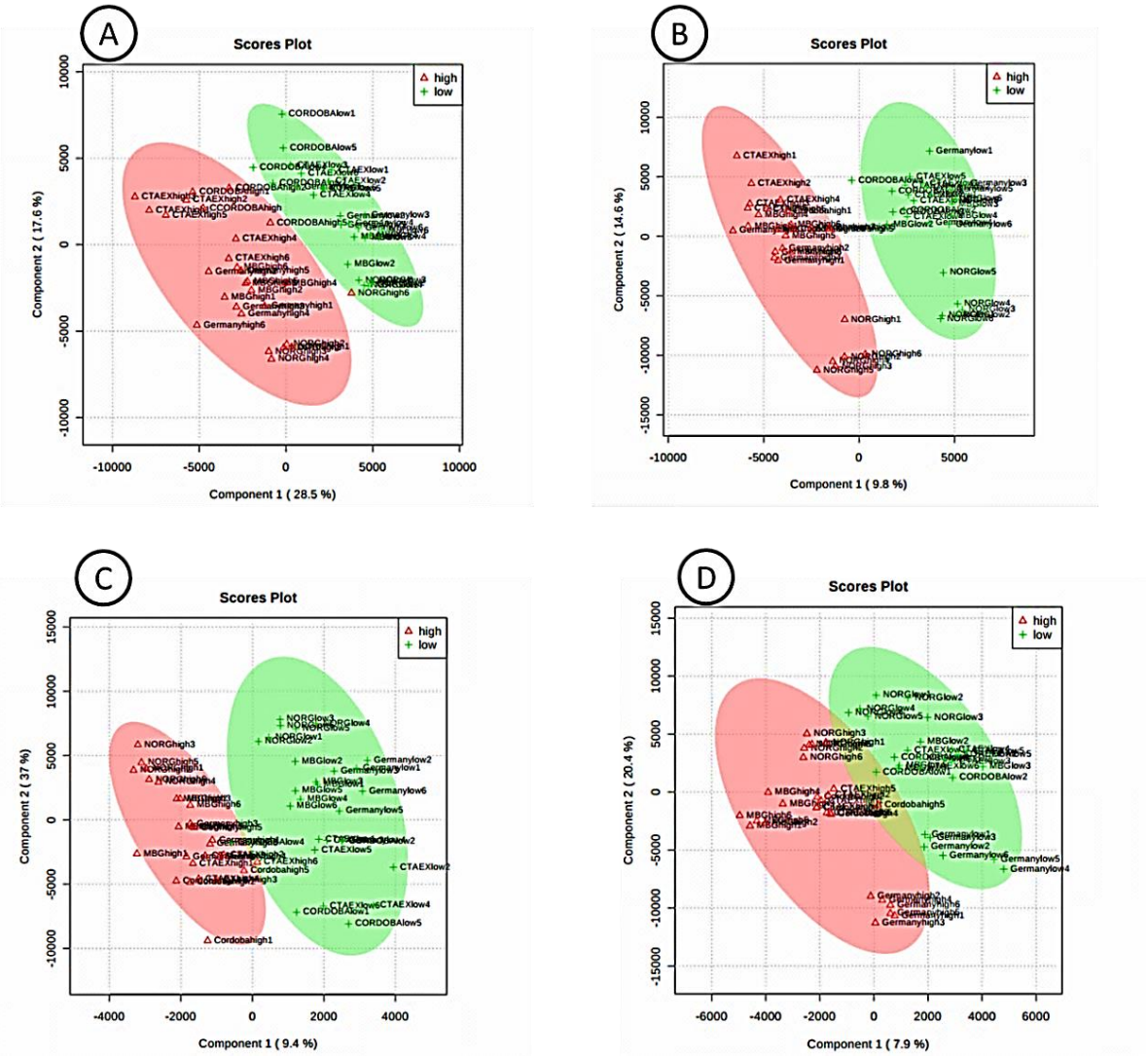

Figure 9. Score plot from a PLS-DA for a divergent selection for GBS and SIN selections in kale (*B. oleracea* L.) at five different locations for both modes (ESI+ and ESI-).A: ESI+ mode for GBS; B: ESI- mode for GBS; C: ESI+ mode for SIN; D: ESI- mode for SIN.

The data in (Table 13) shows the 20 ions with higher VIP-scores (variable importance) at GBS for ESI- mode and SIN selection for ESI- mode. All this information was compared to publicly available databases to assign molecular formula and putative name, but for a precise identification, we must later perform MS/MS analyses and compare the results with standards.

Table 13. Ions with higher VIP-score after PLS-DA analysis for GBS and SIN selections for ESI- mode.

Genotype	Code	Mass m/z $[M-H]^-$	RT (min)	Formula	Putative name
GBS	M1	337.093	10.5	C16H18O8	Coumaroyl quinic acid
	M2	447.053	8.38	C16H20N2O9S2	Glucobrassicin
	M3	609.146	14.3	C27H30O16	Rutin
	M4	755.204	15	C33H40O20	Kaempferol derivative
	M5	593.151	15.2	C27H30O15	Kaempferol derivative
	M6	625.142	14.3	C27H30O17	Myricetin 3-rhamnoside-7-glucoside
	M7	771.198	10.3	C33H40O21	Quercetin 3-glucoside-7-neohesperidoside
	M8	683.466	29.7	C38H69O8P	PA(18:3(6Z,9Z,12Z)/17:0)Phospholipid
	M9	406.030	3.627	C11H21NO9S3	
	M10	755.204	13.1	C33H40O20	Kaempferol derivative
SIN	M11	358.027	1.5	C10H17NO9S2	Sinigrin
	M12	422.025	1.03	C11H21NO10S3	Glucoiberin
	M13	477.065	13.1	C17H22N2O10S2	Neoglucobrassicin
	M14	277.217	27.7	C18H30O2	Fatty acid
	M15	337.093	10.4	C16H18O8	Coumaroyl quinic acid
	M16	279.233	28.7	C18H32O2	
	M17	447.054	8.38	C16H20N2O9S2	Glucobrassicin
	M18	723.213	18.5	C33H40O18	2-Feruloyl-1-sinapoylgentiobiose
	M19	337.093	10.1	C16H18O8	
	M20	355.104	12.2	C20H21ClN2S	N-Dealkylzuclopenthixol

3.4. Effect of environmental conditions on agronomic and metabolomics traits.

Data presented in table 14 shows the results of a multiple linear regression analysis.

In general, one climatic parameter explains most of the variability (around 80% in most traits). Besides, for most of the traits, climatic parameters before harvesting showed the highest importance.

For vegetative growth, when the maximum temperature before harvest, was the main effect and led to decrease fresh weight, as it had a negative value on fresh weight, and precipitation is considered essential for a plant, as it had a positive effect on dry weight, as led to increasing the dry weight. And environmental conditions did not have any significant effect on plant height (Table 14).

For the concentration of metabolites observed that, when the temperature reached a maximum degree absolute in last 30 day, led to increasing the concentration of M3, M14, and M15 metabolites, as it had a positive effect on metabolites concentration. But if the temperature reached a maximum degree absolute during the growth season, it had a negative effect on the concentration of M6 metabolite, as led to decrease in the metabolite concentration (Table 14). And when the temperature reached a maximum in last 30 day led to decrease in the concentration of M4 metabolite and increase in the concentration of M10 metabolite (Table 14).

When the temperature reached a minimum degree absolute in last 30 day, had a negative effect on the concentration of M5 and M15 metabolites, as led to decrease in the concentration of the metabolites. And also, when the temperature reached a minimum

degree absolute during the growth season, led to decrease in the concentration of M19 and M20 metabolites (Table 14).

When the degree temperature was medium at last 30 day, it had a negative effect on the concentration of M7 metabolite, as led to decrease in the concentration of metabolite (Table 14).

When precipitation occurs during the growth season, led to increase in the concentration of M1 and M2 metabolites. And also when precipitation occurs at last 30 day led to increase in the concentration of M8 metabolite, as precipitation had a positive effect on the concentration of metabolite (Table 14).

Table 14. Multiple linear regressions where climatic data were independent variables and fresh weight, dry weight and the concentration of important metabolites were dependent variable. Where 30D was data recorded during 30 days before harvest in each location. Significant at $p \leq 0.15$.

depended variable	climatic data	parameter estimate	partial R-square	F value	*P*
FW	Max temp 30D	-3651074.00	83.66%	15.36	0.03
	Precipitation 30D	-49452.00	14.77%	18.91	0.05
	Max temp absolute 30D	4266267.00	1.55%	157.31	0.05
DW	Precipitation	327.62	78.92%	11.23	0.04
M1	Precipitation	100.16	95.38%	61.97	0.01
M2	Precipitation	1204.35	74.63%	8.83	0.06
	Med temp 30D	-38731.00	24.58%	62.58	0.02
	Precipitation 30D	-1464.26	0.79%	2171.03	0.01
M3	Max temp absolute 30D	27781.00	91.59%	32.68	0.01
	Max temp absolute	-13747.00	8.19%	75.08	0.01
	Precipitation 30D	-272.23	0.22%	3520.70	0.01
M4	Max temp 30D	-113683.00	89.79%	26.39	0.01
	Max temp	124680.00	8.90%	13.56	0.07
	Med temp	-49963.00	1.28%	35.05	0.11
M5	Min temp absolute 30D	-51339.00	94.35%	50.07	0.01
	Max temp absolute 30D	9956.36	4.90%	12.97	0.07
	Med temp	-8906.43	0.76%	7402.96	0.01
M6	Max temp absolute	-242684.00	79.03%	11.31	0.04
	Med temp	410205.00	17.58%	10.38	0.08
	Max temp absolute 30D	-64973.00	3.26%	25.02	0.12
M7	Med temp 30D	-27141.00	86.83%	19.78	0.02
	Precipitation	642.44	10.10%	6.57	0.12
	Radiation	-2757.91	3.02%	59.41	0.08
M8	Precipitation 30D	24048.00	91.67%	33.02	0.01
M10	Max temp 30D	5641747.00	86.00%	18.42	0.02
	Max temp absolute	-1906810.00	11.41%	8.79	0.10
	Max temp absolute 30D	-2151763.00	2.58%	186.78	0.05
M14	Max temp absolute 30D	848953.00	89.98%	26.95	0.01
	Precipitation	17224.00	9.19%	22.22	0.04
	Min temp	583649.00	0.83%	754.99	0.02

Continued

depended variable	climatic data	parameter estimate	partial R-square	F value	*P*
M15	Min temp absolute 30D	-142527.00	83.27%	14.93	0.03
	Precipitation	1307.17	13.25%	7.62	0.11
	Precipitation 30D	-2557.47	3.48%	1301.30	0.02
M16	Max temp absolute 30D	32765.00	77.64%	10.41	0.05
	Min temp absolute 30D	-165557.00	20.71%	25.11	0.04
	Max temp	53544.00	1.65%	1222.83	0.02
M17	Radiation 30D	19730.00	63.85%	5.30	0.10
M18	Radiation 30D	-0.27	62.74%	5.05	0.11
M19	Min temp absolute	-1754.78	78.48%	10.94	0.05
	Max temp absolute 30D	8.75	21.03%	86.72	0.01
M20	Min temp absolute	-107.00	82.66%	14.30	0.03

Discussion

4. Discussion

4.1. Vegetative Growth.

Most traits encountered in plant breeding are quantitatively inherited, whether controlled by few or many genes is influenced significantly by environmental variability (Acquaah, 2012), as that the environmental factors are the main factors which influence plant growth and development with the exception of the genetic factors. The breeding of cultivars for commercial use invariably alters the response of species to environmental conditions because plants have been selected to remove natural variation in the population (Acquaah, 2012) . In this study, we made the evaluation of the adaptation in different location for agronomic performance of a two divergent selection of kale (*B. oleracea* L) and the results obtained from our work showed that the different environments had effect on the FW, DW, and plant height, where phenotypic traits respond differently to a given change in environmental conditions. Both the strength and the direction of correlations among traits are environmentally dependent (Sultan, 1995) and the intensity of selection on each correlated trait will vary among environments(Schlichting, 1986). These results agree with (Sultan, 1995; Via and Lande, 1985) showing that under different environmental, the plants change the morphological traits. The selection for GBS showed significant affected on agronomical trait except for plant height and the selection for SIN revealed significant affected on all agronomical trait but this was reverse the results obtained by (Sotelo et al., 2016) where showed that the effect of selecting for GLs content did not have any effect in agronomical traits, and this Potentially due to environmental impact on varieties , where the presence or absence of

genotype-environment in fitness components thus provides a statistical test to distinguish the case in which genotypes are specialized and have higher performance in certain environments from that in which they are generalized and have equivalent relative performance across environments. If no one genotype has highest fitness in all situations, then genotype x environment interaction suggests the potential for the genetic differentiation of populations under prolonged selection in different environment (Via, 1984).

4.2. Glucosinolate content.

The glucosinolates in two divergent selection for GLs of kales were detected to be sinigrin ,Glucoiberin ,glucobrassicin, 4-hydroxyglucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin and these agree with other results obtained by (Cartea et al., 2008) showed that sinigrin, glucoiberin, glucobrassicin and neoglucobrassicin were present in all the kale varieties from Northwestern Spain while, glucoraphanin and 4-hydroxyglucobrassicin were detected in 60-70% of the varieties. Sarıkamış et al., (2008) also reported that kale plant had glucobrassicin at very high levels which was followed by 4-methoxyglucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin at much lower levels. Glucosinolate biosynthesis in Brassica crops has quantitative inheritance, which is regulated by complex genetic factors and affected by environmental factors (Hirani et al., 2014). In this study we found that the environment had a significant effect on GLs and this agree with the result from Velasco et al., (2007).

In the divergent selection for GBS, results showed that selection for high and low GBS are stable across environments, indicating the genetic base of the inheritance but also significantly affected the content of other glucosinolates like GIB and NeoGBS, one aliphatic and one indolic. This was previously confirmed with the result from Sotelo et al., (2016) in one environment. They showed that the two divergent selections for the leaf GBS content in kale, showed a significant and positive relationship with the content of NeoGBS, and total indolic GLs and this may be back to GBS is the precursor of NeoGBS in the biosynthetic pathway of indolic GLs. Therefore, variations in GBS content motivate a positive response in the leaf content of NeoGBS and indolic GLs (Sotelo et al., 2016).

For the divergent selection for SIN, results showed that HSIN and LSIN varieties had a significantly effected on the content of GIB, SIN, and GBS. According to Sotelo et al., (2016) GLs profile in Brassicaceae can be partially explained by genetic variation in the GSL-ALK locus encoding (2-oxoglutarate-dependent dioxygenase) which catalyzes the conversion of methylsulfinylalkyl GLs to the alkenyl form in plants (Li and Quiros, 2003). In the biosynthetic pathway of GLs, the locus GSL-ALK controls the side chain desaturation and its presence determines the production of the alkenyl GLs SIN and GIB (3C-GLs) and NeOGBS (4C-GLs) (Sotelo et al., 2016)

4.3. Metabolomics.

Changes in plant metabolism are at the heart of plant developmental processes, underpinning many of the ways in which plants respond to the environment (Brunetti et al., 2013). The high variability of the natural environment presents great challenges for

plants in terms of their capabilities to grow, compete with neighboring plants, and respond appropriately to various abiotic and biotic pressures (Walters, 2005). The understanding of the variation in these metabolic traits is critical to understanding of how the environment influences plant growth and how this manifests itself ecologically (Brunetti et al., 2013). In this work we were studying the metabolomics change for the divergent selection of kale under five environmental conditions. The PLS-DA analysis revealed a clear separation between high and low concentration of GBS and high and low concentration SIN at different locations and there was a significant relationship between the metabolome and GBS and SIN selections at different locations. The environment affects the metabolomic far more than genetic variation alone. The studies by Matsuda et al., (2012)on rice and Frank et al., (2012) on maize have shown that variation in the plant's metabolomic due to changes in the plant's environment (such as growing location, seasons) was far greater than the variation caused by differences between strains or genetic modifications. Frank et al., (2012) showed that multivariate data analyses demonstrated a pronounced impact of the factor environment on the metabolite profiles of maize grown in Germany and South Africa. Several factors can affect the natural variability of crop metabolites. The most significant ten metabolites in each selection for ESI- mode were tentatively identified, as, detected Kaempferol derivative in both selections , this result was agree with the result from Farag et al., (2013) showed the derivatives of quercetin and kaempferol were detected in *B. napus* L. and Velasco et al., (2011) showed that kaempferol derivatives were detected in the leaf in *B. napus* L. var. pabularia grown in Spain.

Glucobrassicin was detected in divergent selection for GBS and Glucobrassicin, Sinigrin, Glucoiberin, Neoglucobrassicin were detected in divergent selection for SIN of kale (*B. oleracea* L), this result agree with Velasco et al., (2011) showed that they were from the glucosinolates found in extracts of cabbage (*B. oleracea* capitata group) leaf rape (*B. napus* pabularia group),and kale crops (*B. oleracea* acephala group). Rutin was detected in divergent selection for GBS the result agree with Farag et al., (2013) showed Rutin was detected Inflorescence of *B. napus* L. fatty acid was detected in divergent selection for SIN this agree with the result from Farag et al., (2013) fatty acids was detected in *B. napus.* The most ubiquitous subclass of flavonoids found in Brassica vegetables is derived predominantly from kaempferol and quercetin, whereas isorhamnetin and myricetin (Farag et al., 2013).

4.4. Effect of environmental conditions on agronomic and metabolomics traits.

The environmental factors monitored in this study include temperatures, precipitation, and solar radiation which proved to correlate to variations in metabolite composition and leaves fresh and dry weight and had a negative and positive effect of kale (*B. oleracea* L). Where the maximum temperature led to decrease in fresh weight, this result confirmed with the results from Manske (1998) showed that temperature, an approximate measurement of the heat energy available from solar radiation, is a significant factor because both low and high temperatures limit plant growth. Most plant biological activity and growth occur within only a narrow range of temperatures, between 0 °C and 50 °C.

Temperature regulates several developmental processes in plants, including vegetative growth (Kami et al., 2010). Temperature increases have been found to reduce yields and quality of many crops, most importantly cereal and feed grains (Adams et al., 1998). The maximum temperatures had a positive and negative effect on some of metabolites found in two divergent selection of kale, this result agree with the result from Adams et al., (1998) showed that plants under conditions of stress induced by climate factors (i.e. high temperatures, and high levels of solar radiation) may show changes in the production of different metabolite classes in *T. diversifolia.* Temperature strongly influences metabolic activity and plant ontology (Ramakrishna and Ravishankar, 2011).

Minimum temperature had a negative effect on few metabolites and precipitation had a positive effect on few metabolites , this result agree with results from anther research showed that several climate factors such as precipitation, temperature and solar radiation are well described as being able to influence the metabolites (Adams et al., 1998; Shulaev et al., 2008; Ramakrishna and Ravishankar, 2011; Arbona et al., 2013; Sampaio et al., 2016) .

CONCLUSION

5. CONCLUSION

In this study, the environment had effect on agronomic performance of a divergent selection for leaf glucosinolates content (HGBS; GBS; HSIN; LSIN)) in kale (*B. oleracea* L), as under different environments condition increase the FW, DW, and plant height. Two divergent selections for GLs had effect on FW and DW and selection for SIN had effect on plant height. The GLs pattern was stable among locations, and locations had significant effect on all GLS. In the divergent selection for GBS, results showed that HGBS and LGBS varieties had a significantly effect on the content of GIB, NeoGBS, and GBS GLs. And for the divergent selection for SIN, results showed that HSIN and LSIN varieties had a significantly effected on the content of GIB, SIN, and NeoGBS. The score plot for PLS-DA exhibited an obvious separation between the divergent selections for GLs low and high (SIN) and low and high (GBS). The environmental factors such as temperature and precipitation had significant effect on most of agronomic performance and most of important metabolites were detected in this study.

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