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Institute of Crop Science (340)
Department of Agronomy

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Evaluation of new open pollinating broccoli genotypes (*Brassica oleracea* convar. *botrytis* var. *italica*) specifically bred for organic farming conditions focusing on agronomic performance and glucosinolate content

Dissertation
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List of abbreviations

°C	Degree centigrade
µmol	Micro mol
1-VR	coefficient of determination of cross-validation
4ME	4-methoxyglucobrassicin
ANOVA	Analysis of variance
BLE	The German Federal office for agriculture and Food
cm	Centimeter
CMS	Cytoplasmic male sterility
DW	Dry weight
FAO	Food and Agriculture Organization of the United Nations
FWHI	Fresh weight harvest index
g	Gram
GBS	Glucobrassicin
GI	Glucoiberin
GRA	Glucoraphanin
GS	Glucosinigrin
GSL	Glucosinolate
ha	Hectare
HFW	Head fresh weight
HPLC	High performance liquid chromatography
IFOAM	International Federation of Organic Agriculture Movements
kg	Kilogram

l	Liter
m^2	Square meter
mmol	Millimol
n	Number of samples
N	Nitrogen
n.a.	Not available
n.s.	Not significant
NGB	Neoglucobrassicin
NGO	Non-governmental organizations
NIRS	Near-infrared spectroscopy
OF	Organic farming
OP	Open pollinating
R^2	Coefficient of determination in prediction
RPD	ratio of performance to deviation
SAS	Statistical analysis software
SD	Standard deviation
SEC	Standard error of calibration
SECV	Standard error of cross-validation
SEP	Standard error of prediction
t	Ton
tGSLs	Total glucosinolates

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Chapter 1. Introduction

1. Background

To date, consumers pay more attention to organic products due to the increase of awareness towards the benefits of these products for the environment (Cicia et al., 2002). They believe organic products are produced in a more environmentally friendly manner without the use of certain technologies such as genetic engineering (Zanoli, 2004). Also, consumers expect organic products to be healthier than conventional ones (Roitner-Schobesberger et al., 2008). The data is sometimes in contradiction with the health effects of organic products (Smith-Spangler et al., 2012; Barański et al., 2014). Therefore, organic production is often criticized when it cannot fulfill these expectations.

The contents of health-promoting ingredients are not only due to the production process and environmental conditions but are also very much dependent on the variety used, so that new possibilities for the cultivation of the plant in organic farming are opened up to meet consumer expectations and to produce favorable products. Since organic production is majorly dependent on the cultivars which are specially bred for a conventional farming system (Lammerts van Bueren et al., 2002), breeding special varieties for organic production is in focus. More specifically, the critical attitude of the organic sector to some techniques of conventional plant cultivation, e.g. the use of CMS hybrids, especially for vegetables has shown a need for organic plant breeding (BÖLW, 2013). Since the organic associations have banned the use of CMS hybrids as a form of genetically modified varieties (Bioland e.V., 2013), especial breeding techniques are applied by the organic breeders that comply with the principles of organic farming.

An organic farming system requires organically-derived inputs such as organic seeds (Renaud, 2014). The main focus is on open pollinating (OP) varieties cultivated with methods of classical plant cultivation such as single plant selection, which allow reproduction of the seed. The selection of genotypes and lines usually takes place in on-farm breeding processes. Due to the increased demand for healthy products, development of OP varieties with a focus on the content of health-promoting ingredients could become more important.

2. The purpose of the project

Based on the given background, the German Federal Office for Agriculture and Food (BLE) initiated a project on “Breeding development of open pollinating cultivars of broccoli for organic farming in terms of agronomic characteristics, secondary and bioactive ingredients and

sensory properties". This was a joint project which was done through the cooperation of University of Hohenheim and Kultursaat e. V. (NGO of on-farm breeders) in two parts during five years (2012-2016). The overall aim of the project was to develop new OP broccoli varieties from existing breeding populations of broccoli through on-farm breeding (single plant selection), which meet the requirements of organic farming. The present doctoral thesis focused on the second part of the project during 2014 to 2016. The description of the results of the first part of the project has been already reported by Wolf et al. (2014) and published by BÖLN. In line with the work of Wolf et al. (2014), the second part of the project was designed to test newly bred OP broccoli genotypes, which were adapted to the special requirements of organic farming to release them as final varieties. With this purpose, the research was designed to assess the agronomic performance and chemical quality (with regard to health benefiting compounds content) of the developed OP genotypes. Also, we investigated the possible significant differences between the OP genotypes and the hybrid varieties. This research was based on three field trials and series of laboratory analyses of new bred OP genotypes of broccoli. The investigations were conducted at the organic research station of the University of Hohenheim (Kleinhohenheim).

3. Objectives

The present doctoral dissertation focused on the agronomic performance and health benefitting compounds content of new bred OP genotypes of broccoli. The specific objectives of this thesis were:

- evaluating the agronomic performance of the open pollinating genotypes during two consecutive growing seasons of fall and spring,

- developing a Near Infrared Spectroscopy (NIRS) technic for fast analysis of the glucosinolate content of broccoli heads and to check the accuracy of this methodology,

- determining the glucosinolate concentrations of the OP genotypes during two consecutive growing seasons of fall and spring and evaluating the effect of genotype, growing season and their interactions on the glucosinolates content of the samples.

4. Research design and methodology

For the accomplishment of the objectives described, field experiments were carried out at the organic research station Kleinhohenheim of the University of Hohenheim during fall growing season 2014, fall growing season 2015 and spring growing season 2016.

The field trial of fall 2014 was designed as a randomized complete block with three replications. 12 genotypes were cultivated in this year. Each plot area was 1.5 × 10 m2 and the planting distance between and within the rows was 38 and 50 cm, respectively. The experimental design of the fall experiment 2015 was a randomized complete block design with three replicates, 14 plots per replicate and four rows of plants per plot (in total 80 plants per plot with plant spacing within a row and between two rows 40 and 30 cm, respectively). Plot size was 1.5 × 8 m^2. For the spring experiment, plants were arranged in a resolvable row-column design, which allows accounting for potential trends in both rows and columns. Plots were arranged in 14 rows and three columns (a column here corresponds to a replicate) with a plot size of 2 × 10 m^2. The plant spacing within a row and between two rows was set to be 50 cm, therefore each plot contained 80 plants. In both seasons, eleven OP broccoli genotypes, two F1 hybrids, and one OP variety were cultivated. The plant samples and experimental designs are described more in detail in the second chapter of the present thesis.

Recording the agronomical data and preparing the broccoli samples for further analyses were done in the laboratory of the Institute of Crop Science-Department of Agronomy (340a) at the University of Hohenheim. Glucosinolates content of the samples were determined by NIRS and standard method of HPLC. The implementations of HPLC and NIRS were done in the laboratory of the Institute of Crop Science-Department of Quality of Plant Products (340e) and at the research station of the University of Hohenheim (Ihinger Hof), respectively.

5. Outlines

The present doctorate thesis contains the outcomes of this project in three main articles (Chapter 2 to Chapter 4) which represent the body of the dissertation. **Chapter 2** which was published as paper in *PLOS ONE* journal under the title of *"Agronomic performance of new open pollinated experimental lines of broccoli (*Brassica oleracea *L. var.* italica*) evaluated under organic farming"*, describes the outcomes of evaluation of the agronomic parameters of the broccoli genotypes during two fall and spring growing seasons. **Chapter 3** which is a paper published in *FOOD CHEMISTRY* journal entitled *"Development of a Near-Infrared Spectroscopy Method (NIRS) for fast analysis of total, indole, aliphatic and individual glucosinolates in new bred open pollinating genotypes of Broccoli (*Brassica oleracea *convar.* botrytis *var.* italica*)",* describes the development of a NIRS technology to determine individual and total glucosinolates of broccoli samples. The potential use of NIRS is evaluated in this chapter regardless of the genotype. Finally, **Chapter 4** which was published in *JOURNAL OF AGRICULTURE AND AGRICULTURAL ASPECT* titled *"Total and Individual Glucosinolates*

*of Newly Bred Open Pollinating Genotypes of Broccoli (*Brassica oleracea *convar*. botrytis *var*. italica*) Grown Organically: Effect of Genotype and Growing Season"*, provides the results of glucosinolate determination of samples which were assessed in the second chapter. This comparative research was done to find the genotypes with higher glucosinolates content and to test the seasonal stability of the glucosinolate content of different broccoli genotypes.

For citation of the papers, please use the references given below:

Sahamishirazi, S., Moehring, J., Zikeli, S., Fleck, M., Claupein, W., Graeff-Hoenninger, S. (2018). Agronomic performance of new open pollinated experimental lines of broccoli (*Brassica oleracea* L. var. *italica*) evaluated under organic farming. PLoS ONE 13(5): e0196775. https://doi.org/10.1371/journal.pone.0196775.

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Chapter 2. Agronomic performance of new open pollinated experimental lines of broccoli (*Brassica oleracea* L. var. *italica*) evaluated under organic farming

Sahamishirazi, S., Moehring, J., Zikeli, S., Fleck, M., Claupein, W., Graeff-Hoenninger, S. (2018). Agronomic performance of new open pollinated experimental lines of broccoli (*Brassica oleracea* L. var. *italica*) evaluated under organic farming. PLoS ONE 13(5): e0196775. https://doi.org/10.1371/journal.pone.0196775

Considering the purpose of our research project, the assessment of the agronomic performance was an important area of action to select and introduce proper OP genotypes for further breeding programs. Hence, part of this thesis was designed to cultivate the experimental genotypes in two consecutive seasons to evaluate the performance of each genotype under different seasonal conditions. According to this, Chapter 2 provides outcomes of the performance of the tested experimental genotypes, which have been compared with standard varieties in fall and spring seasons. Through this chapter, different agronomic variables are evaluated and the potential genotypes are listed.

Abstract

In order to develop new open pollinating cultivars of broccoli for organic farming, two experiments were conducted during fall 2015 and spring 2016. This study was aimed at comparing the agronomic performance of eleven new open pollinating breeding lines of broccoli to introduce new lines and to test their seasonal suitability for organic farming. Field experiments were carried out at the organic research station Kleinhohenheim of the University of Hohenheim (Stuttgart-Germany). Different agronomic traits total biomass fresh weight, head fresh weight, head diameter, hollow-stem, fresh weight harvest index and marketable yield were assessed together with commercial control cultivars. The data from both experiments were analyzed using a two-stage mixed model approach. In our study, genotype, growing season and their interaction had significant effects on most traits. Plants belonging to the fall growing season had bigger sizes in comparison to spring with significantly ($p< 0.0001$) higher biomass fresh weight. Some experimental lines had significant lower head fresh weight in spring in comparison to the fall season. The high temperature during the harvest period for the spring season affected the yield negatively through decreasing the firmness of broccoli heads. The low average minimum temperatures during the spring growing season lead to low biomass fresh weight but high fresh weight harvest index. Testing the seasonal suitability of all open pollinating lines showed that the considered fall season was better for broccoli production. However, the change in yield between the fall and the spring growing season was not significant for "Line 701" and "CHE-MIC". Considering the expression of different agronomic traits, "CHE-GRE-G", "Calinaro" and "CAN-SPB" performed the best in the fall growing season, and "CHE-GRE-G", "CHE-GRE-A", "CHE-BAL-A" and "CHE-MIC" and "Line 701" were best in the spring growing season, specifically due to the highest marketable yield and proportion of marketable heads.

1. Introduction

According to FAO statistics [1], the production quantity of cauliflower and broccoli worldwide reached 2.4 million tons in 2014. Broccoli (*Brassica oleracea* L. var. *italica*) is an economically important vegetable. Its production and consumption has a long history in Europe, as it fits into European diets [2]. In Germany, broccoli is currently cultivated on 2170 ha, about 1100 farms are involved in its' production and the average marketable yield is 13.6 t ha^{-1} annually [3]. Broccoli is also an important crop in organic farming (OF), albeit with lower marketable yields compared to conventional farming, with about 10 t ha^{-1} [4]. Briefly, in Germany, shares of organic vegetable production related to total vegetable production is 9%, which is in total

10.392 ha. Also, the percentage of organic vegetable consumption related to the overall vegetable consumption is approximately 6 to 7%.

Today, the broccoli cultivars that are on the market for commercial purposes are almost exclusively F1 hybrids [5]. In OF, F1 hybrids showed an average performance with regard to quality and yield [6]. It is critical in OF to develop F1 hybrids as it requires cytoplasmic male sterility (CMS) derived from Japanese radish by cell fusion as a breeding technique [7]. Some OF organizations even forbid the use of CMS-hybrids, because this practice is seen as a genetic modification that is going against the principles of organic farming [8]. Moreover, man-made hybridization in plant breeding is seen as a practice that is not in line with the principle of plant specific- and genotype integrity as it should be applied in organic breeding [9]. Hence, developing genotypes such as new open pollinating (OP) breed lines, which are considered to be heterogeneous, could be one option for organic farming [5]. Generally, F1 hybrids of broccoli produce small sized plants with big sized and uniform heads [10], which better reflect the demands of consumers and the needs of retailers. The main benefit of the production of F1 hybrids is the stability of plants across different environments [11]. These cultivars are resistant to most abiotic and biotic stressors and typically show a high degree of uniformity in color, buds, firmness and harvesting periods [12]. F1 hybrids are genetically homogeneous [13] but if farmers multiply the seeds of the F1 generation, the resulting F2 generation faces loss of hybrid vigor and is usually so heterogeneous that on-farm seed reproduction has no opportunity. Contrary to this, OP cultivars give farmers the possibility to harvest their own seeds for reproduction [5, 14]. The heterogeneous genotypes are also resistant to the influence of genetic and environmental interactions due to the heterogeneity in their genetic structures [15], also due to better genotype buffering against different growing conditions when compared to homogeneous ones [14]. Furthermore, according to Ciancaleoni et al. [10], OP genotypes show a great variability and are distinguished from each other by differing cold requirements for flower induction, sprouting habit, leaf shape, color and harvesting times. Thus, heterogeneous genotypes are particularly beneficial [14]. On the other hand, as the organic seed market is still not big enough to attract the professional plant breeding companies economically [7, 16, 17], only few cultivars have been specifically bred for OF thus far [5].

According to Renaud et al. [6], in the current market, the existing old OP cultivars of broccoli lack quality traits and uniformity. Also, considering the review study by Lammerts van Bueren et al. [7] on the necessity of breeding for organic and low-input production conditions, significant breeding efforts for the organic sector are required to support the needs of organic farmers. There have been some attempts, such as previous work of Renaud et al. [6] on breeding

OP genotypes of broccoli. In order to develop new OP cultivars of broccoli for organic farming, the University of Hohenheim in cooperation with the NGO Kultursaat e.V. (organization of on-farm breeders) tested and selected commercial cultivars and new experimental lines of broccoli suitable for OF in Germany. The selection criteria were agronomic traits such as; yield level, stability of yield over time and different quality attributes associated with research focused on replacing current cultivars with new OP lines in OF. This study is specifically aimed at evaluating the agronomic performance of experimental genotype populations by comparison with commercial control cultivars in order to: (1) introduce new OP broccoli experimental populations for OF, and (2) to test the seasonal suitability of these OP genotype populations.

2. Materials and Methods

2.1 Plant materials and field trials

Eleven OP breeding lines, two F1 hybrids and one OP cultivar of *Brassica oleracea* var. *italica* were tested under OF conditions during fall 2015 and spring 2016 which are listed in Table 1. The field experiments were carried out at the organic research station of the University of Hohenheim. For detailed description of field trials, please see our previous study Sahamishirazi et al. [18]. The harvest window in fall growing season was six weeks, during which, broccoli heads were harvested seven times. In spring, the harvest window was three weeks with four times of harvest (see Sahamishirazi et al. [18]). Note that for the data described above, the effects caused by the two different experiments, the two different years and the two different growing seasons are totally confounded. To simplify the further description, this confounded effect is called as growing season effect from now on.

2.2 Agronomic traits

2.2.1 Total biomass fresh weight, head fresh weight and head diameter

Total biomass fresh weight was recorded as the total aboveground biomass. This included the weight of stem, leaves, lateral branches and head. After weighing the total biomass, the flower head, formed in the center of the plant, was cut to 18 cm length (including stem) and the head fresh weight was measured. Harvesting was carried out once the head reached a marketable head diameter ≥ 10 cm. The diameter was recorded as the mean of a triplicate measurement of the widest part of the head using a Vernier caliper.

2.2.2 Proportion of hollow-stem

After cutting the heads to 18 cm length the existence of hollow stems was assessed. Presence or absence of a hole in the stem was scored as “positive” and “negative”.

2.2.3 Fresh weight harvest index (FWHI) and marketable yield

Fresh weight harvest index (FWHI) was defined, according to Tan et al. [19], as:

Equation 1:

$$FWHI = \frac{100 \times HFW}{(HFW + \ weight\ of\ residual)}$$

Where, "HFW" is the head fresh weight and the "weight of residual" is the fresh weight of biomass excluding head weight.

In order to calculate the marketable yield, all marketable broccoli heads, which had no quality defects (such as loos buds, brownish color and etc.) and had a minimum diameter ≥10 cm were taken into consideration. Broccoli marketable yield was calculated for each population in tons per hectare as follows:

Equation 2:

$$Marketable\ yield = \frac{total\ weight\ of\ marketable\ broccoli\ heads\ (t)}{area\ (ha)}$$

To assess the performance of each line for production of marketable broccoli heads, the proportion of broccoli plants with marketable heads in relation to the total number of broccoli plants evaluated per genotype population was calculated.

2.3 Statistical analysis

The experimental design of fall 2015 experiment was a randomized complete block design with three replicates each consisting of 14 plots. For the spring experiment 2016, plots were arranged in a resolvable row-column design [20] with 14 rows and three columns (within a column all 14 genotypes were tested, thus it corresponds to a replicate). Note that the effects of different experiments, different years and different growing seasons are totally confounded. Hence, we described and modelled the confounded effect by the growing season but still meant the confounded effect. The data for both the fall and spring experiments were analyzed using a two-stage mixed model approach [21, 22]. The stage one analysis focused on individual experiments. The stage two analysis was across the two experiments, fall and spring. Analysis of the data from the experiment in fall 2015 of the traits; total biomass fresh weight (g), head fresh weight (g), head diameter (cm) and total yield, was conducted using the mixed linear model

$$y_{ijk} = \mu + b_k + g_i + h_j + (gh)_{ij} + e_{ijk}, \quad (1)$$

where g_i, h_j and $(gh)_{ij}$ are the fixed main effects of the i^{th} genotype and j^{th} harvest time as well as the fixed interaction effects between the i^{th} genotype at the j^{th} harvest time, respectively. b_k

is the k^{th} random block effect and e_{ijk} is the error of observation y_{ijk} assuming that error effects from observations from the same plot but different harvest times are potentially correlated with a first-order autoregressive variance-covariance structure. Data from experiment in spring 2016 was analyzed using a similar model, just replacing block effects by row and column effects:

$$y_{ijkl} = \mu + row_k + col_l + g_i + h_j + (gh)_{ij} + e_{ijkl}, \quad (2)$$

in which all effects are defined similar to (1). row_k and col_l are random effects for the k^{th} row and l^{th} column, respectively. For analysis of total yield, both models (1) and (2) were simplified by dropping all effects including harvest time. Note that total yield is the sum of all yields harvested on the same plot, thus no harvest effect can be estimated. For both experiments genotype-by-harvest time least square means of the n^{th} growing season ($\hat{\mu}_{ijn}$) were estimated and subject to an across-growing season analysis with the following second stage model:

$$\hat{\mu}_{ijn} = \mu + g_i + a_n + h_{ln} + (ga)_{in} + (gh)_{jln} + f_{ijln}, \quad (3)$$

where μ is the general intercept, g_i, a_n, and $(ga)_{in}$ are the fixed main effects of the i^{th} genotype, n^{th} growing season, j^{th} harvest time within growing season *n* and the interaction effects between the i^{th} genotype at the n^{th} growing season, respectively. $h_{jn}$and $(gh)_{jln}$ are assumed as random effect of the j^{th} harvest time within growing season *n* and the interaction effects between the i^{th} genotype at the j^{th} harvest time within growing season *n*, respectively. Due to limited degrees of freedom [23], the former was formally taken as fixed in the analysis. f_{ijn} is the approximated error effect estimated in (1) or (2) for genotype-by-harvest time mean $\hat{\mu}_{ijn}$. To use error effects from the first stages, Smith weights [24] were calculated using a SAS macro [22]. We estimate both genotype main effects and genotype-by-growing season means from equation (3). Data measured as a percentage was logit transformed prior to analysis. Residuals were checked graphically for normality of distribution, homogeneity of variance and potential outliers. If they latter were non-plausible, they were excluded from data previous to statistical analysis. No means of across growing seasons for cultivar "Miranda" was calculated as it did not produce any broccoli heads in spring 2016. After finding significant differences via F-test, a multiple t-test with α=0.05 was used to compare genotype means within or across growing seasons. Note that this testing approach is called ANOVA or F test protected post hoc testing meaning that the F test ensures the family-wise error rate of 5% while t tests only ensure the comparison-wise error rate. To visualize which genotypes perform best for which trait and to show the correlations between traits, principal component analysis was performed using variety means across growing seasons of total yield and the other traits. From this analysis the first two dimensions were plotted as a biplot [25, 26, 27]. All statistical analysis of both experiments

were determined by using SAS version 9.4. Additionally, graphics were generated using SigmaPlot 12.0.

3. Results and Discussion

3.1 Growing and climate condition

In general, cultivation period of fall and spring season lasted 15 and 11 weeks after transplanting. The 15 weeks of growth in fall 2015 included ten weeks of vegetative and generative growth and five weeks of harvesting. In spring 2016, the 11 weeks of growth contained seven weeks of vegetative and generative growth and four weeks of harvesting. The shorter cultivation time during the spring season was due to higher temperatures from the beginning of head formation to the end of harvest [28], which potentially accelerated plant development in whole. In the fall growing season 2015, the average daily temperature decreased from 22 °C, at the transplanting time in August, to 7 °C at the end of harvest in November (Figure 1a). Throughout the spring season 2016, the average daily temperature increased from 9 °C to 20 °C during April to July (from transplanting to the end of harvest). The average daily air temperature values were higher in fall season 2015 than in spring season 2016 during the stages of growth and head formation up to the beginning of harvesting. The sum of precipitation was recorded throughout both seasons (Figure 1c). According to the Figure 1c, precipitation was much higher in spring 2016 in comparison to fall 2015 during the whole growing season. Specifically, the highest precipitation was in the fifth and the seventh week after transplanting. Regarding the average relative humidity, the range was similar for both seasons from 60% to 90%, although the changing trend of the relative humidity during both seasons was much different (Figure 1d) based on the amount of precipitation.

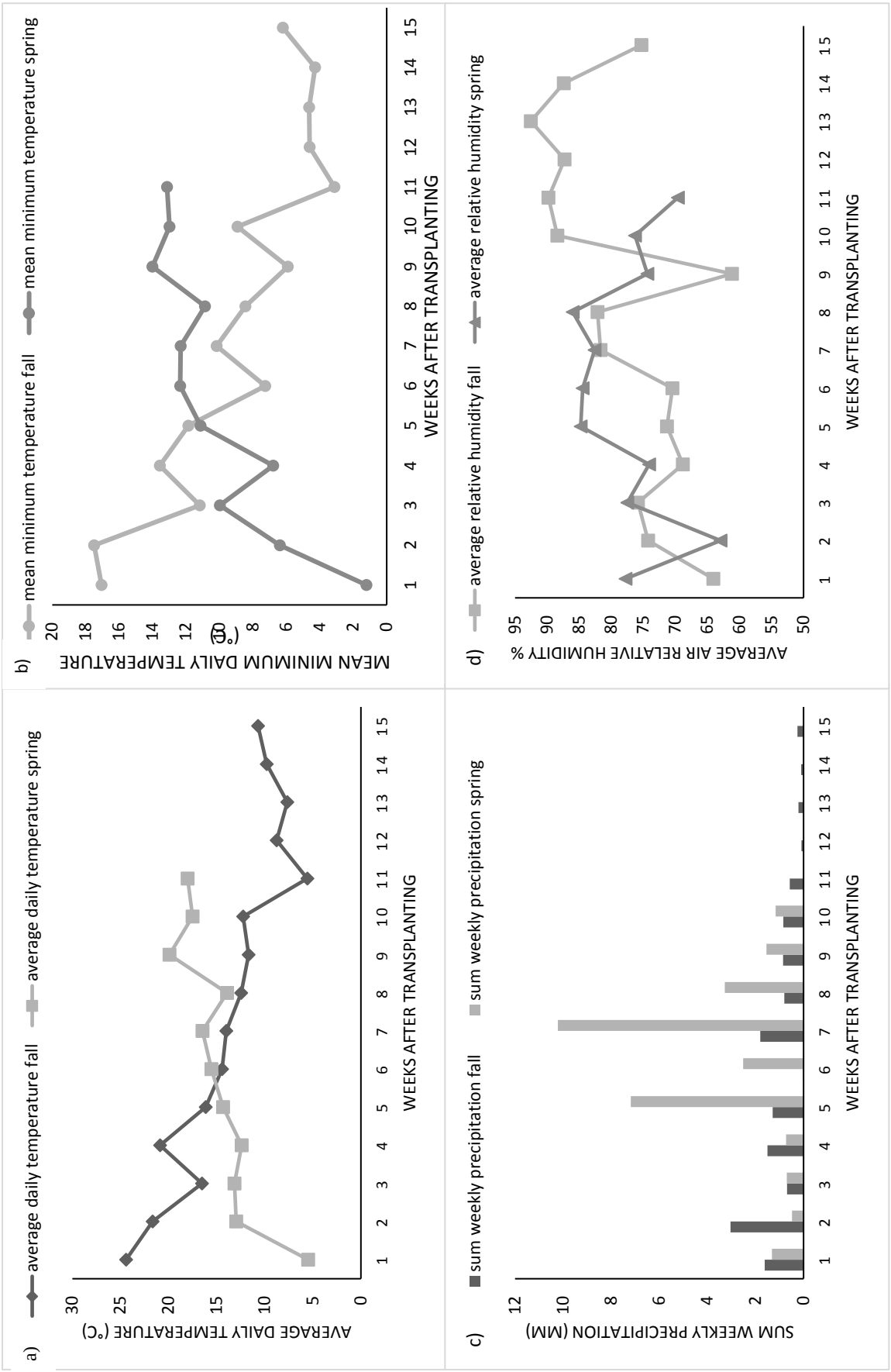

Figure 1. The average daily temperature (a), mean minimum daily temperature (b), sum weekly precipitation (c) and average air relative humidity (d) in the region of Hohenheim during fall 2015 and spring 2016 from transplanting to the end of harvest

Of the three commercial control cultivars planted in spring 2016, “Miranda” (OP) failed to produce any heads. Similar to the study reported by Farnham et al. [29], some broccoli cultivars did not form heads due to the high temperatures. We assume that “Miranda” showed a similar response and therefore was sensitive to high temperatures during the spring trial. Even though the central stem was formed, no head was produced at all. Nevertheless, in fall 2015, “Miranda” performed very well with a mean biomass weight of 1278 g and mean head weight of 275.7 g (Table 1).

3.2 Total biomass fresh weight

Generally, plants in the fall growing season 2015 were bigger in size in comparison to plants in spring 2016. According to Table 1, total biomass fresh weight per broccoli plant in fall 2015 ranged from 928 g (Line 124) to 1700 g (Marathon F1). This range in spring 2016 was significantly lower than in the fall season 2015, between 568 g (TH-LIM-20-68) and 966 g (CHE-BAL-A). Across the two growing seasons the “CHE-BAL-A” had significantly higher biomass weight than the commercial cultivars, as well as the other lines except for “TH-COA” and “CHE-MIC” (Table 1). According to the study by Tan et al. [19], the decrease of the average minimum temperatures during the growing season led to a decrease of biomass fresh weight. Likewise, in the current study, the mean minimum temperatures of the growing season in fall 2015 were higher than in spring 2016 during the first five weeks after transplanting (Figure 1b). The lower minimum temperatures in spring (-4 °C to 3.3 °C) resulted in significantly lower biomass weight in comparison to fall (6.6 °C to 8.1 °C) for all genotype populations and also lead to shorter cultivation periods (Table 1). Results from ANOVA, in Table 2, showed significant effects ($p<0.0001$) of genotype × growing season interaction and growing season × harvest interaction on biomass fresh weight.

Table 1. Comparison of biomass fresh weight (g), head fresh weight (g), Diameter (cm), proportion of hollow stem (%), Fresh Weight Harvest Index (FWHI %), marketable yield (t ha^{-1}) and share of marketable heads of broccoli samples within fall 2015 and spring 2016.

	Genotype	Season	Biomass fresh weight (g)	Head fresh weight* (g)	Diameter (cm)	Proportion of hollow stem[1] %	FWHI (%)	Marketable yield (t ha^{-1})	Marketable heads (%)
Commercial control cultivars	Batavia F1	fall	1434.92 ± 52.46 ^{a}	358.67 ± 11.97	11.84 ± 0.23	18.07	25.53 ^{b}	15.32 ± 1.3 ^{a}	87.86
		spring	804.61 ± 48.77 ^{b}	274.61 ± 15.28	15.5 ± 1.29	0.6	35.51 ^{a}	7.79 ± 0.6 ^{b}	68.33
	Marathon F1	fall	1700.85 ± 61.66 ^{a}	317.68 ± 13.74	11.94 ± 0.29	11.36	18.07 ^{b}	12.82 ± 1.3 ^{a}	79.21
		spring	797.77 ± 61.67 ^{b}	260.49 ± 18.88	13.16 ± 1.6	0	32.82 ^{a}	8.46 ± 0.6 ^{b}	84.37
	Miranda	fall	1277.82 ± 58.74	275.67 ± 13.42	11.79 ± 0.26	28	22	10.14 ± 1.3	79.17
		spring	n.a. [2]	No heads	No heads	No heads	n.a.	No heads	No heads
Experimental genotype population lines	CHE-BAL-A	fall	1356.95 ± 55.03 ^{a}	312.51 ± 11.97	12.01 ± 0.24	13.06	23 ^{b}	10.51 ± 1.3 ^{a}	68.19
		spring	966.12 ± 51.59 ^{b}	287.55 ± 16.24	12.79 ±1.37	6.62	31.04 ^{a}	6.55 ± 0.6 ^{b}	61.25
	CAN-SPB	fall	1124.59 ± 55.57 ^{a}	273.63 ± 12.22	12.38 ± 0.25	25.5	24.89 ^{b}	15.83 ± 1.3 ^{a}	76.12
		spring	693.03 ± 56.46 ^{b}	245.45 ± 17.65	13.00 ± 1.48	0	35.39 ^{a}	5.38 ± 0.6 ^{b}	60.41
	Calinaro	fall	971.94 ± 54.5 ^{a}	274.63 ± 11.86	11.92 ± 0.24	0	28 ^{b}	11.72 ± 1.3 ^{a}	73.64
		spring	648.53 ± 50.14 ^{b}	247 ± 15.76	13.13 ± 1.33	0	39.03 ^{a}	3.75 ± 0.6 ^{b}	42.5
	TH-COA	fall	1431.52 ± 59.63 ^{a}	272.87 ± 12.8	12.31 ± 0.26	35.83	19.02 ^{b}	6.72 ± 1.3 ^{a}	62.23
		spring	915.41 ± 53.98 ^{b}	229.11 ± 16.95	12.1 ± 1.42	2.33	25.48 ^{a}	2.06 ± 0.6 ^{b}	31.25
	CHE-GRE-A	fall	1141.4 ± 52.34 ^{a}	250.22 ± 11.38	12.38 ± 0.23	22.76	23.41 ^{b}	10.04 ± 1.3 ^{a}	67.3
		spring	687.16 ± 48.53 ^{b}	204.59 ± 15.26	12.64 ± 1.29	0.6	30.74 ^{a}	6.57 ± 0.75 ^{b}	67.91
	CHE-GRE-G	fall	1260.88 ± 57.37 ^{a}	305.5 ± 12.51	12.32 ± 0.25	14.06	24.72 ^{b}	15.56 ± 1.3 ^{a}	81.54
		spring	786.67 ± 48.72 ^{b}	253.98 ± 15.25	12.15 ± 1.28	0.6	33.15 ^{a}	6.23 ± 0.52 ^{b}	70.83
	TH-LIM-19-28	fall	1128.89 ± 56.46 ^{a}	276.41 ± 12.26	11.86 ± 0.25	0	25.29 ^{b}	7.16 ± 1.3 ^{a}	65.68
		spring	591.75 ± 52.2 ^{b}	223.04 ± 16.31	12.16 ± 1.37	0	38.7 ^{a}	3.2 ± 0.6 ^{b}	45
	TH-LIM-20-68	fall	952.97 ± 52.86 ^{a}	255.02 ± 11.53	11.64 ± 0.22	30.63	27.31 ^{b}	9.5 ± 1.3 ^{a}	66.34
		spring	568.18 ± 50.15 ^{b}	210.57 ± 15.75	13.09 ± 1.33	0.6	37.13 ^{a}	2.06 ± 0.6 ^{b}	32.08
	Line 124	fall	928.05 ± 49.66 ^{a}	253.3 ± 10.78	11.33 ± 0.22	18.4	28.13 ^{b}	8.55 ± 1.3 ^{a}	66.83
		spring	675.38 ± 50.17 ^{b}	242.33 ± 15.74	12.44 ± 1.33	0.6	36.94 ^{a}	3.42 ± 0.6 ^{b}	40.41
	Line 701	fall	1395.4 ± 67.7 ^{a}	328.46 ± 14.75	11.68 ± 0.30	13.03	23.84 ^{b}	4.04 ± 1.3 ^{a}	38
		spring	676.16 ± 56.88 ^{b}	257.29 ± 17.79	11.69 ± 1.49	0	38.79 ^{a}	6.34 ± 0.6 ^{a}	75.41
	CHE-MIC	fall	1212.84 ± 57.04 ^{a}	294.95 ± 12.69	12.23 ± 0.25	34.43	24.75 ^{b}	9.44 ± 1.3 ^{a}	70.32
		spring	866.37 ± 52.77 ^{b}	248.38 ± 16.4	12.83 ± 1.38	0.34	30.17 ^{a}	6.54 ± 0.6 ^{a}	72.91

Means of one genotype in one column followed by different letters significantly different from each other ($p < 0.05$).

1 Incidence of hollow stem is rated as 0=No, 1= Yes, the probability of incidence of hollow stem is analyzed.

2 Not available

* No letter display was created simple means for this variable, as the marginal means of genotypes across growing seasons should be compared (see Table 3)

Table 2. Results from the analysis of variance for different agronomic traits.

Effects	Biomass fresh weight	Head weight	Diameter	Hollow stem	FWHI[1]	Marketable yield
Genotype	<0.0001	<0.0001	n.s. [2]	n.s.	<0.0001	<0.0001
Growing season	<0.0001	<0.0001	n.s.	n.s.	<0.0001	<0.0001
Genotype × Growing season	<0.0001	n.s.	n.s.	n.s.	0.0232	<0.0001
Growing season × harvest	<0.0001	0.5086	<0.0001	n.s.	0.0478	-

[1] Fresh Weight Harvest Index
[2] Not significance

3.3 Head diameter

Diameter measurements of marketable heads indicated a range from 11.33 cm (Line 124) to 12.38 cm (CAN-SPB) and from 11.69 cm (Line 701) to 15.5 cm (Batavia F1) in fall 2015 and spring 2016, respectively (Table 1). However, according to Table 2, for this trait no significant effects for genotype, growing season and their interactions were found ($p > 0.05$). Yet, a significant effect of harvest time within growing seasons on head diameter was observed ($p < 0.0001$), which was caused by higher diameter of broccoli heads harvested later in the growing season.

3.4 Proportion of hollow stems

According to Table 1, the proportion of hollow stem in fall 2015 ranged from 0% (Calinaro, TH-LIM-19-28) to 36% (TH-COA). The range in spring 2016 was from 0% (Calinaro, CAN-SPB, TH-LIM-19-28, Line 701 and Marathon) to 7 % (CHE-BAL-A). Similar to the head diameter, and according to the results of ANOVA (Table 2), this trait was not significantly influenced by genotype, growing season and their interactions ($p > 0.05$). The comparison of the proportion of hollow stem for each genotype across the two consecutive growing seasons showed a non-significant decrease of the proportion of hollow stems in spring compared to fall. Occurrence of hollow stem as a physiological disorder is not desirable in broccoli as it has negative effects on the shelf life. Environmental factors like rapid growth rate [31, 32], high nitrogen fertilization [33, 34] and lower plant density [35] increase the incidence of hollow stem. While the level of nitrogen fertilization was set to 300 kg ha^{-1} in both cropping periods, plant density was lower in spring season. Conversely, in the current research, the proportion of hollow stem was less in spring in comparison with fall growing season.

3.5 Fresh weight harvest index (FWHI) and marketable yield

According to Table 1, the FWHI ranged from 18% (Marathon F1) to 28% (Calinaro) in fall 2015. The range in spring 2016 was between 26% (TH-COA) and 39% (Calinaro) which was higher than fall. Outcomes of ANOVA (Table 2) showed significant effect of genotype × growing season interaction and harvest date on FWHI trait ($p = 0.0232$ and $p = 0.0478$, respectively). According to Tan et al. [19], low average minimum temperatures during the growing season lead to low biomass fresh weight but high FWHI. Also, Kaluzewicz et al. [28] stated that FWHI increased with later planting time. In the current study, the mean minimum temperatures during fall 2015 were higher than in spring 2016 during the first five weeks after transplanting (Figure 1b). Therefore, the lower air temperatures in spring could result in lower biomass weight and significantly higher FWHI in comparison to fall. We have to consider that in 2016 only spring cropping was applied, therefore the air and soil temperature was lower during day and night. Although soil temperatures have not been monitored in our study, we are sure that soils are colder in April than in July. The warmer the soil (with comparable water and air conditions) the more the soil will be mineralized. In case of the OP breeding lines "CHE-BAL-A", "CAN-SPB" and "Calinaro", significantly lower biomass weight resulted in significantly higher FWHI as the head weight was not significantly different across fall and spring. The genotype populations with higher FWHI values are useful for commercial production as they produced heavy broccoli heads in combination with low residual weight. Hence, the OP breeding lines could be good choices for cropping as they reached the highest FWHI values in both fall 2015 and spring 2016.

Results of evaluation of marketable yield of each genotype population are shown in Table 1. In growing season fall 2015, the marketable yield ranged from 4.0 t ha^{-1} (Line 701) to 15.8 t ha^{-1} (CAN-SPB). The range of marketable yield decreased in growing season spring 2016 and varied from 2.1 t ha^{-1} (TH-COA) to 8.5 t ha^{-1} (Marathon F1). All genotypes had significantly higher yield in fall 2015 compared to spring 2016. However, the yield reduction between the fall and the spring growing seasons was not significant for "Line 701" and "CHE-MIC" (Table 1). Statistical analysis (Table 2) showed significance of genotype × growing season interactions for marketable yield of broccoli heads ($p < 0.0001$). Similar to results reported by Pek et al. [37], significantly higher yields were achieved in our study during the fall growing season except for "Line 701". Our results were in line with the outcomes of Elwan and Abd-Elhamed [38] which showed higher broccoli yields in the fall compared to spring. Likewise, Tan et al. [30] observed lower yields in spring compared to fall in previous studies. The significant effect of growing season may be caused by the high dependency of broccoli yield on temperatures

[28]. Higher temperature in spring compared to fall results in a decrease of photosynthetic rate and increase in respiratory losses which may lead to yield losses [39]. Higher yields will be obtained when the temperature ranges between 15 to 25 °C during an early stage after cultivation and during the phase which proceeds to harvest [28, 40]. According to a study by Kaluzewicz et al. [28], the longer the broccoli plants are exposed to the temperatures of 15 - 25 °C, the higher the yield. The same authors found that temperatures between 25 to 30 °C during the harvest period results in lower yield. More precisely, according to previous studies and conforming to practical experiences, high temperature during the harvesting period affected the firmness of broccoli heads negatively, specifically formation of loose broccoli heads increased when the temperature rose above 18 °C [28, 36]. Similarly, we observed the negative effect of higher temperature, which resulted in loose broccoli heads in the samples of the spring growing season, hence obtained lower yield.

3.6 Head fresh weight

Generally, the experimental lines had significantly lower head fresh weight in spring in comparison to the fall season (Table 1). Tan et al. [19] reported that the overall quality of broccoli heads was mostly influenced by genotype but only slightly by the environment [29]. In this regard, we observed significant effects of genotypes on the head fresh weight in our study (Table 2). The effect of growing season was significant on this trait as well. According to Table 2, since the interaction of genotype and growing season did not affect the head weight significantly, the values across growing seasons is provided for this trait in Table 3. Comparison of the mean head fresh weight of genotypes across fall and spring growing seasons showed that the OP lines “CHE-BAL-A” and “Line 701” had significantly heavier heads than the other lines except for “CHE-GRE-G” and “CHE-MIC”. The performance of these two lines regarding head weight trait were similar to the commercial control cultivars.

Table 3. Comparison of the mean values of head fresh weight (g) of different broccoli samples across growing seasons (fall 2015 and spring 2016).

	Genotypes	Head fresh weight (g)
Commercial control cultivars	Batavia F1	316.64 [a]
	Marathon F1	289.08 [abc]
	Miranda	n.a.[1]
Experimental genotype population lines	CHE-BAL-A	300.03 [ab]
	CAN-SPB	259.54 [defg]
	Calinaro	260.82 [cdef]
	TH-COA	250.99 [defg]
	CHE-GRE-A	227.41 [g]
	CHE-GRE-G	279.74 [bcd]
	TH-LIM-19-28	249.73 [efg]
	TH-LIM-20-68	232.79 [fg]
	Line 124	247.81 [efg]
	Line 701	292.88 [ab]
	CHE-MIC	271.66 [bcde]

Means in one column followed by different letters significantly different from each other ($p < 0.05$).
[1] Not available

This research assessed the agronomic performance of OP breeding lines compared to control cultivars (hybrids and released OP) during fall 2015 and spring 2016 growing seasons as well as across both growing seasons. For the latter, correlations between traits and genotype-by-trait interactions can be seen in the biplot (Figure 2). The biplot represents 77.27% of the total variance and therefore just approximate correlations between traits or genotype-by-trait interactions. The FWHI and diameter are negatively correlated, yield and head weight are positively correlated. Biomass weight and yield showed high positive correlations as their red arrows pointing in the same direction. Batavia had positive interaction effects with yield as its projection on trait arrows is positive (in the direction of the arrow). In general, the plot shows that correlations between traits are most often low and vary from negative to positive correlations.

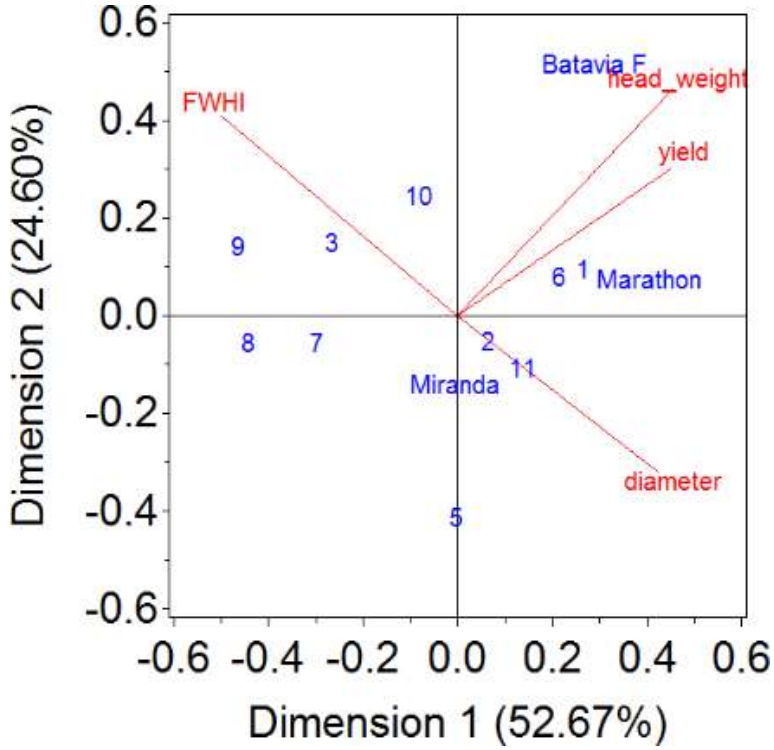

Figure 2. Biplot of genotype-by-trait means across growing seasons. Arrows denote traits, lines names denote experimental lines.

1: CHE-BAL-A, 2: CAN-SPB, 3: Calinaro, 4: TH-COA, 5: CHE-GRE-A, 6: CHE-GRE-G, 7: TH-LIM-19-28, 8: TH-LIM-20-68, 9: Line 124, 10: Line 701, 11: CHE-MIC.

Overall, the environmental conditions in growing season fall 2015 resulted in significantly higher yields, head and biomass fresh weight compared to the growing season spring 2016. The results showed that despite a large variability within the newly bred OP lines, some of the OPs already performed similar to the hybrid cultivars, frequently used in organic farming, regarding different agronomic traits such as head fresh weight, head diameter and etc. In the fall growing season, all of the OP lines showed 23% to 73% lower yields compared to the hybrid cultivars except for "CHE-GRE-G" and "CAN-SPB" which had non-significant different yield as hybrids. In the growing season spring 2016, all the OP lines showed 16% to 73% lower yield in comparison with hybrids. Considering the yield of the different broccoli lines, testing the seasonal suitability of all OP lines showed that the considered fall season was better suited for cultivation and production. Based on the expression of the different agronomic traits measured, OP lines "Line 701", "CHE-BAL-A", "CHE-GRE-G", "Calinaro" and "CAN-SPB" performed best for cultivation in the fall growing season. However, focusing on yield performance of the experimental lines only, we would like to emphasize on "CHE-GRE-G", "CAN-SPB" and "Calinaro" for cultivation in fall growing season. These lines had the highest ranking for marketable yield and proportion of marketable heads. Additionally, they had the shortest duration of harvest. Specifically, "CHE-GRE-G", "CAN-SPB" performed the best in growing season fall and the yields of these two experimental lines were even higher than those of the

control hybrid and released OP cultivars. In addition, suitable lines for the spring growing season based on general agronomic performance could be “Calinaro”, “CHE-MIC”, “Line 701” and “CHE-GRE-G”. Experimental lines “CHE-GRE-A”, “CHE-BAL-A” and “CHE-MIC” and “Line 701” show highest marketable yield and portion of marketable heads in the spring growing season. However, these lines still lack the requested head firmness. Therefore, this trait should be taken into account in further breeding.

Out of the experimental lines, “CHE-GRE-G” and “Calinaro” have been released and are being cultivated by local farmers and home gardeners. We would like to encourage the breeders that further genetic improvement of the proposed experimental lines would result in final broccoli cultivars, which are specifically bred for organic farming. However, when selecting lines for future breeding, other traits in addition to agronomic performance, such as health associated compounds (see Sahamishirazi et al. [18]) and sensory quality should be considered.

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Chapter 3. Development of a Near-Infrared Spectroscopy Method (NIRS) for fast analysis of total, indole, aliphatic and individual glucosinolates in new bred open pollinating genotypes of Broccoli (*Brassica oleracea* convar. *botrytis* var. *italica*)

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Considering the objective of our research project, determination of glucosinolates content of broccoli samples lead us to the development of a methodology research. In this context, we developed a fast analysis for glucosinolates compounds of broccoli samples by applying near-infrared spectroscopy techniques. A fast screening methodology would be beneficial especially for the breeders of the broccoli genotypes to test their most promising genotypes with regard to their glucosinolates content. For this purposes, we analyzed data which belonged to the broccoli samples of fall season 2014. Chapter 3 describes the accuracy of the implementation of NIRS methodology for the determination of individual and total glucosinolates of broccoli regardless of their genotype, through calibration with HPLC standard method. Additionally, this study was designed to obtain the relative calibration equation for further assessment of glucosinolates level of samples of the following years (fall 2015 & spring 2016) which will be described in Chapter 4.

Abstract

This study describes the development of near-infrared spectroscopy (NIRS) calibration to determine individual and total glucosinolates (GSLs) content of 12 new bred open pollinating genotypes of broccoli (*Brassica oleracea* convar. *botrytis* var. *italica*). Six individual GSLs were identified using high performance liquid chromatography (HPLC). The NIRS calibration was established based on modified partial least squares regression with reference values of HPLC. The calibration was analyzed using coefficient of determination in prediction (R^2) and ratio of preference of determination (RPD). Large variation occurred in the calibrations, R^2 and RPD due to the variability of the samples. Derived calibrations for total-GSLs, aliphatic-GSLs, glucoraphanin and 4-methoxyglucobrassicin were quantitative with a high accuracy (RPD = 1.36, 1.65, 1.63, 1.11) while, for indole-GSLs, glucosinigrin, glucoiberin, glucobrassicin and 1-methoxyglucobrassicin were more qualitative (RPD = 0.95, 0.62, 0.67, 0.81, 0.56). Overall, the results indicated NIRS has a good potential to determine different GSLs in a large sample pool of broccoli quantitatively and qualitatively.

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is an economically important vegetable. This popular crop is of value due to its abundant source of vitamins, minerals and beneficial phytochemicals, as well as its particularly strong anti-cancer sulfur-containing glucosides called glucosinolates (Wang, Gu, Yu, Zgao, Sheng & Zhang, 2012; Kushad et al., 1999). *Brassica* species are rich in glucosinolates and are a significant group of cultivated plants in the world (Rosa & Rodrigues, 2001). Epidemiological studies (Hidgon, Delage, Williams & Dashwood, 2001) have shown that the consumption of vegetables of *Brassica* species, especially broccoli and cauliflower, can possibly reduce the risk of cancer (Wang et al., 2012), because glucosinolates and their respective compounds act as cancer-chemoprevention agents (Shapiro, Fahey, Wade, Stephenson & Talalay, 2001).

Glucosinolates belong to a class of secondary plant metabolites (Rosa & Rodrigues, 2001), which are derived from an amino acid (R) and glucose (Hernandez-Hierro et al., 2012). The R substituent might be an alkyl or alkenyl side chain which itself may contain substituent sulphur or hydroxyl groups (Font, Del Rio-Celestino, Rosa, Aires & De Haro-Bailo, 2005b). Alternatively, the R substituent derives from different amino acids such as Methionin, Leucine, Iso-leucine, Valine, Tryptophan and Phenylalanin (Wang et al., 2012). The glucosinolates could be aliphatic, indolic or aromatic (Rosa & Rodrigues, 2001) depending on whether their amino acid precursor is methionine, tryptophan or an aromatic amino acid (tyrosine or phenylalanine),

respectively (Padilla, Cartea, Velasco, De Haro & Ordas, 2007). Broccoli contains mainly indole (brassicin, 1-methoxyglucobrassicin, 4-methoxyglucobrassicin) and aliphatic (sinigrin, progoitrin, glucoraphanin, gluconapin, etc.) glucosinolates (Lewis & Fenwich, 1987; Kushad et al., 1999; Baik, Juvik, Jeffery, Wallig, Kushad & Klein, 2003; Bellostas, Kachlicki, Sorensen & Sorensen, 2007; Barbieri, Pernice, Maggio, De Pascale & Fogliano, 2008; Wang et al., 2012). The indole group is categorized as the most important within glucosinolates (Rosa & Rodrigues, 2001) and glucoraphanin is considered as the major aliphatic glucosinolate (Kushad et al., 1999; Schonhof, Krumbein & Brueckner, 2004; Barbieri et al., 2008; Wang et al., 2012). Renaud et al. (2014) detected that the cultivars of broccoli which had the highest concentrations of glucoraphanin contained the lowest concentration of glucobrassicin and neoglucobrassicin.

Generally, determination of glucosinolates in plant material is done by applying various chemical methods such as standard separations, chromatographic and spectrometric methods (Schulz, 2004; Krueger & Schulz, 2007; Cozzolino, 2009) like High Performance Liquid Chromatography (HPLC), Gas-Liquid Chromatography (GLC) and Mass Spectrometry (MS) (Biston, Dardenne, Cwikowski, Marlier, Severin & Wathelet, 1988; Prestera, Fahey, Holtzclaw, Abeygunawardana, Kchinski & Talalay, 1996; Font, Del Riaeo, Fernandez-Martinaenez & De Haro-Bailo, 2004; Font, Del Rio-Celestino, Cartea & De Haro-Bailo, 2005a; Font et al., 2005b). HPLC, which is the most common way to analyze glucosinolates, proceeds by calibration of substances in a defined amount. The Reverse phase HPLC quantitative analysis of desulfurized glucosinolates is an official reference method approved by the European Union since 1990 (Matthaeus & Luftmann, 2000), established by Spinks, Sones & Fenwick (1984) and has been extensively used by many researchers since then (Chen et al., 2014). However, this method is time-consuming and expensive as the preparation of the samples implies a two-day preparation.

Near-Infrared Spectroscopy has proven to be a fast, low cost analysis method that does not require the use of hazardous chemicals (Chen et al., 2014). NIRS is being used for monitoring and assessing the composition and quality of food products. The infrared (IR) wavelength region is between the visible (VIS) and the microwave wavelengths of the electromagnetic spectrum (McClure, 2003) (wavelength: 750-2500 nm) (Huck, 2014). IR has a great potential for analytical work and is the most promising technique for molecular spectroscopy (Cozzolino, 2009).

Determination of glucosinolate content by NIR spectroscopy has been done by many researchers on different samples of *Brassica* Species, such as leaves of leaf rape (Font et al., 2005a), seeds of broccoli (Bellostas et al., 2007), seeds of Indian mustard (Font et al., 2004)

and seeds of canola (Elahi, Duncan & Stasolla, 2016); intact seeds of *Brassica* species (Velasco & Becker, 1998; Petisco, Garcia-Criado, Vazquez-de-Aldanaa, De Haro & Garcia-Ciudad, 2010), also kale (Chen et al., 2014), cabbage (Font et al., 2005b) and broccoli (Hernandez-Hierro et al., 2012 & 2014). Although, there exists quite an evidence for determining glucosinolates by NIRS within the *Brassica* species, most of the studies focused on seeds or on other species than broccoli. To our knowledge, there are two studies by Hernandez-Hierro et al. that use spectral procedures to determine glucosinolates on broccoli heads, one by using near infrared spectroscopy (2012) and the other one through near infrared hyperspectral imaging (2014). Our study, similar to the first study by Hernandez-Hierro et al. (2012), assessed glucosinolates content of broccoli heads through NIRS calibration. In contrast to that study which was only on two cultivars of broccoli, our research developed NIRS for fast analysis of glucosinolates content of 12 new bred open pollinating (OP) genotypes of broccoli (regardless of type of genotype) cultivated over fall growing season in 2014, thus offering a broad variability within the expected amount of glucosinolates due to breeding. The current study is part of a project done by the University of Hohenheim in cooperation with the organization of on-farm breeders (NGO of Kultursaat e.V.) which aimed at breeding and developing new bred OP genotypes of broccoli for organic production in Germany.

Considering the previous studies applying NIRS for different plants, this research aimed (1) to examine the potential use of NIRS methodology to determine total glucosinolates and (2) to test the accuracy of this method in predicting individual glucosinolate (GSL) profiles of broccoli heads.

2. Materials and Methods

2.1 Plant samples

This study was conducted with 100 broccoli samples. Samples were taken from plots in our field experiment. As our samples were new bred open pollinating genotypes of broccoli (Table 1), there existed an inhomogeneity between plants on each plot. In order to get along with the given variability and to get more representative samples out of each plot, each sample was prepared by a mixture of three heads per genotype for each sampling date, therefore out of 300 broccoli heads 100 samples were provided. The field trial was carried out at the organic division of the Research Station for Agriculture of the University of Hohenheim (Kleinhohenheim, Stuttgart, Germany). The altitude of the field is about 435 m above sea level. The long-term annual average precipitation and the long-term annual average temperature are 700 mm and 8.8 °C, respectively.

In order to ensure an adequate basic supply of nitrogen (N), a preceding crop of one-year clover grass was used as green manure and incorporated into the soil. The minimal nitrogen content of the soil was determined two weeks after planting and two weeks before head formation. The necessary amount of slow-release Bioilsa fertilizer (7% N, 7% P and 7% K) was applied to set the soil nitrogen content at 300 kg N ha^{-1} for broccoli growth. Freshly harvested plants of broccoli were collected at the time when the head size reached a diameter > 10 cm. Broccoli heads were cut to 18 cm length, chopped to very small pieces, immediately freeze dried with liquid nitrogen, milled into powder (1 mm) and analyzed by HPLC and NIRS.

Table 1. List of 12 new bred open pollinating genotypes of broccoli and their origin

	New bred open pollinating genotypes	Origin
Experimental genotypes	**Line Balimo**	
	CHE-BAL	Kultursaat
	CHE-LIM	Kultursaat
	Line Geba	
	CHE-GEB	Kultursaat
	Line Greenia	
	CHE-GRE	Kultursaat
	CHE-MIC	Kultursaat
	Line Calabrese	
	TH-CAN-FK	Kultursaat
	TH-CAN-FS	Kultursaat
	CHE-CAL	Kultursaat
	Calabrese-spaet	Kultursaat
	Line Atlanta	
	CHE-ATL	Kultursaat
	Line Coastal	
	CN-COA	Kultursaat
	Line Primo	
	CN-PRI	Kultursaat

2.2 Chemicals

For the HPLC, methanol (MeOH, 70%), sodium acetate, DEAE-Sephadex A-25, Imidazole format and sulfatase (1:10) were obtained from Sigma-Aldrich, Darmstadt, Germany. Glucotropaeolin (Benzylglucosinolate, Sigma-Aldrich) was also used as internal standard solution (5 mmol/l) in GSL determination by HPLC.

2.3 Near Infrared Spectroscopy (NIRS) Analysis

Near Infrared spectroscopy was applied by using a Model 5000 NIRS spectrometer (ISI Company). 2.5 to 3 mg of the freeze dried and pulverized broccoli samples were placed in a cuvette Double determination of each sample was performed by an average of two readings to reduce the sampling error. An individual spectrum was the average of 16 scans and 32 reference scans for each sample. The spectrums were obtained at each 2 nm intervals in the wavelength range of 400 to 2498 nm. Based on the analysis of each spectrum, random selected samples, out of the whole data set, were categorized into two groups in order to carry out further investigations. Specifically, the NIRS software randomly chose 30 samples (25 % of the whole data set) for validation set and the rest of the samples (70 samples) were assigned to calibration set. Validation and calibration was performed with the software WIN ISITM (Windows Infra Soft International) which, based on the study of Hernandez-Hierro et al. (2012), approved instrument control, spectra acquisition and also pretreatment and development of quantitative and qualitative models.

2.4 HPLC Analysis

Liquid chromatography was used to perform glucosinolate identification and separation. A Merck-Hitachi High Performance Liquid Chromatography system (HPLC, Darmstadt, Germany) was used for chromatographic analysis. Merck-Hitachi HPLC employed an L-7100 solvent delivery pump, an L-7200 auto-sampler, a Smartline column-holding oven (25 °C), a D-700 communicator module, and a DAD L-7450A Detector. A Phenomenex Kinetex™ 5µm C18 100 Å column (150mm length, 4.6 mm diameter) was used for glucosinolate separation. Data was analyzed using D-7000 HSM software (Darmstadt, Germany). Elution was performed with mobile phase A (water) and mobile phase B (acetonitrile). The optimum column temperature was set at 30 °C. At a flow rate of 0.4 ml/min and a detection limit of 0.5 mmol/L, the gradient conditions were set as follows: solvent A volume at 2% for 0 to 5 minutes and solvent B volume at 45% for 5 to 45 minutes. The detector monitored glucosinolates at 229 nm. Glucotropaeolin was used as an internal standard for quantitation of extraction recovery and the glucosinolates content were expressed as µmol. All samples were analyzed in duplicate.

The quantification of total and individual glucosinolate content was also performed with High Performance Liquid Chromatography (HPLC). In this study the principle of HPLC analysis was based on the European Standard of glucosinolate analysis DIN EN ISO 9167-1. The adaptation of the method for analysis of GSL in broccoli involved the modified methods of Chio et al. (2014). Initially, glucosinolates were extracted with methanol. Afterwards, extracts were purified and desulfurized with Ion-exchange method and later on were used for specific determination of the single glucosinolates (Glucoiberin, Glucoprogoitrin, Glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin) by HPLC (Figure 1). More precisely, 300 mg sample powder which were previously scanned by NIRS was mixed with 2 ml methanol (70 %) and 20 µl glucotropaeolin solution (5 mmol/l) as an internal standard. Afterwards, each solution was shaken in vortex. Thereafter, samples were incubated in a water bath at 75°C for 10 min and then cooled to room temperature and centrifuged for further 10 minutest (4800 rpm). The supernatant was decanted into 5 mL volumetric flask. This process was repeated twice for the remaining pellet, and the supernatants were pooled together. To prepare the ion exchange columns, for each obtained extract a Pasteur pipette and a snap cap glass with a volume capacity of 10 ml were required. 0.5 ml DEAE Sephadex A-25th was pipetted each time and was loaded onto the column. Then, the columns were washed with 2 ml Imidazole format solution (6 mol/l) and rinsed twice with 1 ml of double-distilled water. For desulfurization 1 ml extract was applied to the prepared column. Purification was effected with 1 ml of sodium acetate buffer twice. Then 75 µl of diluted sulfatase was applied. The pillars stood standing for 16 hours. The Desulfoglucosinolate obtained were eluted twice with 1 ml water, taken into a syringe to be transferred into a brown vial with a blue edge filter. Quantification of glucosinolate was performed by High Performance Liquid Chromatography (HPLC). In the first minute, the mobile phase consisted of 99 % distilled water and 1 % acetonitrile. Henceforth acetonitrile gradually reached 99 % within 21 minutes. The flow rate was 1 ml min^{-1} in a wavelength range of 229 nm. Each glucosinolate concentration was calculated by means of internal standard (Glucotropaelin) and was expressed as micromoles per gram of dry weight (DW).

Calibration of NIRS samples with standard method of HPLC were performed based on the method used by Hernandez-Hierro et al. (2012) through modified partial least squares regression (MPLS). According to this method, “the set of calibration samples is divided into a series of subsets in order to perform cross-validation to set the number of PLS factors, reduce the likelihood of overfitting and remove chemical outliers” (Hernandez-Hierro et al., 2012,). We decided to pick one general calibration instead of single calibrations for each genotype to

establish a fast method that shows an acceptable accuracy. In our study, six PLS factors were used for each individual glucosinolate. The PLS factors were set by the software WIN ISITM automatically. Since the calibration model uses statistics to set the PLS factor, the reason for the similarity of the PSL factors for all glucosinolates is the number of samples and factors. More precisely, equal number of samples (70) and factors (e.g. genotype and growing season) used for calibration resulted in the same number of PLS factors for all glucosinolates. Also the outliers of each glucosinolate were detected and are indicated in Table 2. The prediction ability of the calibration was determined based on the coefficient of determination in prediction, the standard error of cross-validation and the ratio of the standard deviation of the reference chemistry data to the standard error of cross-validation.

3. Results and Discussion

3.1 HPLC

Six main glucosinolates (GSLs), namely glucoiberin (GI), glucosinigrin (GS), glucoraphanin (GRA), glucobrassicin (GBS), 4-methoxyglucobrassicin (4ME) and neoglucobrassicin (NGB), were detected from the samples by means of HPLC in different retention times (Figure 1).

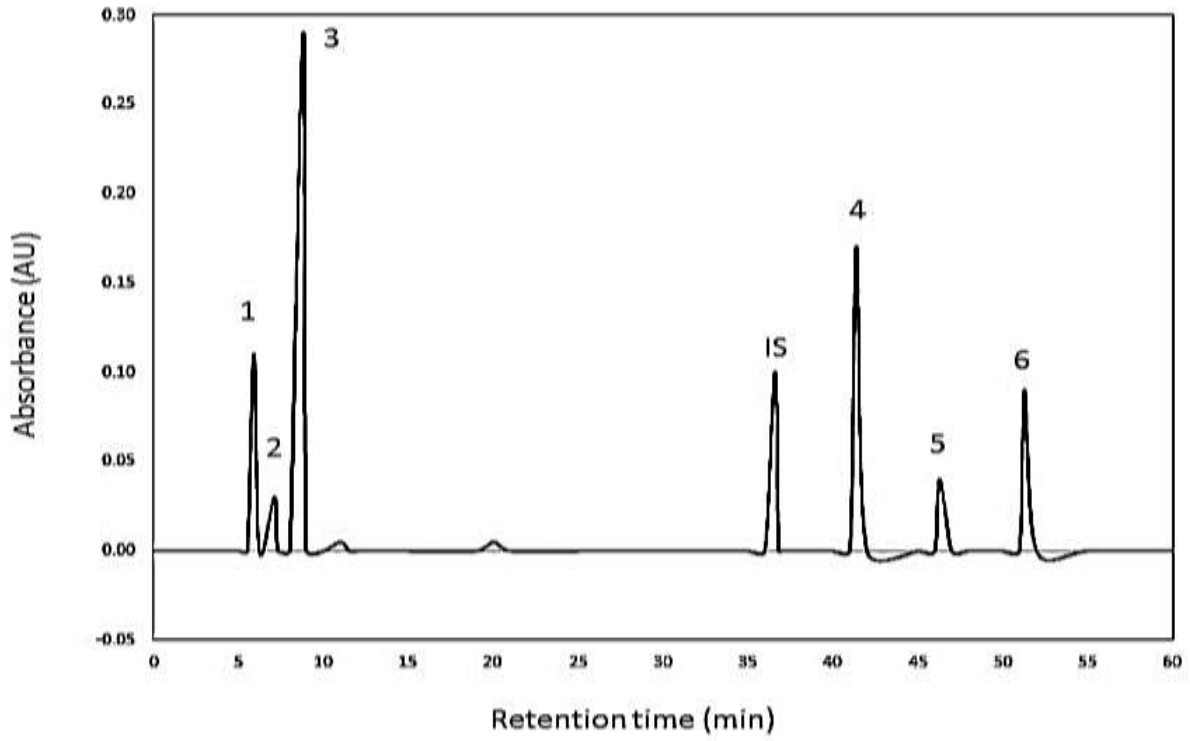

Figure 1. HPLC identification and separation chromatograph of individual qlucosinolates in 12 new bred open pollinating genotypes of broccoli monitored at 520 nm. Peaks: (1) Glucoiberin /5.9 min, (2) Glucosinigrin /7.1 min, (3) Glucoraphanin /8.8 min, (4) Glucobrassicin/ 41.4 min, (5) 4-Methoxyglucobrassicin/46.3 min, (6) 1-Methoxyglucobrassicin/ 51.3 min, (IS=Internal Standard) Glucotropaeolin/ 36.6 min.

The accuracy of the HPLC analysis is highly dependent on the concentration of each GSL in the samples (William, 1987). GRA was identified as the predominant GSL in broccoli and is valued for its powerful chemo preventive effects (Shapiro et al., 2001; Liu. Hirani, McVetty, Daayf, Quiros & Li, 2012). In agreement to previous studies, in this study, the highest amount of GSLs belonged to GRA with a mean value of 1.1 µmol g^{-1} DW which formed 34 % of the total GSLs (tGSLs). GRA ranged between 0.03 to 2.87 µmol g^{-1} DW. Followed by that, GBS had 17 % share of tGSLs with mean value of 0.55 µmol g^{-1} DW and range of 0.21 to 0.73 µmol g^{-1} DW. In our study, the share of GRA and GBS were respectively 4 % more and 25 % less in comparison with the samples of Hernandez-Hierro et al. (2012). The other constituents from the highest to the lowest amount were 4-ME, NGB, GS and GI with mean content of 0.43, 0.40, 0.37 and 0.12 µmol g^{-1} DW, respectively. The ranges were between 0.41 to 0.45 µmol g^{-1} DW for 4-ME, 0.02- 0.72 µmol g^{-1} DW for NGB, 0.36-0.37 µmol g^{-1} DW for GS and 0.2-0.36 µmol g^{-1} DW for GI. Some other single GSLs were at the limit of detection, and were finally not considered as individual GSLs due to an extremely low amount. In addition to the individual GSLs, the amount of two groups of indole and aliphatic GSLs also tGSLs were determined in this research. The indoles ranged between 0.19 to 3.16 µmol g^{-1} DW with a mean content of 1.56 µmol g^{-1} DW, aliphatics ranged from 0.21 to 4 µmol g^{-1} DW with a mean content of 1.59 µmol g^{-1} DW and the range of tGSLs was between 0.43 to 6 µmol g^{-1} DW with a mean content of 3.27 µmol g^{-1} DW.

Outcomes showed about 36 % of indole GSLs composed by GBS, 28 % by 4ME and about 26 % by NGB. In the aliphatic group, nearly 68 % of the share belonged to GRA, 24 % to GS and 8 % to GI. In general, the proportion of two groups of indole and aliphatic GSLs were relatively similar with an average composition of about 48 % and 49 % of the tGSLs, respectively (Table 2). Comparison of the range and average of GBS, GRA, NGB and tGSLs with the study of Hernandez-Hierro (2012) showed lower concentration of GSLs in the samples of our study, except for 4ME. According to the other study of Hernandez-Hierro (2014), environmental effects, variation in growing season and type of soil, type of cultivar and harvest conditions influence the concentration of GSLs in broccoli samples. Considering the effect of type of cultivars, narrow range and low concentration of individual and total GSLs of the current research could be due to the fact that the samples were experimental breeding genotypes and not commercial cultivars. Additionally, the low concentration of GSL in this study could be due to the type of the samples, which were from all parts of the broccoli head. Based on a statement of Hernandez-Hierro (2014), GSLs mostly appear to be in the external part of the broccoli florets. As well, the amounts of GRA and GBS obtained in this study were less than the reported

ones by Kushad et al. (1999), which could be also due to smaller broccoli size (10 cm diameter) and heterogeneous sample pool. Kushad et al., (1999) used 50 broccoli heads with 15-20 cm diameter, while we used 300 heads with head sizes > 10 cm. The potential longer development period to obtain bigger heads may lead to increased GSLs content in comparison with samples of our current research.

3.2 NIRS

A NIRS calibration model was set up and evaluated by means of cross-validation on 70 samples for each GSLs which is shown in Table 2. This table indicates the estimated performance of the NIRS calibration model, which comprised minimum, maximum and mean content of different GSLs and various statistical parameters such as standard deviation (SD), standard error of cross-validation (SECV), standard error of calibration (SEC), ratio of performance to deviation (RPD) which is the ratio of the standard deviation of the reference chemistry data to the standard error of cross-validation (SD $SECV^{-1}$), standard error of prediction (SEP), the coefficient of determination of cross-validation (1-VR) and the coefficient of determination in prediction (R^2).

Prediction of GSL content by using coefficient of determination (R^2) via NIRS correlations methodology (Figure 2 & Table 2) was used in this research and has been applied by different authors (Font et al., 2004/2005a/2005b; Hernández-Hierro et al., 2012). We have used R^2 and SECV to show the accuracy of our calibration. The prediction ability of the calibration models was assessed by using the RPD. The same statistics were considered by Chen et al. (2014) and Shenk & Westerhaus (1996) for evaluation of accuracy of NIRS calibration. However, according to Batten et al. (1998), the accuracy of calibration can be indicated by the achieved R^2 and SEC values. In the current study, calibration of NIRS data with HPLC for tGSLs had a coefficient of determination of cross-validation and prediction of 0.55 and 0.69, respectively. Also, the SEC and SEP were 0.9 and 1.25 µmol g^{-1} DW. The SECV was 1.17 µmol g^{-1} DW and RPD was 1.36. Comparison of the outcomes of our study regarding tGSLs content with study of Hernandez-Hierro et al. (2012) showed lower values for all statistics except for SEP and RPD, which is probably due to the broader given variation in the used cultivars and the number of cultivars we used

Table 2. NIRS calibration and validation statistics developed for determination of glucosinolate content of 12 new bred open pollinating genotypes of broccoli

	Calibration (µmol g^{-1} DW) n=70										Validation (µmol g^{-1} DW) n=30			
GSLs	**Outliers**	**PLS**	**Min**	**Max**	**Mean**	**SD**	**SEC**	**SECV**	**1-VR**	**RPD**	R^2	**SEP**	**Slope**	**Bias**
GI	8	6	0.02	0.36	0.12	0.13	0.11	0.2	0.31	0.67	0.38	0.17	1	7.4
GS	6	6	0.36	0.37	0.37	0.004	0.003	0.007	0.05	0.62	0.44	0.004	1	0.01
GRA	3	6	0.03	2.87	1.1	0.7	0.49	0.54	0.66	1.63	0.71	0.99	1.09	3.19
GBS	6	6	0.21	0.73	0.55	0.24	0.21	0.3	0.11	0.81	0.24	0.33	0.99	16.58
4ME	3	6	0.41	0.45	0.43	0.01	0.01	0.01	0.21	1.11	0.34	0.01	1	0.14
NGB	7	6	0.02	0.72	0.4	0.39	0.34	0.68	0.03	0.56	0.25	0.49	1.05	-2
Indole	4	6	0.19	3.16	1.56	0.8	0.58	0.85	0.26	0.95	0.50	0.91	1	0.43
Aliphatic	3	6	0.21	4	1.59	0.9	0.48	0.58	0.64	1.65	0.76	0.96	1	2.15
TGS	4	6	0.43	6	3.27	1.5	0.9	1.17	0.55	1.36	0.69	1.25	1	36.31

n: number of samples used for developing the NIRS calibration and validation; GI: Glucoiberin; GS: Glucosinigrin; GRA; Glucoraphanin, GBS; Glucobrassicin; 4ME; 4-methoxyglucpbrassicin; NGB; 1-methoxyglucobrassicin; TGS; total glucosinolates; PLS: Partial Least Squares regression factors; SD: standard deviation of the reference data obtained by HPLC; SECV: standard error of cross-validation; 1-VR: coefficient of determination of cross-validation; RPD: ratio of performance to deviation; R^2: coefficient of determination in prediction; SEP: standard error of prediction

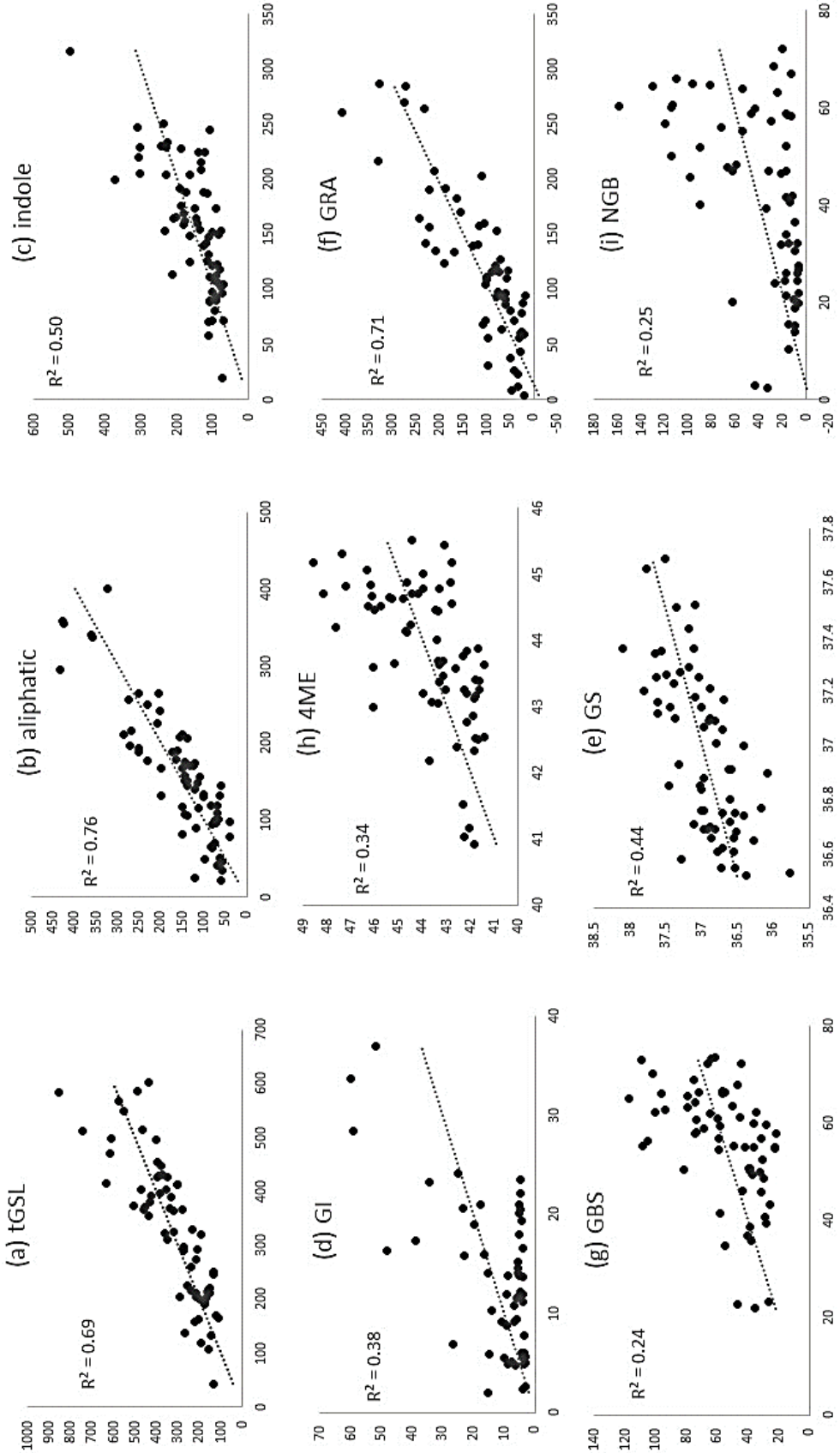

Figure 2. Calibration scatter plots (n=70), (a) total glucosinolates, (b) aliphatic glucosinolates, (c) indole glucosinolates, (d) Glucoiberin, (e) Glucosinigrin, (f) Glucoraphanin, (g) Glucobrassicin, (h) 4-methoxy-glucobrassicin, (i) 1-methoxy-glucobrassicin. Abscissa axis: predicted values of glucosinolates by NIRS (µmol 100g^{-1} DW); vertical axis: laboratory values of glucosinolates by HPLC (µmol 100g^{-1} DW).

For aliphatic GSLs results showed 1-VR= 0.64 and R^2= 0.76. There was a high correlation and prediction as the deviation between NIRS and HPLC values was low (SEC= 0.48 & SEP= 0.96 µmol g^{-1} DW) in the calibration of aliphatic groups. The SECV was 0.48 µmol g^{-1} DW and the RPD was 1.65. The calibration and cross-validation of indole GSLs showed R^2= 0.50, SEC= 0.58 µmol g^{-1} DW, SEP= 0.91 µmol g^{-1} DW and SECV= 0.85 µmol g^{-1} DW. As the difference between the NIRS and HPLC values in cross-validation analysis (SECV) was higher for indole GSLs in comparison to the aliphatic group, a lower coefficient of determination (R^2= 0.50) and prediction reliability was found for indole GSLs compared to aliphatic GSLs (RPD = 0.95).

In the study of Font et al. (2005b) R^2 of GSLs content (total and single GSLs) in the leaves of *Brassica oleracea* L. generally fluctuated from 0.77 to 0.90, while tGSLs achieved the highest R^2. Quantifications of total and single GSL of *Brassica napus* ssp. *pabularia* resulted in R^2 from 0.4 to 0.89 (Font et al., 2005a). In mustard seeds R^2 ranged between 0.33 - 0.86 (Font et al., 2004). Hernández-Hierro et al. (2012) reported the possibility of determining total and individual GSLs in broccoli with a R^2 ranging from 0.40 to 0.89 for each GSL (GBS= 0.89; GRA= 0.4; 4ME= 0.69; NGB= 0.68) and 0.73 for the tGSLs content. In our study, the R^2 of single GSLs ranged between 0.25-0.71 and were as following; GRA= 0.71, GBS= 0.24, 4ME = 0.34, NGB= 0.25, GS= 0.44 and GI= 0.38. Calibration of GSLs measurements showed SEC of 0.49 µmol g^{-1} DW for GRA and 0.21 µmol g^{-1} DW for GBS. The RPD values for GRA and GBS were 1.63 and 0.81, respectively. Furthermore, results of calibration showed SEC of 0.15 µmol g^{-1} DW for 4ME, 0.34 µmol g^{-1} DW for NGB, 0.35 µmol g^{-1} DW for GS and 0.11 µmol g^{-1} DW for GI with a RPD of 1.11, 0.56, 0.62 and 0.67, respectively.

Previous studies on different species of the *Brassica* family beside broccoli (Font et al., 2005a; Liu et al., 2006; Chen et al., 2014) revealed the successful implementation of cross-validation for evaluation of the performance of NIRS equations. Outcomes of cross-validation are shown in Table 2 and exhibit a range of RPD between 0.56 (NGB) and 1.65 (aliphatic). The low content of GSL in broccoli samples would lead to wrong reference values detected by HPLC. In the current study, the low content of GI, GS, GBS, 4-Me and NGB might cause an error during the detection process by HPLC. The consequences would appear in the diminishing correlation with spectral data of NIRS (Chen et al., 2014; Figure 2d, 2e, 2g, 2h and 2i). Specifically, as HPLC analysis is dependent to the concentration of GSLs, the extremely low concentration of GI and NGB in the samples resulted in a low accurate validation (Figure 2d and 2i). According to Williams (1987) and Font et al. (2005a), the differences shown by these ratios for the different GSLs could be explained by the fact that the SECV value is limited by the degree of correlation between HPLC measurements and NIRS predictions. Amongst all type

of GSLs, the broader range and higher R^2 shown by the tGSLs, aliphatic GSLs and GRA with respect to other GSLs led to larger accuracy of NIRS determination and a higher RDP. However, when the range is narrow and the variance in reference data is low, the values for R^2 and the RPD cannot be very high (Font et al., 2004), which is the case for the GI, GS, GBS, 4-ME and NGB.

Considering the study of Hernandez-Hierro et al. (2012), the NIRS methodology shows a good potential for determination of individual and total GSLs. Since the obtained RPD for tGSLs, aliphatic GSLs, GRA and 4ME was approximately similar to the achievements of Hernandez-Hierro et al. (2012), the performance of the calibration model was remarkable for determination of these GSLs in our study. As Oblath et al. (2016) indicated, calibrations with low RPD could be applied for quick screening of the samples to determine high or low GSL content, for the rest of the GSLs, a proper and rather qualitative calibration model was achieved as indicated by the low RPD values.

As the current study was done on 12 new bred OP genotypes, the calibration was influenced by a large variation in GSLs content which resulted in obtaining lower R^2 and RPD in comparison to the previous studies. Further, as Rosa & Rodriguez (2001) indicated that the season of cultivation and the type of cultivar have a significant impact on the GSLs content of broccoli further studies will have to evaluate, if NIRS calibrations can be improved, if individual calibrations for each cultivar are developed.

4. Conclusion

The outcomes of the present study indicated a good potential of NIRS in determining tGSLs, aliphatic GSLs and GRA in 100 samples out of 300 broccoli heads. The prediction of indole group, GBS, 4-ME, NGB, GI and GS was more qualitative. In general, the development of NIRS calibrations will allow researchers in the fields of plant breeding and health applications to quickly identify the main GSLs in broccoli without performing HPLC analysis. However, for determining tGSLs with a high accuracy HPLC analysis is necessary. Also, we recommend to run HPLC analysis on samples after NIRS screening for getting more precise results when difficulties in applying a calibration model for quantitative analysis arise. For later studies, applying NIRS calibration on samples with more homogeneity regarding cultivar and growing season may result in more accurate calibration equations. Additionally, using more than one calibration equation, also, separating calibration equations into two separate ones for higher and lower ranges as well as for individual cultivars may improve the calibration and result in a more precise analysis of a broad range of data. Finally, new bred genotypes may not be the ideal

samples to test a feasibility and accuracy of a method as the content of the compounds is affected by breeding progress. This is an ongoing project and more data will be added to the calibration on the following years.

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Chapter 4. Total and Individual Glucosinolates of Newly Bred Open Pollinating Genotypes of Broccoli (*Brassica oleracea* convar. *botrytis* var. *italica*) Grown Organically: Effect of Genotype and Growing Season

Sahamishirazi, S., Moehring, J., Zikeli, S., Fleck, M., Claupein, W., Graeff-Hoenninger, S. (2018). Total and Individual Glucosinolates of Newly Bred Open Pollinating Genotypes of Broccoli (*Brassica oleracea* convar. *botrytis* var. *italica*) Grown Organically: Effect of Genotype and Growing Season. *Journal of Agriculture and Agricultural Aspect*: JAAA-123. DOI: 10.29011/2574-2914. 000023.

Based on the obtained calibration equations in Chapter 3, the current chapter evaluates the GSLs content of the samples which were examined in the second chapter. Through this chapter we assessed the amount of individual and total GSLs and addressed the effect of genotype, growing season and their interaction on GSLs content within and across growing seasons. We planned to select OP genotypes with high concentration of GSLs which are stable across different growing seasons.

Abstract

Considering the demand for broccoli cultivars suitable for organic production and the prohibition of cultivating CMS-F1 hybrid cultivars under organic farming condition, current study evaluated glucosinolate content of eleven newly bred open pollinating genotypes of broccoli by comparison with F1 hybrid cultivars over two growing seasons. Effect of genotype, growing season and their interaction on glucosinolates was assessed as well. The results indicated the determination of six individual glucosinolates including glucoiberin, glucosinigrin, glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Glucoraphanin was the major glucosinolate with the largest share in total-glucosinolates (more than 70%) and significantly higher concentration in fall. Total-glucosinolates and glucoraphanin ranged from 3.46 to 3.60 µmol g^{-1} DW and 1.44 to 1.69 µmol g^{-1} DW, respectively. We observed significant reduction in concentration of glucoraphanin, glucoiberin, 4-methoxyglucobrassicin and neoglucobrassicin in all genotypes in spring compared to fall growing season as the result of growing season significant effect. In contrast, glucobrassicin content of open pollinating genotypes was mostly stable across growing seasons. The genotype × growing season interaction did not affect the concentration of glucosinigrin and total-glucosinolates. Genetic factor affected the concentration of all glucosinolates significantly and resulted in differences in individual glucosinolates content of open pollinating genotypes and F1 hybrid cultivars. However, the level of total-glucosinolates of newly bred open pollinating genotypes was similar to F1 hybrid cultivars (3.46 - 3.60 µmol g^{-1} DW). A study on the agronomic performance of the open pollinating genotypes supplements the outcomes of this study and helps breeders and farmers to select the promising genotypes.

1. Introduction

In relation to the potential prevention of cancer and other diseases, species of the *Brassica* family are often in focus. Broccoli (*Brassica oleracea* L. var. *italic*) is considered as an important vegetable with health-promoting properties [1]. It is a cool-season crop, which is grown in temperatures ranging from 15 to 18°C [2]. Moreover, it is a favorite vegetable, consumed mostly cooked in Germany [3]. The composition of broccoli is 88.5% water, 3.8% protein, 0.2% fat, 2.7% available carbohydrates, 3.0% dietary fibers and 1.1% minerals [4]. On average, per 100 g of broccoli 58 mg of calcium, 15 µg of iodide, 459 µg of zinc and 700 ng of selenium are present. Additionally, broccoli contains vitamin C (94 mg $100g^{-1}$), folic acid (114 µg $100g^{-1}$) and many antioxidant compounds, such as carotenoids, tocopherols and Glucosinolates (GSLs) [4]. Including a high portion of *Brassica* species in diets showed a great reduction in the risk of some diseases like cancer [5]. High GSLs contents and their respective compounds, which derived from an amino acid and glucose [6], act as cancer-chemoprevention agents [7]. Depending on the type of the amino acid; methionine, tryptophan and phenylalanine [8], the GSLs can be divided into three classes of aliphatic, indole and aromatic [9], respectively. Broccoli mainly contains indole and aliphatic GSLs [4]. The concentrations of aliphatic GSLs are mostly affected by genotype while the concentration of indoles is more affected by environment and genotype × environment interactions [10,11].

Due to current horticultural practices, broccoli cultivars that are on the market are almost exclusively F1 hybrid [12]. In organic production, due to the restrictions of the principles-according to the rules of International Federation of Organic Agriculture Movements (IFOAM)-, it is forbidden to develop F1 hybrid by using Cytoplasmic Male Sterility (CMS) [13]. Therefore, developing new Open Pollinating (OP) cultivars could be in favor of organic farming since it gives the farmers the possibility to produce their own seeds for reproduction [12]. OP cultivars are less homogeneous and differ from F1 hybrids in terms of composition [14]. Often, they are expected to contain higher amounts of health-benefitting secondary plant metabolites (such as glucosinolates, phenolics and flavonoids) compared to hybrid cultivars [13,15].

Based on the information given above, we conducted a research study on the GSL composition of newly developed OP genotypes of broccoli, which were specifically bred for organic production (through on-farm breeding and single plant selection). Our current study is part of a larger project on the development of new OP cultivars of broccoli for organic farming in Germany. In this regard, we conducted two experiments during the 2015 fall growing season and during the 2016 spring growing season. We evaluated the results statistically over two

different seasons to express the impact of growing season on the performance of the newly bred lines as well as the GSL pattern. Doing so, we were able to test the stability of the tested cultivars over the two growing seasons. We determined the GSL content of eleven OP genotypes and compared them with commercial control cultivars. In addition, we tested the effect of head weight, genotype and genotype × growing season interaction on GSLs content within and across growing seasons. Finally, we intended to select genotypes for the different growing seasons (fall and spring) based on their GSL content.

2. Materials and Methods

2.1 Plant Materials and Field Trials

Three commercial cultivars (F1 hybrids: "Batavia" and "Marathon", released OP: "Miranda") and eleven newly bred OP genotypes of broccoli (experimental lines) were our plant materials (listed in Table 2). The field trials were done under organic farming practices during fall growing season 2015 and spring growing season 2016 at the organic research station of the University of Hohenheim (Kleinhohenheim), Stuttgart, Germany (alt. 435 m, lat. 48.7, long. 9.2, long-term annual average precipitation and temperature 700 mm and 8.8°C). The soil type was sandy-loamy-clay. Broccoli seeds of fall and spring experiments were sown on July 10th, 2015 and March 21st, 2016, respectively. The seeds were pre-germinated in a greenhouse for two days at 18°C. Afterwards they were placed in another chamber of the greenhouse for further germination and grown at the same daily temperature matching that of the outdoors for 3-4 weeks. Seedlings were transplanted in the field at the stage of 3-4 true leaves and 10 cm stem length, 25 and 35 days after sowing for fall and spring experiments, respectively.

In order to ensure an adequate basic supply of Nitrogen (N) in the field, a preceding crop of one-year clover grass was used as green manure and incorporated into the soil. The nitrogen content of the soil was determined two weeks before planting. Soil samples were taken from two different depths (30 cm and 60 cm) and the nitrogen content was determined according to the $CaCl_2$ extraction method by the Association of German Agricultural Research and Research Institutes (VDLUFA). We applied necessary amount of slow-release Maltaflor fertilizer (5% N, 5% P and 5% K) to the field in order to keep the nitrogen content at 300 kg N ha^{-1}. The plants were covered by crop protection nets ((S48), with mesh sizes of 0.8×0.8 mm²), to protect against flea beetles (*Pyllotreta* ssp.) and swede midge (*Contarinia nasturtii*) until the first harvest. Irrigation was done directly after transplanting on 26.04.2016 (10 l m^{-2}) and on 20.05 2016 (15 l m^{-2}). In the 2015 fall growing season, the average daily temperature decreased from 22°C, at the transplanting time in August, to 7°C at the end of harvest in November. Throughout

the 2016 spring season, the average daily temperature increased from 9°C to 20°C during April to July (from transplanting to the end of harvest). The average daily air temperature values were higher in fall season than spring season during the stages of growth and head formation up to the beginning of harvesting. At the time of harvesting broccoli heads in spring, the temperature was higher in comparison with fall growing season. The sum of precipitation was much higher in spring 2016 in comparison to fall 2015 over growing season with noticeable amount in the fifth and the seventh week after transplanting. Regarding the average relative humidity, the range was similar for both seasons from 60 % to 90 %, although the changing trend of the relative humidity during both seasons was different based on the amount of precipitation.

Harvesting of the fall and spring experiment was done between 63-103 and 51-72 days after planting, respectively (Table 1). During harvest time, plots were visited regularly. On each assessment date, three individual heads (which were representative for the whole plot) were picked for further analysis of GSL contents. Overall, each plot was assessed three to five times. To account for spatial trends in the field, the experimental design of the fall experiment was a randomized complete block design with three replicates, 14 plots per replicate. For the spring growing season 2016, planting direction and height gradient were orthogonal (due to slope of the field), therefore plants were arranged in a resolvable row-column design, which allowed to account for trends in both directions [16]. Again, plots were arranged in 14 rows and 3 columns, where a column corresponds to a complete replicate.

Table 1: Harvesting period of broccoli heads in fall 2015 and spring 2016.

Growing season	Harvesting period	Harvesting window	Sampling interval
Fall 2015	07.10.-16.11.	6 weeks	7 times
Spring 2016	15.06.-06.07.	3 weeks	4 times

2.2 Sample Preparation

At each harvest, three broccoli heads were harvested fresh from each plot. The indicator of harvest was a head diameter of >10 cm. The stem was detached, and the heads were halved for sampling. The half heads were chopped into very small pieces and were placed into four bottles. Afterwards, they were frozen with liquid nitrogen, freeze-dried for one week, milled into 1 mm powder, stored at -20°C and finally mixed to one composite sample per plot per harvest. To analyze the GSL content, the samples were prepared similar to our previous study, Sahamishirazi et al. [17].

2.3 Glucosinolates Determination

The amount of total and individual GSLs was determined according to our former study by Near Infrared Spectroscopy (NIRS) [17]. NIRS was done using NIRS Model 5000 NIRS

spectrometer (ISI Company, Germany). The amount of GSLs content was measured as previously described by Hernandez-Herrero et al. [6] and Sahamishirazi et al. [17]. The spectrums were obtained in the wavelength range of 400 to 2498 nm using the WIN ISI™ (Windows Infra Soft International, Germany).

2.4 Statistical Analysis

The data of both experiments was analyzed using a two-stage mixed model approach [19,20]. This approach accounts for all specifics of each experiment in stage one and calculates the means across growing seasons in stage two. The approach allows the handling of different designs in different trials while producing nearly identical results. For both experiments, the least square means of the genotype-by-harvest time from the first stage were estimated and subjected to an across-growing season analysis with the following second stage model:

$$\hat{\mu}_{ijn} = \mu + g_i + a_n + h_{ln} + (ga)_{in} + (gh)_{jln} + f_{ijn} \quad (1),$$

where μ is the general intercept, g_i, a_n and h_{jn} are the fixed main effects of the i^{th} genotype, n^{th} growing season and j^{th} harvest time within growing season n, respectively. Note that the effect of growing season is a confounded effect of experiment, year (2015 and 2016) and season (fall and spring). $(ga)_{in}$ and $(gh)_{jln}$ are assumed as random interaction effects between the i^{th} genotype and the n^{th} growing season or the i^{th} genotype and the j^{th} harvest time within growing season n, respectively. f_{ijn} are the error effects estimated in the first stages for genotype-by-harvest time means $\hat{\mu}_{ijn}$. To use error effects from the first stages, Smith weights [21] were calculated using a SAS macro [20]. We estimated both genotype main effects and genotype-by-growing season means from equation (1). Residuals were tested graphically for normality and homogeneity of variance. No means of across growing seasons for cultivar "Miranda" were calculated, as this cultivar did not produce any heads in spring 2016. After finding significant differences via F-test, a multiple t-test with □ =0.05 was used to compare genotype means within or across growing seasons. Note that we also tried to extend the analysis of the first stage by adding a co-variable head weight, but it was non-significant for all traits. The rationale for adding this co-variable is that we want to avoid differences in head weight as reasons for differences in the content of glucosinolates. All statistical analysis was determined using SAS version 9.4.

3. Results and Discussions

Determination of GSL contents resulted in detection of six individual GSLs including three aliphatic (glucoiberin: GI, glucosinigrin: GS, glucoraphanin: GRA) and three indoles

(glucobrassicin: GBS, 4-methoxyglucobrassicin: 4ME, neoglucobrassicin: NGB) similar to the study of Fachmann et al. [4]. The complete information on GSL contents of cultivars and genotypes of this study are provided in Table 2. Total glucosinolates (tGSLs) of each genotype, which is the sum of their individual GSLs, are also listed in the same table. In spring 2016, "Miranda" did not produce proper heads, which could have been the result susceptibility to high temperature at the time of head formation. Therefore, the concentrations of total and individual glucosinolates are not available for this cultivar. In line with the findings of Charron et al. [22] and Renaud et al. [15], in our study GRA, GBS and NGB were the dominating GSLs in all broccoli genotypes of both growing seasons. The proportions of the dominant individual GSLs in tGSLs were: GRA 36 to 41 %, GBS 16 to 19 % and NGB 16 to 18 %. In fall 2015 as well as spring 2016, the share of aliphatic GSLs in the samples were mostly higher than the indole ones. However, in spring the shares of the dominant GSLs in tGSLs were lower (GRA 31 to 35 %, GBS 19 to 24 % and NGB 12 to 15 %).

Table 2. Comparison of individual and total glucosinolates content (µmol g^{-1} DW) and head weight (g) of broccoli samples in: a) fall growing season 2015, b) spring growing season 2016.

a) 2015	Genotypes	GI [1]	GS [2] *	GRA[3]	GBS [4]	4-ME [5]	NGB [6]	tGSLs [7] *	Head weight
Commercial control cultivars	Batavia F1	0.1956 $^{a\ A}$	0.3683	1.69 $^{a\ A}$	0.69 $^{bd\ A}$	0.4557 $^{cd\ A}$	0.67 $^{cd\ A}$	4.06	358.67 ± 11.97
	Marathon F1	0.1949 $^{ab\ A}$	0.3670	1.64 $^{abc\ A}$	0.68 $^{bd\ A}$	0.4518 $^{d\ A}$	0.64 $^{de\ A}$	3.98	317.68 ± 13.74
	Miranda	0.1492 $^{e\ A}$	0.3660	1.44 $^{f\ A}$	0.74 $^{ab\ A}$	0.4615 $^{b\ A}$	0.71 $^{ac\ A}$	3.86	275.67 ± 13.42
Experimental open pollinating genotypes	CHE-BAL-A	0.1731 $^{cd\ A}$	0.3658	1.56 $^{cde\ A}$	0.70 $^{bd\ A}$	0.4632 $^{b\ A}$	0.7 $^{ac\ A}$	3.96	312.51 ± 11.97
	TH-CAN-SPB	0.1633 $^{de\ A}$	0.3685	1.51 $^{ef\ A}$	0.70 $^{bd\ A}$	0.4615 $^{b\ A}$	0.7 $^{ac\ A}$	3.9	273.63 ± 12.22
	Calinaro	0.1789 $^{ad\ A}$	0.3659	1.57 $^{bde\ A}$	0.70 $^{bd\ A}$	0.4630 $^{b\ A}$	0.69 $^{bc\ A}$	3.96	274.63 ± 11.86
	TH-COA	0.1937 $^{ab\ A}$	0.3660	1.64 $^{abc\ A}$	0.66 $^{cd\ A}$	0.4705 $^{a\ A}$	0.69 $^{bc\ A}$	4.02	272.87 ± 12.8
	CHE-GRE-A	0.1738 $^{bcd\ A}$	0.3677	1.55 $^{cde\ A}$	0.73 $^{ab\ A}$	0.4608 $^{b\ A}$	0.70 $^{ac\ A}$	4.01	250.22 ± 11.38
	CHE-GRE-G	0.1802 $^{ad\ A}$	0.3658	1.56 $^{bde\ A}$	0.68 $^{bd\ A}$	0.4631 $^{b\ A}$	0.70 $^{ac\ A}$	3.95	305.5 ± 12.51
	TH-LIM-19-28	0.1978 $^{a\ A}$	0.3668	1.66 $^{ab\ A}$	0.65 $^{d\ A}$	0.4600 $^{bc\ A}$	0.64 $^{de\ A}$	3.98	276.41 ± 12.26
	TH-LIM-20-68	0.1807 $^{ad\ A}$	0.3658	1.60 $^{ad\ A}$	0.65 $^{d\ A}$	0.4642 $^{b\ A}$	0.70 $^{bc\ A}$	3.96	255.02 ± 11.53
	Line 124	0.1860 $^{ac\ A}$	0.3670	1.53 $^{df\ A}$	0.65 $^{d\ A}$	0.4601 $^{bc\ A}$	0.63 $^{e\ A}$	3.83	253.3 ± 10.78
	Line 701	0.1599 $^{de\ A}$	0.3656	1.54 $^{cde\ A}$	0.73 $^{abc\ A}$	0.4642 $^{b\ A}$	0.73 $^{ab\ A}$	3.99	328.46 ± 14.75
	CHE-MIC	0.1487 $^{e\ A}$	0.3638	1.46 $^{ef\ A}$	0.77 $^{a\ A}$	0.4634 $^{b\ A}$	0.74 $^{a\ A}$	3.94	294.95 ± 12.69

b) 2016	Genotypes	GI [1]	GS [2] *	GRA[3]	GBS [4]	4-ME [5]	NGB [6]	tGSLs [7] *	Head weight
Commercial control cultivars	Batavia F1	0.1298 $^{ab\ B}$	0.3711	1.03 $^{ab\ B}$	0.65 $^{bc\ A}$	0.4307 $^{ef\ B}$	0.41 $^{bc\ B}$	3.04	274.61 ± 15.28
	Marathon F1	0.1306 $^{ab\ B}$	0.3710	1.01 $^{ab\ B}$	0.64 $^{bc\ A}$	0.4360 $^{ce\ B}$	0.42 $^{bc\ B}$	2.98	260.49 ± 18.88
	Miranda	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	No heads
Experimental open pollinating genotypes	CHE-BAL-A	0.1213 $^{bc\ B}$	0.3704	1 $^{ab\ B}$	0.69 $^{ab\ A}$	0.4330 $^{def\ B}$	0.44 $^{ab\ B}$	3.09	287.55 ± 16.24
	TH-CAN-SPB	0.1324 $^{ab\ B}$	0.3707	1.09 $^{a\ B}$	0.64 $^{bc\ A}$	0.4421 $^{ac\ B}$	0.42 $^{ac\ B}$	3.07	245.45 ± 17.65
	Calinaro	0.1307 $^{ab\ B}$	0.3705	1.05 $^{ab\ B}$	0.73 $^{a\ A}$	0.4269 $^{f\ B}$	0.42 $^{ac\ B}$	3.12	247 ± 15.76
	TH-COA	0.1029 $^{c\ B}$	0.3699	0.96 $^{ab\ B}$	0.74 $^{a\ A}$	0.4480 $^{a\ B}$	0.48 $^{a\ B}$	3.07	229.11 ± 16.95
	CHE-GRE-A	0.1338 $^{ab\ B}$	0.3710	1.05 $^{ab\ B}$	0.63 $^{bc\ B}$	0.4346 $^{ce\ B}$	0.4 $^{bc\ B}$	3.04	204.59 ± 15.26
	CHE-GRE-G	0.1496 $^{b\ B}$	0.3716	1.07 $^{a\ B}$	0.63 $^{bc\ A}$	0.4324 $^{ef\ B}$	0.38 $^{c\ B}$	3.01	253.98 ± 15.25
	TH-LIM-19-28	0.1239 $^{bc\ B}$	0.3713	1.01 $^{ab\ B}$	0.63 $^{bc\ A}$	0.4396 $^{bcd\ B}$	0.4 $^{bc\ B}$	2.96	223.04 ± 16.31
	TH-LIM-20-68	0.1361 $^{bc\ B}$	0.3714	1.02 $^{ab\ B}$	0.6 $^{c\ A}$	0.4448 $^{ab\ B}$	0.41 $^{bc\ B}$	2.97	210.57 ± 15.75
	Line 124	0.1283 $^{bc\ B}$	0.3714	0.94 $^{b\ B}$	0.68 $^{ab\ A}$	0.4341 $^{cf\ B}$	0.4 $^{bc\ B}$	2.92	242.33 ± 15.74
	Line 701	0.1150 $^{ac\ B}$	0.3698	1.07 $^{ab\ B}$	0.75 $^{a\ A}$	0.4353 $^{ce\ B}$	0.48 $^{a\ B}$	3.20	257.29 ± 17.79
	CHE-MIC	0.1238 $^{bc\ B}$	0.3704	1.05 $^{ab\ B}$	0.7 $^{ab\ B}$	0.4348 $^{ce\ B}$	0.43 $^{ab\ B}$	3.08	248.38 ± 16.4

[1]GI: Glucoiberin, [2]GS: Glucosinigrin, [3]GRA: Glucoraphanin, [4]GBS: Glucobrassicin, [5]4ME: 4-methoxyglucobrassicin, [6]NGB: 1-methoxyglucobrassicin, [7]tGSLs: total glucosinolates.
Means with the same letters were not significant ($p < 0.05$). Lowercase letters: comparison of genotypes within one year; Uppercase letters: comparison based on years within one genotype; n.a.: not available
* no letter display was created simple means for this variable, as the marginal means of genotypes across growing seasons should be compared(see Table 4)

To analyze the main effects (growing season, genotype, head weight) and their interactions (genotype × growing season), the output of the mixed model analysis for different GSLs are resented in Table 3. According to this table, the content of total and individual GSLs generally differed with growing season except for GBS. Variation due to genotype effect was significant for all individual GSLs and tGSLs, which is consistent with the results of Rosa and Rodrigues [23], Vallejo et al. [24], Schonhof et al. [25], Farnham et al. [11] and other former studies [10,15,32].

Table 3: Results of analysis of variance for the individual and total glucosinolates content.

Effects	**GI [1]**	**GS [2]**	**GRA[3]**	**GBS [4]**	**4-ME [5]**	**NGB [6]**	**tGSLs [7]**
Growing season	***	***	***	*NS*	***	***	***
Genotype	**	*	*	***	***	***	*
Genotype × Growing season	*	*NS*	*	*	**	*	*NS*

[1]GI: Glucoiberin, [2]GS: Glucosinigrin, [3]GRA: Glucoraphanin, [4]GBS: Glucobrassicin, [5]4ME: 4-methoxyglucobrassicin, [6]NGB: 1-methoxyglucobrassicin, [7]tGSLs: total glucosinolates.
*NS= non-significant; *, **, *** significant at a ≤ 0.05, 0.01, 0.001 by ANOVA*

Since a lower level of GSLs content was observed in broccoli samples of spring season compared to fall season - similar to the results of Renaud et al. [15], the interaction of genotype × growing season was evaluated to check the possible effects. The effect was significant on all individual GSLs except GS and tGSLs. The interaction between the genotype and growing season illustrated the dependency of the relative performance of genotypes on the growing season or the dependency of difference between the growing seasons and the genotype. To test whether the weight of broccoli head has significant effects on GSLs content, the effect of head weight was evaluated on individual and total GSLs.

The results showed that none of the individual and total GSLs were influenced by head weight. In this regard, our findings were in line with the study of Farnham et al. [11] who reported no correlation between head weight and GSLs content of their broccoli samples. However, it was in contrast with the statement of Renaud [27] on the positive correlation between head weight and GRA. Similar to the study of Farnham et al. [11] and in contrast to the findings of Rosa and Rodriguez [23] our results indicated no dilution effect on GSL content of broccoli samples.

Glucoraphanin: Mainly, GRA represented the largest percentage of GSLs in broccoli, between 50 % and 80 % of total GSLs, therefore, it is considered as the key GSL [6,23,24,28,29,30,31,32]. Generally, concentrations of GRA in the broccoli samples of our study were similar to the amount of GRA of some experimental lines found by Vallejo et al.

[23], some accessions tested by Kushad et al. [33] and in range with the GRA content of the study of Wang et al. [5]. GRA formed more than 70 % of the aliphatic glucosinolates in the samples of the fall and spring growing seasons. GRA content is greatly influenced by genotype [15,37] and less affected by environment and genotype × environment [37] since genetic factor is important in phenotypic expression of GRA [10]. In this study, in addition to the effect of genotype, we found significant effects of growing season and genotype × growing season interaction on GRA content of our broccoli samples. In fall, among the OP genotypes, GRA ranged from 1.46 µmol g^{-1} DW (CHE-MIC) to 1.66 µmol g^{-1} DW (TH-LIM-19-28). In this season, the GRA concentration of experimental genotypes was significantly lower when compared to the commercial cultivars, except for "TH-LIM-19-28", "TH-LIM-20-68" and "TH-COA". All commercial cultivars and experimental genotypes had significantly lower GRA content in spring 2016 compared to fall 2015 (Tables 2a and 2b). In the spring growing season (Table 2b), GRA ranged from 0.94 µmol g^{-1} DW (Line 124) to 1.09 µmol g^{-1} DW (TH-CAN-SPB) among experimental genotypes. There were no significant differences between the commercial cultivars and the experimental genotypes except between "CHE-GRE-A", "TH-CAN-SPB" and "Line 124".

Glucobrassicin: Up to 75 % (in fall) and 45 % (in spring) of indole glucosinolates belonged to the sum of GBS and NGB. In fall 2015 (Table 2a), "CHE-MIC" had significantly higher GBS contents than the tested commercial ones and all OP genotypes except "CHE-GRE-A" and "Line 701". In spring 2016, only "CHE-GRE-A" and "CHE-MIC" had significantly lower GBS contents when compared to fall 2015 (Tables 2a and 2b). "Calinaro", "TH-COA" and "Line 701" had significantly higher GBS contents than commercials. Similar to the outcomes of Renaud et al. [15], our findings showed that the level of GBS in OP genotypes tended to be higher than in hybrids. The comparison of the concentration of GBS of our samples with previous studies showed a lower level of GBS in samples of both growing seasons compared to the study of Vallejo et al. [24], Charron et al. [22] and Renaud et al. [15]. The lower concentration of GBS could be due to a higher level of GRA [15]. GBS was not significantly affected by growing season therefore its concentration was stable across growing seasons in most of the genotypes.

Neoglucobrassicin: NGB ranged from 0.63 µmol g^{-1} DW (Line 124) to 0.74 µmol g^{-1} DW (CHE-MIC) in fall 2015 (Table 2a). "Line 124" had a significantly lower concentration of NGB compared to the most of the samples in fall 2015. All of the commercial cultivars and the experimental genotypes had significantly lower NGB contents in spring 2016 compared to fall 2015 (Table 2a and 2b). In spring 2016 (Table 2b), NGB ranged from 0.38 µmol g^{-1} DW (CHE-

GRE-G) to 0.48 µmol g^{-1} DW (Line 701 and TH-COA). "TH-COA" and "Line 701" had significantly higher content of NGB than both commercial cultivars. The NGB contents of our samples were in range of the amount reported by Vallejo et al. [24]. Since, indole GSLs content is mostly influenced by environment rather and genotype × environment rather than genotype effects [37], differences in NGB content of the samples could be explained by different environmental conditions due to significant effect on regulating indole GSLs expression [10]. This could describe the higher NGB content of our samples compared to the work of Kushad et al. [33]. Moreover, different growing locations influence the content of GSL due to differences in nitrogen fertilizers, type of soil, spaces between plants and harvest date [33,34,35,36]. The rest of individual glucosinolates were available in smaller quantities in all genotypes and both growing seasons (Table 2a and 2b).

4-Methoxyglucobrassicin: The concentration of 4ME was in line with the amount and ranges previously reported in other studies [5,7,22,24,33]. All the commercial cultivars and the experimental genotypes had significantly lower 4ME content in spring 2016 compared to fall 2015 (Tables 2a and 2b). The concentration of 4ME was significantly higher in "TH-COA" within fall and spring growing season compared to the other experimental lines and commercial cultivars.

Glucoiberin: Effect of genotype is high on synthesis of aliphatic GSLs due to its significant effect on regulating aliphatic indole GSLs expression [10,37]. Therefore, in contrast to the study of Charron et al. [22], we could detect GI in broccoli samples of our study. GI levels of both seasons were in agreement with the outcomes of Wang et al. [5]. In fall 2015, GI ranged from 0.15 µmol g^{-1} DW (CHE-MIC) to 0.20 µmol g^{-1} DW (TH-LIM-19-28). In the same growing season, the concentration of GI was significantly lower in "CHE-MIC" compared to other OP genotypes except "Line 701". The range of GI in spring decreased to 0.10 µmol g^{-1} DW (TH-COA) and 0.14 µmol g^{-1} DW (CHE-GRE-G). All the commercial cultivars and the experimental genotypes had significantly lower GI contents in spring 2016 compared to fall 2015 (Tables 2a and 2b). This could be due to higher temperature at the time of harvesting in the spring growing season. According to Rosa and Rodriguez [23], higher temperatures cause the increase of degradation of GSLs, hence reducing their concentrations in samples through stimulating myrosinase activity.

Glucosinigrin: Since the interaction of genotype and growing season did not affect the concentration of GS significantly, the level of this GSL across growing seasons is provided in Table 4 in which no differences in concentration of GS between the OP genotypes and F1 hybrid

cultivars is observed. The levels of GS content of the current study were similar to the outcomes of Wang et al. [5].

Total Glucosinolates: Production of broccoli under organic farming affected the GSLs content of broccoli heads negatively. Studies showed lower GSLs level in organic broccoli compared to conventionally grown broccoli due to the optimum production conditions in conventional farming [38]. The comparison of the tGSLs content of the broccoli genotypes of the current study with former studies [7,24, 29,30] showed our findings were in line with the ranges achieved by the previous researchers. The outcomes of GSLs determination showed no significant differences between the tGSLs content of each genotype within both growing seasons (Tables 2a and 2b). However, since there was a significant genotype main effect, tGSLs of genotypes were significant across both seasons (Table 4). “Line 124” had significantly lower tGSLs content value compared to both F1 hybrid cultivars and other OP genotypes except for “CHE-MIC” and “TH-CAN-SPB”. In addition to the effect of genotype, climatic conditions could have affected the concentration of tGSLs by influencing the stimulation of myrosinase activity [23]. Other factors such as soil fertilization [39] also showed positive impacts on GSL content of *Brassica* vegetables

Table 4: Comparison of broccoli genotype main effects across two consecutive seasons (fall 2015 and spring 2016) for the mean concentration of glucosinigrin and total glucosinolates (µmol g^{-1} DW).

	Genotypes	Glucosinigrin	Total glucosinolates
Commercial control cultivars	Batavia F1	0.3697 [b]	3.55 [a]
	Marathon F1	0.3690 [ab]	3.48 [ab]
	Miranda	n.a.	n.a.
Experimental open pollinating genotypes	CHE-BAL-A	0.3681 [bc]	3.53 [ab]
	TH-CAN-SPB	0.3695 [b]	3.49 [abc]
	Calinaro	0.3682 [bc]	3.54 [a]
	TH-COA	0.3679 [bc]	3.55 [a]
	CHE-GRE-A	0.3693 [ab]	3.52 [a]
	CHE-GRE-G	0.3686 [bc]	3.48 [ab]
	TH-LIM-19-28	0.3690 [ab]	3.47 [ab]
	TH-LIM-20-68	0.3686 [bc]	3.46 [ab]
	Line 124	0.3692 [ab]	3.37 [c]
	Line 701	0.3677 [abc]	3.60 [a]
	CHE-MIC	0.3670 [bc]	3.51 [ab]

Means with the same letters were not significant ($p < 0.05$).
n.a.: not available

4. Conclusion

Six individual GSLs were detected in the broccoli samples of this study. Among them, GRA, GBS and NGB were the main individual GSLs. There was a similar range of total and individual GSLs contents among the experimental genotypes and the commercial cultivars. We observed a significant effect of genotype on all individual GSLs and tGSLs contents of our broccoli samples. The interaction of genotype × growing season was significant on all indole GSLs, the main aliphatic GSL and GI. Generally, the GSLs content of the samples was higher when broccoli was cultivated in the fall growing season; however, the difference in the level of GSLs contents across seasons was significant only for GRA, NGB, 4Me and GI. Marketable head weight of broccoli genotypes showed no significant effect on GSL content of our samples. The OP genotypes performed similar to the F1 hybrid cultivars considering the content of tGSLs. Since the concentration of GSLs in the OP genotypes were mostly in the same ranges in each

growing season, selection of specific genotypes was not noteworthy. A study on the agronomic performance of the genotypes supplements the outcomes of this study and helps breeders and farmers to pick genotypes, which perform well in both yield and quality.

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Chapter 5. General discussion

As it was initially represented, organic farming is loaded with the varieties obtained from conventional breeding (Lammert van Beuren et al., 2011). These varieties are bred for high input conditions and lack special traits when cultivated in organically low input environments (Wolfe et al., 2008; Murphy et al., 2007; Lammert van Beuren et al., 2002). For example, cultivating varieties adapted to conventional high input conditions yielded lower under organic production conditions (Navazio & Zystro, 2014). Therefore, there is a need to breed specific varieties organically. Furthermore, the existence of some restrictions in organic rules regarding the limitations of reproducing hybrids organically (IFOAM, 2014), emphasizes on the necessity of breeding specific broccoli varieties for organic farming. According to Lammert van Beuren et al. (2011), the issue which differentiates the goals of organic breeding programs from the conventional ones is the fact that traits should be expressed under low-input conditions in organic farming.

On this basis, this doctorate study was designed as a part of the BLE project to investigate newly bred OP genotypes of broccoli obtained from on-farm selection by the organic breeders. These breeders used two approaches; 1) mass selection based on morphological traits and sensory properties of individual plants, 2) single plant selection according to testing and selecting the off-springs based on morphological traits and sensory attributes. Both approaches have been tested in different field trials within the whole project (Fleck et al., 2017). In general, mass selection is a simple method but rather limited, because the selection is based on the appearance of the plants (Navazio & Zystro, 2014). Quality improvement by progeny selection is more effective, but also more labor and area intensive (Acquaah, 2012). The breeders prefer to improve the homogeneity and agronomic and sensory traits by testing the progenies of the single plants.

This thesis presents the studies on different agronomical and chemical properties of the newly bred OP genotypes of broccoli. In addition, the effects of genotype, growing season and their interactions on the mentioned attributes in each genotype were assessed. Two hybrid varieties were considered as the comparison references for evaluating the OP genotypes. The aim of this study was to identify the OP varieties of broccoli, which can perform similar to hybrids and introduce them to farmers as the substituent of hybrids for organic broccoli production. For this purpose, we evaluated the agronomic parameters in the genotypes during two different growing seasons as described in Chapter 2. The assessed agronomic parameters were important traits for the production of broccoli. At the end of that chapter the genotypes, which performed best with regard to different agronomic parameters were highlighted.

According to a survey study by Renaud (2014), organic farmers listed agronomic traits such as "head size" and "yield" as important agronomical traits of broccoli. The marketable broccoli head size is the diameter of at least 10 cm with a maximum stem length of 20 cm (UNECE-standard FFV-48, 2010). Since organic farmers are not allowed to use synthetic fertilizers to increase the yield (Messmer et al., 2012; Wolfe et al., 2008), cultivating varieties with stable performance over different conditions is in priority over varieties which yield high under optimal conditions (Renaud et al., 2014). Due to the importance of yield, marketable yield was one of the agronomic parameters that were assessed in the newly bred OP genotypes. The outcomes showed that the genotype × growing season interaction significantly affected the marketable yield. In both fall and spring growing seasons, the marketable yield of OP genotypes was compared to the hybrids (Batavia F1 and Marathon F1). Cultivation of the same hybrid varieties over spring by Herbener (2011) resulted in marketable yields of approximately 9 t ha^{-1} which were higher than our study (8 t ha^{-1}). However, over fall season the yield was higher (up to 15 t ha^{-1}). Generally, higher marketable yield was achieved in fall 2015 than spring 2016. Figure 1 shows the differences between the yield level of the OP genotypes and each hybrid in percentage. In the study of Renaud et al. (2014), the OP varieties were the least stable varieties among all the experiments and had the lowest yield level compared to other varieties. In contrast, in our study the OP genotypes "CAN- SPB" and "CHE-GRE-G" had a similar yield level as Batavia F1, which was higher than Marathon F1. In this season, the poorest yield performance was observed in "Line 701", which had the largest differences with both hybrids. The genotypes "Calinaro", "CHE-BAL-A", "CHE-GRE-A", "TH-LIM-20-68" and "CHE-MIC" had respectively the lowest variances with the yield level of hybrids (between 9 and 16 t ha^{-1}).

In spring 2016, the marketable yield level of both OP genotypes and hybrids decreased to a great extent. "CHE-GRE-G", "CHE-GRE-A", CHE-BAL-A", "CHE-MIC" and "Line 701" with the marketable yield of 6 t ha^{-1}, had the highest yield level compared to other OP genotypes in spring 2016. In the spring experiment, broccoli plants grew under cooler growing temperatures compared to fall experiment (as shown in Chapter 2). Since cool temperatures result in slower N mineralization rate, it could be one of the reasons of decrease in the yield level of organic broccoli production over spring (Renaud et al., 2014). In addition, the development of plants might have been accelerated due to higher temperatures in the later cultivation period of spring season which gave the plants less time to build up yield. The possible reasons of lower yield level in spring are discussed more in Chapter 2.

Production of broccoli needs high N supply and regular irrigation (Pasakdee et al., 2007). The recommended N demand of broccoli is level of approximately 300 kg ha^{-1} (Scharpf, 1991). Applying a certain amount of organic fertilizers could enhance the yield impressively (Abd El-Moniem et al., 2012). In a conventional broccoli production, there is no limitation in adding nitrogen input and applying approximately 400 kg ha^{-1} nitrogen fertilizer resulted in marketable yield over 24 t ha^{-1} (Castellanos et al., 1999). As it was mentioned previously, synthetic fertilizers are banned in organic farming, therefore, applying nitrogen fertilizers is not that simple under organic conditions. One of the main limiting factors of growing organic crops is the nitrogen content of the soil (ADAS, 2006) which can be provided by cultivating legumes in a crop rotation or by applying manure or other organic fertilizers (IFOAM, 2011). Overall, it is difficult to meet the nitrogen requirement of broccoli plants under organic conditions, hence nitrogen use efficiency might be an important breeding goal for the future.

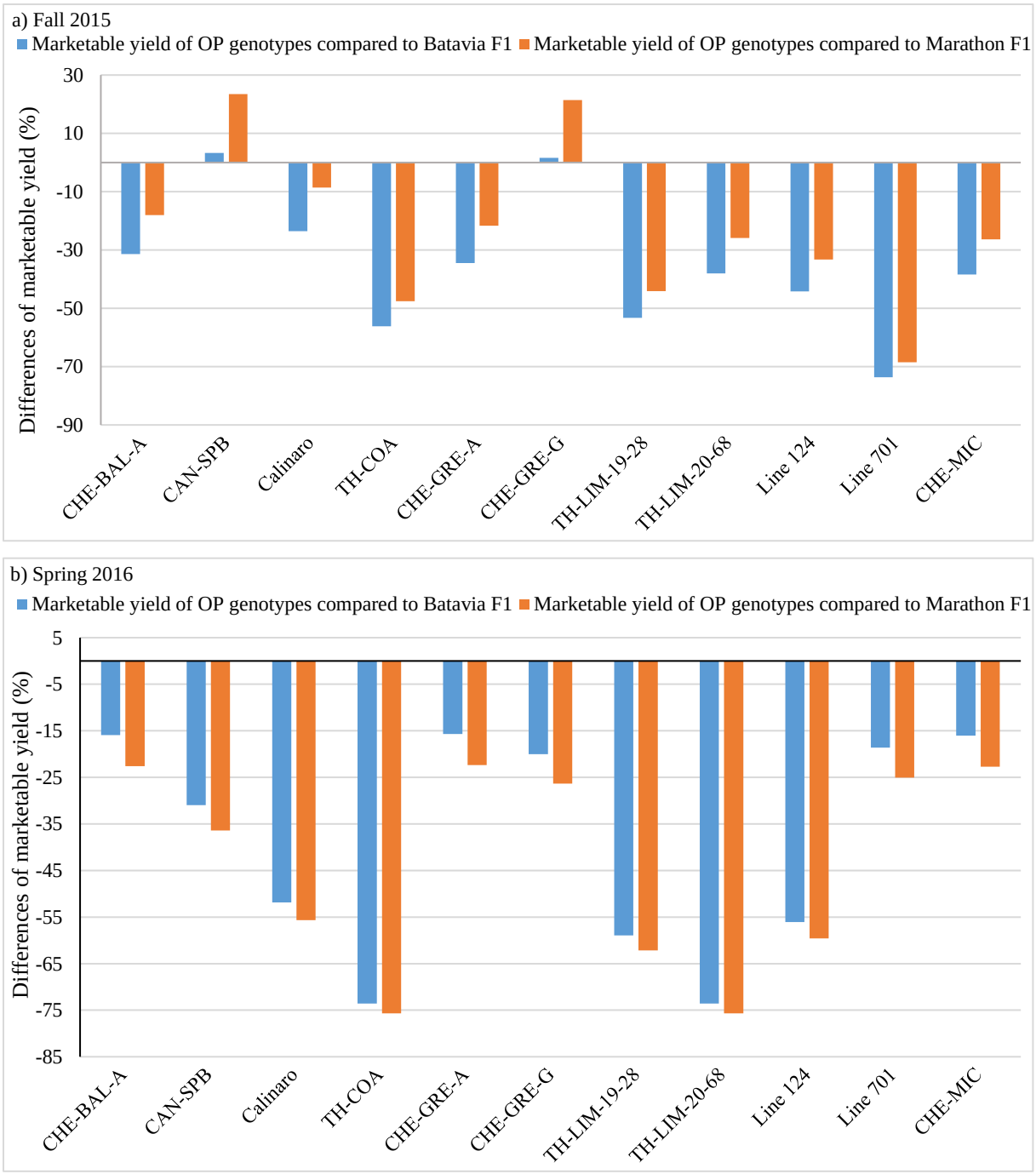

Figure 1. Differences of marketable yield (%) of the open pollinating genotypes compared to hybrids in a) fall 2015 b) spring 2016

Regarding marketable yield, the outcomes of the production of some of the genotypes at Bingenheim over spring 2016 (Fleck et al., 2017) indicated that some breeding lines of "CHE-GRE", "CHE-MIC" had higher marketable yield levels than others. However, they had a lower yield level compared to Batavia F1 (Figure 2).

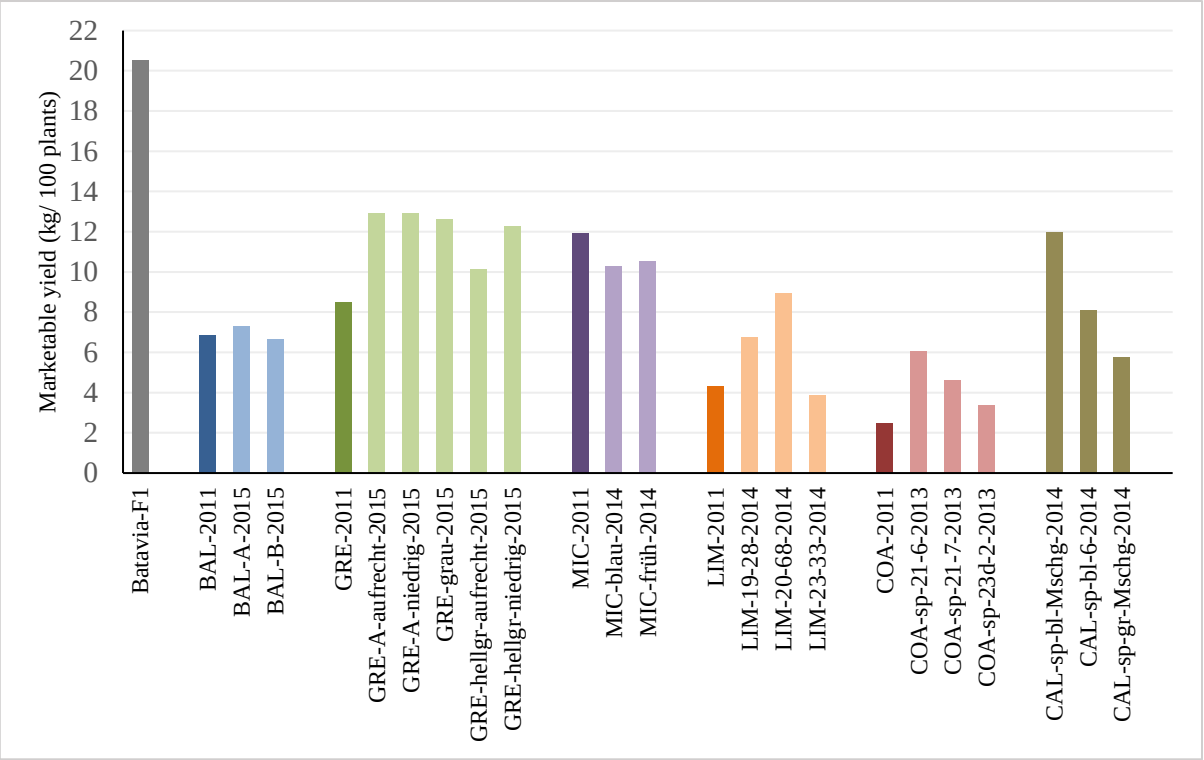

Figure 2. Marketable yield (kg/ 100 plants) of different open pollinating breeding line of broccoli in comparison with hybrid Batavia F1 cultivated at Bingenheim over spring 2016 (Fleck et al., 2017)

Among the breeding lines listed in Figure 2, other breeding progress was attained with respect to uniformity of the broccoli heads specifically in the "LIM" group. Also, improvements were observed in the "COA" and "CAL" groups which achieved middle to high range of head uniformity (Fleck et al., 2017).

Furthermore, referring to the report of Wolf et al. (2014), OP genotypes showed progress in achieving favorable head weights during the first part of the project. Higher head weight was demonstrated in OP genotypes (similar to Batavia F1) especially over their two last cultivation periods, which could indicate a breeding progress through the selection over 2012 and 2013. In the scope of the whole project, the illustration of improvement in two OP genotypes of "Calinaro" and "CHE-GRE-G" over five years through on-farm breeding resulted in releasing both genotypes as OP varieties of broccoli for cultivation in order to enter the market.

In addition to agronomic properties, organic farmers showed interest in knowing the cultivars with higher nutritional values to increase the production of them (Renaud et al., 2010). In this case, the better cultivars e.g. in terms of health-benefitting compounds are known by producers and launching a marketing strategy would inform and sensitize the consumers to buy that specific cultivar. A previous study on tomato showed e.g. that cherry tomatoes had higher levels of flavonol content (Crozer et al., 1997) and a higher concentration of lycopene (Commission

of the European Communities, 1993; Lis, 2017) in comparison with the normal tomato. Therefore, cultivation of cherry varieties would be in favor of consumers as well as farmers (Lis, 2017). Likewise, the production of the apple cultivar "Santana" is in favor of farmers and consumers who are allergic to apples due to "scab resistance" properties and "low allergenic traits", respectively (Nuijten et al., 2015).

The importance of the health benefiting compounds (GSLs) content of the OP genotypes in the selection of suitable genotypes was the basis of Chapter 4. Since a fast screening methodology would be beneficial especially for the breeders of the broccoli genotypes to test their most promising genotypes according to the GSLs content, a methodological study on fast determination of GSLs was developed and aimed at checking the accuracy of the used method with regard to determination of individual and total GSLs (Chapter 3). The calibration equation obtained from this chapter was used for determination of GSLs (Chapter 4).

Generally, broccoli is a valuable vegetable due to the existence of GSLs as chemopreventive compounds (Fahey et al., 2001; Latte et al., 2011). On this basis, the level of GSLs content which is associated with genetic variation (Robbins et al., 2005) could be an important trait for breeders in broccoli breeding programs. For this purpose, we evaluated the accuracy of NIRS which is a fast, low-cost technique (Chen et al., 2014; Oblath et al., 2016). It can help to predict the GSLs content of broccoli heads to help broccoli breeders test and select their most favorable genotypes over breeding procedures. The detail findings of applying NIRS on broccoli samples are reported in Chapter 3. Comparable to the only similar study on determination of GSLs content of samples of broccoli heads with NIRS (Hernandez Hierro et al., 2012), we also found a good potential of NIRS in a quantitative and qualitative analysis of GSLs.

Assessment of GSLs profile of OP genotypes of broccoli showed a small variation in the composition of tGSLs of each genotype and the hybrid varieties (Figure 3). We found a significant effect of genetic variation on GSLs content of our broccoli samples similar to the study of Rosa and Rodrigues (2001), Vallejo, Tomas-Barberan & Garcia-Viguera (2002), Schonhof et al. (2004) and Farnham et al. (2004). A former study showed that cultivation conditions of broccoli plants change the concentration of GSLs of broccoli heads (Robbins et al., 2005). More specifically, production of broccoli under organic farming and water stress affected the GSLs content of broccoli heads negatively and resulted in lower GSLs level compared to conventionally grown broccoli (Robbins et al., 2005). Combining the outcomes of the evaluation of agronomic and GSLs properties of OP genotypes indicated a similar range of GSLs concentration in the OP genotypes compared to the hybrids Hence, since all the OP

genotypes had similar level GSL content in each growing season, farmers can choose the best yield performing genotype for cultivation.

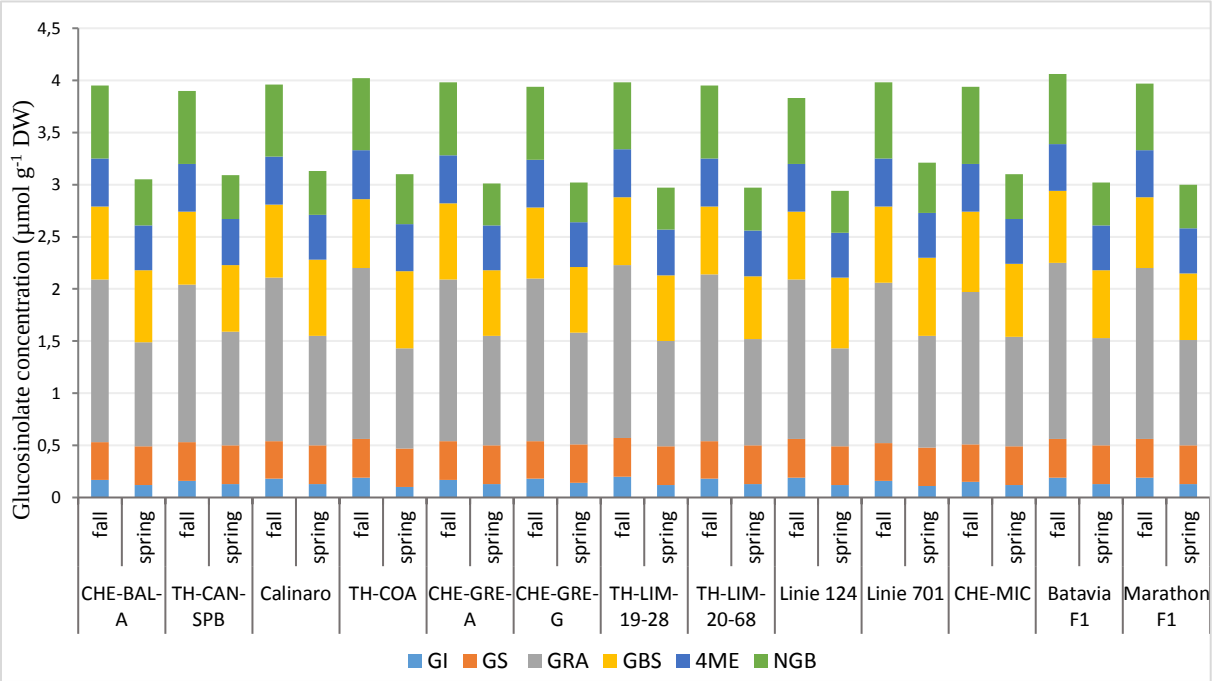

Figure 3. Glucosinolate composition (µmol g^{-1} DW) of open pollinating genotypes and hybrid varieties of broccoli in fall 2015 and spring 2016

In addition to the agronomic performance and GSLs level of the OP genotypes, other aspects like sensory quality should be considered to select genotypes for further breeding procedures. Consumers expect organic products to have a good quality and taste (Lammert van Beuren et al., 2007). The consumer's opinion on the quality of vegetables establishes according to the sensory characteristics (Lappalainen et al., 1998; Jiménez-Guerrero et al., 2012). Since different sensory traits such as appearance and taste affect consumer's decision on purchasing a product (Sandell et al., 2014), evaluation of sensory attributes of the product is of interest. In this regard, to find the OP genotypes acceptable by consumers, a preference sensory evaluation (a hedonic test consist of 27 participants with 10-cm-line scale) was performed in form of a master thesis (Frank, 2016) in the framework of this project. The goal of the sensory study was to find consumers perception on the degree of liking and purchase decision of some OP genotypes. The preference tests were done to find the preferable product (Vaclavik & Christian, 2008). Three OP genotypes ("TH-CAN-SPB", "CHE-GRE-A", "CHE-MIC") and one control hybrid variety ("BATAVIA F1") cultivated in fall 2015 were evaluated with regard to eight main attributes. According to Frank (2016), the attributes were specified through descriptive tests by a trained panel of eight assessors. The panelists were first trained to be able to distinguish "sweetness", "sourness", "bitterness" and "saltiness", also evaluate the intensity of the tastes.

Afterward, the training was followed by testing organic broccoli from the market and characterizing the ‘appearance’, ‘texture’ and ‘flavor’. Then, through group discussion and by use of ISO lists the panelists specified eight main attributes in three categories of flavor (overall taste, sweetness, bitterness, pungent taste and broccoli-like taste), texture (crispiness and granularity of buds) and appearance.

As illustrated in Figure 4 (Frank, 2016), “CHE-MIC” was the most likable compared to the other OP genotypes and therefore appeared in the outer rows of the diagram. Interestingly, the overall taste and appearance of “CHE-MIC” were liked more than the hybrid variety. Furthermore, the crispiness, bitterness, sweetness and broccoli-like taste of “CHE-MIC” were as pleasant as “BATAVIA F1” for the assessors. Regarding consumers’ preferences, evaluation of the results of purchase decision indicated that the consumers would buy “CHE-MIC” and “CHE-GRE-A” since they liked the overall taste, sweetness, broccoli-like taste and crispiness of both. However, as different sensory attributes, especially the overall taste and the pungent taste, of “TH-CAN-SPB” were not in favor of the consumers, they did not show any preferences in buying this specific genotype.

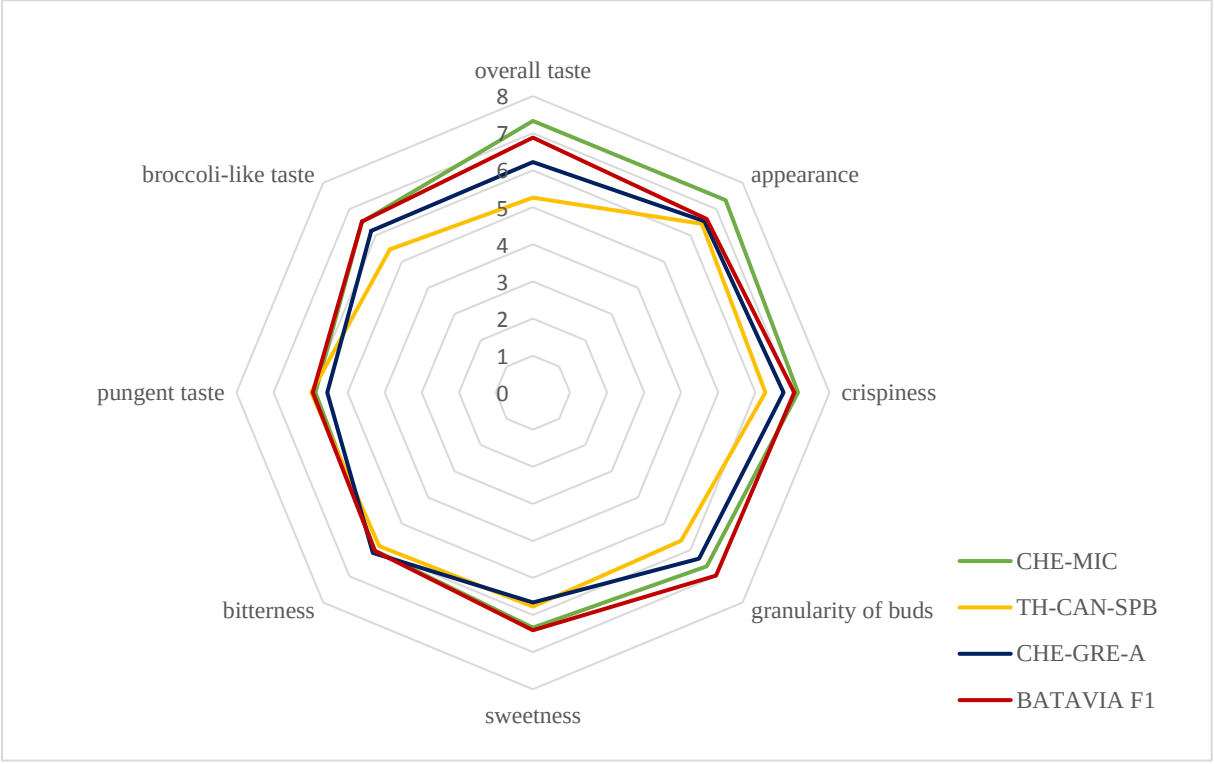

Figure 4. Mean values of the Degree of liking (cm) of the eight main attributes marked on a 10-cm-line scale in hedonic test (n=27). 0 cm: “dislike extremely”, 5 cm: “neither like nor dislike”, 10 cm: “like extremely” (Frank, 2016)

To date, few studies have been done on preferences of consumers towards broccoli based on their sensory quality (Johansen et al., 2016). Two former studies on broccoli by Schonhof et al. (2004) and Brueckner et al. (2005) showed broccoli samples with a high degree of sweetness,

juiciness, crispiness and intensity of broccoli-like flavor were more likable and acceptable by the consumers. More specifically, there was a great preference for broccoli samples with the high degree of sweetness and low intensities of bitterness and pungent taste (Brückner et al., 2005). Although bitter broccoli is not in favor of consumers, however, the intensity of the bitterness (especially "CHE-MIC" which was the bitterest) of our broccoli samples was low and did not affect the degree of liking negatively.

Considering previous studies which showed specific GSLs (GBS, NGB, and GS) contribute to bitter taste of broccoli (Schonhof et al., 2004; Beck et al., 2014), the link between the bitterness and GSL content of the broccoli samples were evaluated within the master thesis (Frank, 2016). The result was in line with the mentioned studies since GBS and NGB levels were higher in the bitterest genotype "CHE-MIC" compared to the other genotypes.

Finally, based on the positive sensory assessment of CHE-MIC and CHE-GRE-A, since both genotypes showed good agronomical performance (as shown in Chapter 2) and had considerable content of GSLs (as shown in Chapter 4), they seem to be acceptable when released to the market as organic varieties. According to different characteristics of these two specific OP genotypes, they might have a high marketability potential which can attract the consumers of organic products. It should be noted that since the sensory quality of broccoli is under the influence of environmental conditions such as temperature and climate condition, to produce crispy and juicy broccoli heads growing conditions with lower temperature and longer days' period are required (Johansen et al., 2016).

At the end, it is noteworthy to point out the good progress, which has been achieved by the breeders during the whole period of the project. Yet, there is a potential for further developing cultivars to meet the still open traits (agronomic and sensory parameters) such as firmness, head weight, sweetness and overall taste. Even further breeding towards higher yield is required since yield is always an issue. Likewise, to optimize the favorable OP genotype, further breeding is suggested. Finally, to clarify additional nutritive and health benefiting compounds of broccoli such as vitamins and polyphenols and the order of magnitude in the newly bred cultivars, supplementary studies will have to be conducted.

Summary

Currently, a considerable share of varieties being used in the organic vegetable production are developed for conventional high-input production systems, and broccoli is no exception. In addition, F1 hybrids are cultivated in organic broccoli production to a great extent because of high quality and yield. Two main restrictions of cultivating the mentioned categories of varieties in organic farming are; 1) ban of using cytoplasmic male sterility (CMS) in organic agriculture for reproduction of F1 hybrids of broccoli and limitations of farmers to produce their own seeds, 2) absence of special traits of these varieties which result in weaker performance when being cultivated under organically low-input conditions. In contrast to hybrids, cultivation of open pollinating broccoli varieties gives the opportunity of reproducing seeds to organic farmers. Therefore, developing new open pollinating broccoli varieties, which have the same quality (agronomical, chemical and sensorial) as F1 hybrids, through organic breeding programs (on-farm breeding) would allow the organic broccoli farmers to replace the hybrids with varieties adapted to organic production conditions.

With this in mind, the German Federal office for Agriculture and Food (BLE) initiated a project on "Breeding development of open pollinating cultivars of broccoli for organic farming in terms of agronomic characteristics, secondary and bioactive ingredients and sensory properties". This was a joint project which was done through the cooperation of University of Hohenheim and Kultursaat e. V. (NGO of on-farm breeders) in two parts during six years (2011-2016). The present doctoral thesis, which was a part of the mentioned project, aims at 1) investigating the agronomic performance of the newly bred open pollinating genotypes of broccoli, 2) developing a Near-Infrared Spectroscopy (NIRS) method for fast analysis of total, indole, aliphatic and individual glucosinolates content of broccoli samples; and 3) determining the total and individual glucosinolate content of the newly bred open pollinating genotypes of broccoli.

For investigations on agronomic performance, two field experiments were carried out by cultivating eleven newly bred open pollinating genotypes, two F1 hybrids and an open pollinating variety of broccoli over two growing seasons of fall 2015 and spring 2016. Evaluation of the effect of genotype, growing season and their interactions on agronomic parameters were targeted in this study. According to our findings, assessment of agronomic variables indicated that although there were distinctions in different parameters such as head firmness, head shape and total biomass fresh weight among the newly bred open pollinating genotypes, some genotypes performed similar to hybrid varieties in organic farming. However, most of the open pollinating genotypes had 16 % to 73 % lower yields compared to the hybrid varieties depending on growing season. Generally, the "marketable yield" of the genotypes was

under the significant effect of "genotype × growing season interaction". Head weight was significantly affected by growing season which resulted in significantly lower head weight of some genotypes in the spring compared to the fall season. Overall, cultivation of the genotypes in fall season led to significantly higher marketable yields, head weight and total biomass weight, as well as firmer heads in contrast to the spring season. Considering the performance of different agronomic parameters, we recommend genotypes "TH-CAN-SPB", "Calinaro", "CHE-GRE-G" for both fall and spring growing season. Other genotypes such as "CHE-GRE-A", "CHE-BAL-A" and "CHE-MIC" and "Line 701" are also recommended for cultivation in spring growing season specifically due to the high marketable yield and share of marketable heads.

In addition, this thesis aimed at testing a fast analytical technique for determination of glucosinolates content in order to help breeders to quickly test their most favorable genotypes during breeding procedures based on glucosinolates content. For this purpose, the accuracy of NIRS technic was tested, regardless of type of genotype, for fast analysis of the individual and total glucosinolates content of broccoli samples. NIRS calibration was developed by reference method of High Performance Liquid Chromatography (HPLC) based on modified partial least squares regression, to measure individual and total glucosinolates content of open pollinating genotypes of broccoli regardless of the type of genotype. The calibration was analyzed using coefficient of determination in prediction (R^2) and ratio of preference of determination (RPD). Large variation occurred in the calibrations, R^2 and RPD due to the variability of the samples. Derived calibrations for total glucosinolates (RPD = 1.36), aliphatic glucosinolates (RPD = 1.65), glucoraphanin (RPD = 1.63) and 4-methoxyglucobrassicin (RPD = 1.11) were quantitative with a high accuracy, while for indole glucosinolates (RPD = 0.95), glucosinigrin (RPD = 0.62), glucoiberin (RPD = 0.67), glucobrassicin (RPD = 0.81) and neoglucobrassicin (RPD = 0.56) they were more qualitative. Overall, the results showed a good potential of NIRS in determination of different glucosinolates in a large sample pool of broccoli quantitatively and qualitatively. The achieved calibration equations were used to measure glucosinolates content of the broccoli samples of following years.

To evaluate the health beneficial value of the open pollinating genotypes, the glucosinolates content of them were determined. The determination was done by the tested NIRS technic. Six individual glucosinolates were detected in the broccoli samples similar to findings of the previous chapter. Glucoraphanin (1.44-1.69 µmol g^{-1} DW), glucobrassicin (0.63-0.77 µmol g^{-1} DW) and neoglucobrassicin (0.38-0.74 µmol g^{-1} DW) had the highest share and were the main individual glucosinolates. Total glucosinolates content ranged from 3.46 to 3.60 µmol g^{-1} DW

across both growing season. Significant effect of genotype and growing season existed on the total glucosinolates content of broccoli samples. All individual glucosinolates were affected by genotype. The effect of growing season was significant on all individual glucosinolates, except for glucobrassicin. The interaction of genotype × growing season was significant on all indole glucosinolates, glucoraphanin and glucoiberin. Generally, the glucosinolates content of the samples were higher when broccoli genotypes were cultivated in the fall growing season, however the difference in the level of glucosinolates contents across seasons was significant only for glucoraphanin, neoglucobrassicin, 4-methoxyglucobrassicin and glucoiberin. The open pollinating genotypes showed a similar range of glucosinolates compared to the tested hybrids and performed as good as the hybrids. Since total glucosinolates were nearly similar in all open pollinating genotypes across seasons, all are recommended for cultivation in both growing seasons. It is important to note that this study only focused on a single health beneficial compound (glucosinolate) in broccoli heads. To provide a full insight into the nutritive and health benefiting compounds of broccoli such as vitamins and polyphenols, supplementary studies will have to be conducted.

All in all, releasing new open pollinating broccoli varieties out of this pool of genotypes and replacing the present varieties with them seemed beneficial due to the well adapted agronomic performance and high health value with regard to glucosinolates content under organic farming conditions.

Zusammenfassung

Derzeit werden im ökologischen Gemüsebau vor allem Sorten genutzt, die für den konventionellen Anbau entwickelt wurden. Brokkoli bildet hier keine Ausnahme. Im ökologischen wie im konventionellen Anbau werden bei Brokkoli zudem vor allem F1-Hybride angebaut, um einen hohen Ertrag und eine gute Produktqualität sicherzustellen. Dabei treten speziell im Ökologischen Landbau folgende Herausforderungen auf: 1) einige Öko-Anbauverbände (z.B. Demeter e.V., Bioland e.V.) verbieten die Sorten, deren Züchtung auf dem Einsatz von cytoplasmatisch-männlicher Sterilität (CMS) für die Reproduktion von F1-Hybriden beruht, 2) der Einsatz von Hybriden ermöglicht nicht den Nachbau von eigenem Saatgut durch die Landwirte und 3) die aktuell auf dem Markt befindlichen Sorten sind nicht an die Low-Input-Bedingungen im Ökologischen Landbau angepasst, was gerade bei einer N-intensiven Kultur wie Brokkoli häufig mit verminderten Erträgen einhergeht. Darüber hinaus wird im verfügbaren Sortensortiment bislang kein Augenmerk auf gesundheitsfördernde Inhaltsstoffe und mögliche Unterschiede zwischen den Sorten gelegt. Bei Brokkoli spielen jedoch Glucosinolate als gesundheitsfördernde Inhaltsstoffe eine große Rolle, da ihnen eine krebsvorbeugende Wirkung nachgesagt wird. Ziel ist es daher, für die Bedingungen des Ökologischen Landbaus neue samenfeste Brokkoli-Sorten durch ökologische Zuchtprogramme on-farm zu entwickeln. Idealerweise zeichnen sich diese Sorten durch ähnlichen Eigenschaften (agronomisch, chemisch und sensorisch) wie F1-Hybriden aus, könnten diese somit im Ökologischen Landbau ersetzen.

Vor diesem Hintergrund wurde in Rahmen des „Bundesforschungsprogramms für Ökologischen Landbau und andere Formen der Nachhaltigen Landwirtschaft“ das Projekt „Züchterische Entwicklung von samenfesten Sorten von Brokkoli für den biologischen Landbau in Bezug auf agronomische Merkmale, sekundäre und bioaktive Inhaltsstoffe und sensorische Eigenschaften“ gefördert. Es fand in Kooperation zwischen der Universität Hohenheim und Kultursaat e.V. (Verein zur On-Farm-Züchtung ökologischer Gemüsesorten) statt und lief über zwei Förderperioden von insgesamt sechs Jahren (2011-2016). Die vorliegende Doktorarbeit, welche ein Teil dieses Projektes war, umfasst 1) die Erfassung und Bewertung der agronomischen Parameter neu gezüchteter samenfester Brokkoli-Genotypen unter den Anbaubedingungen des Ökologischen Landbaus; 2) die Entwicklung einer Nahinfrarotspektroskopie (NIRS)-Methode für die schnelle Analyse der Gesamtglucosinolatgehalte, der indolischen und aliphatischen Fraktion sowie der einzelnen Glucosinolate für Brokkoli; und 3) die Bestimmung der Glucosinolate (Gesamtgehalte,

indolische und aliphatische Fraktion, Einzelsubstanzen) der neu gezüchteten samenfesten Brokkoli-Genotypen.

Für die Untersuchung der agronomischen Parameter wurden zwei Feldexperimente mit elf neuen samenfesten Genotypen, zwei F1 Hybriden und einer samenfesten Sorte über zwei Anbauzeiträume im Herbst 2015 und im Frühjahr 2016 durchgeführt. In diesen Versuchen wurden die Faktoren „Genotyp“, „Anbauzeitraum“, „Erntezeitpunkt“ und deren Interaktionen untersucht. Die Untersuchungen zeigten, dass einige der neuen samenfesten Brokkoli-Genotypen ähnliche Merkmale aufwiesen wie die Hybriden. Hinsichtlich der Merkmale, z.B. Festigkeit der Blume, Blumenform sowie Gesamtbiomasseertrag (Frischgewicht) konnten signifikante Unterschiede zu den Hybriden gezeigt werden. Weiterhin wiesen die meisten der samenfesten Genotypen in Abhängigkeit vom Anbauzeitraum um 16 % bis 73 % geringere Erträge im Vergleich zu den Hybridsorten auf. Grundsätzlich beeinflusste die Interaktion „Genotyp × Anbausaison“ den marktfähige Ertrag. Das Gewicht der Blume wurde signifikant vom Anbauzeitraum beeinflusst; im Vergleich zum Herbstanbau führte der Frühjahrsanbau bei den meisten Genotypen zu signifikant niedrigeren Blumengewichten. Insgesamt wurden im Herbstanbau signifikant höhere Erträge an marktfähigen Blumen, höhere Blumengewichte, eine höhere Gesamtbiomasse sowie festere Blumen als im Frühjahrsanbau ermittelt. Hinsichtlich ihrer agronomischen Parametern können die Genotypen „TH-CAN-SPB”, „Calinaro” und „CHE-GRE-G” für die Herbst- und Frühjahrsanbau empfohlen werden, während die Genotypen „CHE-GRE-A“, „CHE-BAL-A“, „CHE-MIC“ und „Linie 701“ besser für den Frühjahrsanbau geeignet scheinen.

Um in der ökologischen Brokkoli-Züchtung auf gesundheitsfördernde Inhaltsstoffe selektieren zu können, ist es nötig, eine schnelle und kostengünstige Methode einzuführen, mit der die Glucosiolatgehalte in den Einzelpflanzen bestimmt werden können. Im Rahmen dieser Arbeit wurde daher mit Hilfe der Referenzmethode der Hochleistungsflüssigkeitschromatographie (HPLC) eine Kalibrierung für einzelne Glucosinolate sowie für den Gesamtgehalt an Glucosinolaten für NIRS entwickelt. Die Kalibrierung erfolgte über eine Regressionsfunktion (modifizierte Methode der kleinsten Quadrate) wobei die Güte des Fits durch das Bestimmtheitsmaß (R^2) und die „ratio of preference of determination“ (RPD) geprüft wurde. Aufgrund der Heterogenität der Proben traten große Schwankungen bei der Kalibrierung auf. Die ermittelten Kalibrierungen für den Gesamtglucosinolatgehalt (RPD = 1,36), die aliphatischen Glucosinolate (RPD = 1,65), Glucoraphanin (RPD = 1,63) und 4-Tethoxyglucobrassicin (RPD = 1,11) waren quantitativ von einer hohen Genauigkeit, während die Kalibrierungen bei den indolischen Glucosinolaten (RPD = 0,95), Glucosinigrin (RPD =

0,62), Glucoiberin (RPD = 0,67), Glucobrassicin (RPD = 0,81) und Neoglucobrassicin (RPD = 0,56) nur für eine qualitative Bestimmung geeignet waren. Insgesamt zeigen die Ergebnisse, dass NIRS zur quantitativen und qualitativen Bestimmung verschiedener Glucosinolate bei einer hohen Anzahl von Brokkoli-Proben geeignet ist.

Die Kalibrierungen wurden in den Folgejahren genutzt, um die Glucosinolatgehalte und damit den Gehalt an gesundheitsfördernden Inhaltstoffen in den neuen samenfesten Brokkoli-Genotypen zu analysieren. Glucoraphanin (1,44-1,69 µmol g^{-1} Trockemasse (TM), Glucobrassicin (0,63-0,77 µmol g^{-1} TM) und Neoglucobrassicin (0,38-0,74 µmol g^{-1} TM) waren die dominierenden Glucosinolate in allen getesteten Genotypen und Hybriden. Der Gesamtgehalt an Glucosinolaten reichte von 3,46 bis 3,60 µmol g^{-1} TM in beiden Anbauzeiträumen, wobei die Faktoren „Genotyp“ und „Anbausaison“ statistisch signifikant waren. Alle einzeln untersuchten Glucosinolate waren vom Faktor „Genotyp“ beeinflusst, der Faktor „Anbausaison“ war bei allen außer bei Glucobrassicin signifikant. Die Interaktion Genotyp × Anbausaison war bei den indolischen Glucosinolaten sowie bei Glucoraphanin und Glucoiberin signifikant. Grundsätzlich war der Glucosinolatgehalt im Herbstanbau höher, jedoch war der Unterschied nur für Glucoraphanin, Neoglucobrassicin, 4-Methoxyglucobrassicin und Glucoiberin signifikant. Die samenfesten Genotypen zeigten ähnliche Glucosinolatgehalte wie die untersuchten Hybriden. Die Gesamtglucosinolatgehalte waren in allen samenfesten Genotypen in beiden Anbauzeiträumen ähnlich. Daher kann die Auswahl der anzubauenden Sorten bzw. Genotypen unabhängig vom Glucosinolatgehalt erfolgen und die Landwirte können anhand der geprüften agronomischen Merkmale sowie des möglichen Ertragspotenzials ihre Sorten selektieren. Es ist jedoch wichtig darauf hinzuweisen, dass diese Studie sich nur auf die gesundheitsfördernden Verbindungen der Glucosinolate konzentrierte. Um einen vollen Einblick in die Nähr- und gesundheitsfördernden Stoffe in Brokkoli, wie Vitamine und Polyphenole zu bieten, müssen ergänzende Studien durchgeführt werden.

Zusammenfassend bleibt festzustellen, dass einige der untersuchten samenfesten Brokkoli-Genotypen geeignet sind, derzeit gängige Hybrid-Sorten im Ökologischen Landbau zu ersetzen, da sie gute agronomische Eigenschaften aufweisen, hohe Gehalte an gesundheitsfördernden Glucosinolaten aufweisen und an die speziellen Bedingungen des Ökologischen Landbaus angepasst sind.

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Publications

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