Preharvest and postharvest factors affecting the quality profile of onion landraces

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Faculty of Agricultural Sciences
University of Hohenheim

Institute of Crop Science
University of Hohenheim

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Maria Luisa Romo Pérez

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weitere Berichterin bzw. Prüferin: Frau Jun.-Prof. Dr. Sandra Schmöckel

"It is hard to imagine a civilization without onions; in one form or another their flavor blends into almost everything in the meal except the desert"

Julia Child

Content

Chapter 1 – General introduction

1. General introduction

According to the Food and Agriculture Organization (FAO), onion *(Allium Cepa* L.*)* is, after tomato, the second most cultivated vegetable in the world. In 2018, total production of onion - including greens and shallots - reached 100 million tons, an increase of almost 100% over last 20 years (FAOSTAT, 2020). As one of the oldest crops known to man, onions were disseminated around the world and have over the centuries adapted to different climates and conditions, both in bulb and inflorescence. Because of that, more so than with many other crops, we today have a wide range of onion genotypes and local varieties in existence (Brewster, 2008). For instance, genotypes from tropical zones adapted to short days as well as high temperatures and genotypes from temperate zones on the other hand prefer longer days and initiate bulb formation at > 14 hours of daylight (Khokhar, 2017).

To consumers making a purchasing decision, most important characteristics of onions are often their appearance and freshness - specifically the shape, size, color, uniformity and firmness (Camelo and FAO, 2004). On the other hand, one cannot disregard yield and onion nutritional attributes, flavor and aroma – which is equally important for both consumer markets and industry applications – and a focus of many research and breeding programs. Preharvest factors such as genotype, soil characteristics, as well as postharvest factors including storage conditions, have been shown to affect external quality characteristics and the nutritive value of onion bulbs (Bettoni et al., 2016; Coolong and Randle, 2003; Kopsell and Randle, 1997; Pérez-Gregorio et al., 2014; Petropoulos et al., 2016; Randle, 2000; Randle and Bussard, 1993; Randle et al., 1995; Sekara et al., 2017; Shock et al., 1998). Genotype, soil condition, organic farming systems, as well as storage conditions, are the principal factors discussed in this thesis.

1.1. Onion varieties and the organic farming

It is difficult to trace onions to a single source. Over centuries, they have adapted to different temperatures and photoperiods, creating a wide range of genotypes and landraces. These local varieties and landraces evolved naturally to adapt to diverse environmental conditions found around the world (Brewster, 2008). With the modernization of agriculture, breeders have increasingly focused on growing F_1 hybrids – a narrow genetic base – and abandoned old local varieties, endangering biodiversity (Cramer, 2001). Modern varieties are mainly bred to perform well in conventional systems that rely on chemical inputs for fertilization and plant protection. These varieties are often ill-equipped to meet the standards of the modern organic sector, where the application of fertilizers and synthetic pesticides is not permitted. Furthermore, in Europe, certification organizations for organic and biodynamic agriculture, such as Demeter International e.V., Bio Suisse, and Bioland e.V. prohibited the use of hybrid grain seeds for field crops (with an exception of maize) as well as use of hybrids that are the result of protoplasm and cytoplasm fusion techniques (Bioland, 2019; BioSuisse, 2020; Demeter-International, 2019). In order to enrich organic farming systems, new varieties are required that fit the standards of organic agriculture (Osman et al., 2008). Moreover, with growing concern over complaints that modern commercially produced fruits and vegetables are losing their typical flavor characteristics, landraces are again drawing the interest of farmers and consumers alike due to their genetic variability and distinct aroma properties (Klee and Tieman, 2013; Petropoulos et al., 2015; Tieman et al., 2012; Tieman et al., 2017).

1.2. Quality aspects of and health benefits of onion bulbs

Onions are highly valued for their unique flavor and nutritional properties and can be consumed raw, fresh or used as dehydrated products for food processing. Three compounds in particular are responsible for these characteristics: flavor- and tear-inducing sulfur compounds, powerful antioxidative flavonoids, as well as non-structural carbohydrates (Griffiths et al., 2002) (Figure 1).

Onions are high in non-structural carbohydrates like sucrose, glucose, fructose, and fructans. The latter act as prebiotics in the human body, since they cannot be digested in the upper intestine and serve as an important source of energy for probiotic bacteria in the caeo-colon. Non-structural carbohydrates make up to 80% of the bulb dry matter content and their composition varies immensely depending on the variety (McCallum et al., 2006).

Two flavonoid types are of the utmost importance in genus *Allium*: the anthocyanins, present in the red-colored skin of some onion varieties, and flavonols. Quercetin is the most important flavonol in onions and represents more than 95 % of the total flavonoid content (Rodriguez Galdon et al., 2008). Several studies already reported cardioprotective and anti-cancirogenic effects of quercetin in onions (Griffiths et al., 2002; Knekt et al., 1996; Sharma et al., 2018). Moreover, like vitamins A, C and E, quercetin acts as a radical scavenger and can therefore prevent damage to DNA by free radicals (Li et al., 2020).

Apart from non-structural carbohydrates and flavonoids, onion health properties are often attributed to *S*-alk(en)yl-L-cysteine sulphoxides (ACSOs). These also act as precursors for volatile sulfur compounds, which are in turn responsible for the characteristic pungent and tear-inducing effect in onion plants (Block, 1992; Griffiths et al., 2002).

Non-structural Carbohydrates

Glucose, fructose, sucrose and **fructans**
Fructans: storage carbohydrates in onions
Fructans: source of energy for beneficial bacteria. Act as prebiotics

Flavonoids

Two major flavonoid types in genus *Allium*: Anthocyanins and flavonols (incl. **quercetin**)
Quecetin: powerful antioxidant
Quecetin: Agent for lowering LDL cholesterol and blood pressure
Quercetin: Anticarcinogenic properties

***S*-alk(en)yl-L-cysteine sulfoxides (ACSOs)**

Precursors of flavor, pungency and lachrymatory effect in onions
Antifugal and antibacterial properties
Prevention of thrombotic cardiovascular diseases

Figure 1 Summary of the most important compounds in onion bulbs and their health promoting benefits.

1.3. Onion storage life and postharvest quality

Onions have been successfully cultivated around the world for centuries and marketed more widely than other vegetables because of their storability (Griffiths et al., 2002). Onion bulbs are normally harvested while dormant and a prolonged dormancy is desired before the regrowth phase begins. Depending on the genotype, storability of onions can be prolonged through post-harvest technologies, for instance cold or controlled-atmosphere (CA) storage. The principle of these technologies is to regulate relative humidity and temperature of a storage room. In CA-storage the gas atmosphere is controlled and monitored as well. Both technologies aim to slow down respiration and metabolic processes in onions during long storage periods (Brewster, 2008; Petropoulos et al., 2017). These processes can be delayed but not completely stopped and sooner or later sprouting occurs. When onion bulbs break their dormancy, the onion base plate (sink) needs nutrients to start internal sprouting and these must come from the onion scales (source). During the sprouting process, onions undergo a source-sink transition, which is reflected in metabolic changes such as increasing water loss, reducing sugar concentrations and respiration (Sharma et al., 2016). An important factor for a long dormancy phase is to choose proper variety. Quality monitoring of different onion varieties before and after harvest is of paramount importance in developing modern varieties with good yield stability and long storability. Markers in onion bulbs that indicate prolonged storability are: high dry matter content, high pungency, high level of soluble solids, as well as high fructan concentrations (Brewster, 2008).

1.4. Salinity and mechanisms of tolerance in onion plants

Preharvest abiotic factors such soil salinity can lead to changes in yields and quality aspects of onions plants. Salinity perturbs plant metabolism by lowering the availability of substrates or inhibiting enzyme function. Plants cope with such adverse conditions by reconfiguring their metabolic network in order to maintain essential metabolic functions and adopt a new homeostatic equilibrium (D’Amelia et al., 2018; Obata and Fernie, 2012). Salinity results in the formation of Reactive Oxigen Species (ROS), which can damage lipids, proteins and DNA. In order to protect and repair the damage caused by, *e.g.* salinity, plants possess an antioxidative defense mechanism, including enzymatic and non-enzymatic antioxidants (Ahmad et al., 2008). Previous studies demonstrated that plants responded to the increased production of ROS by inducing cysteine biosynthesis as a protection against high Na^+ accumulation, which in turn led to increased glutathione levels (Ruiz and Blumwald, 2002). Glutathione, a tripeptide (gly-cys-glu) and sulfur-containing compound, is one of the most important components in the metabolite profile of onions. Besides playing a significant role for storage and transport of reduced sulfur and antioxidative defense, it is also a starting point in the biosynthetic pathway that leads to the production of flavor precursors ACSOs (Figure 2). As such, changes in glutathione levels, as induced by increased Na^+ accumulation, could be closely connected with alterations in pungency and aroma properties of onions.

Onions, as well as other members of the *Alliaceae* family reported low threshold values (not greater than 1.21 mS cm^{-1}) for yield reduction, making them, next to *Vicia faba* L., one of the most salt sensitive crops (Allen et al., 1998; Brewster, 2008). In contrast to the well-known metabolic acclimatization of field crops (*e.g.,* maize, wheat, beans, etc) to salinity, information about stress-related modifications on the metabolome of vegetables, in particular onions, is scarce. In order to understand onion responses to salinity, it is necessary to study its physiology and metabolism, as well as reactions of the antioxidative defense system to increased Na^+ accumulation.

Metabolomics is a powerful tool that enables a comprehensive compositional analysis and the detection of changes in the onion metabolism caused by abiotic factors, such as salinity. Several techniques, including gas chromatography mass spectrometry (GC×GC-MS), possess higher sensitivity and separation performance, making it useful for the detection of a large number of complexes metabolites (Weinert et al., 2015; Wojciechowska et al., 2014). Furthermore, physiological changes in plants (*e.g.* sprouting during storage) are reflected in plant metabolism and therefore can also be observed by metabolomics. In this thesis, metabolomic techniques accompanied with other targeted methods were used to identify changes during cold storage and due to abiotic factors (*e.g.* salinity), as well as to describe and compare the chemical composition of different onion landraces.

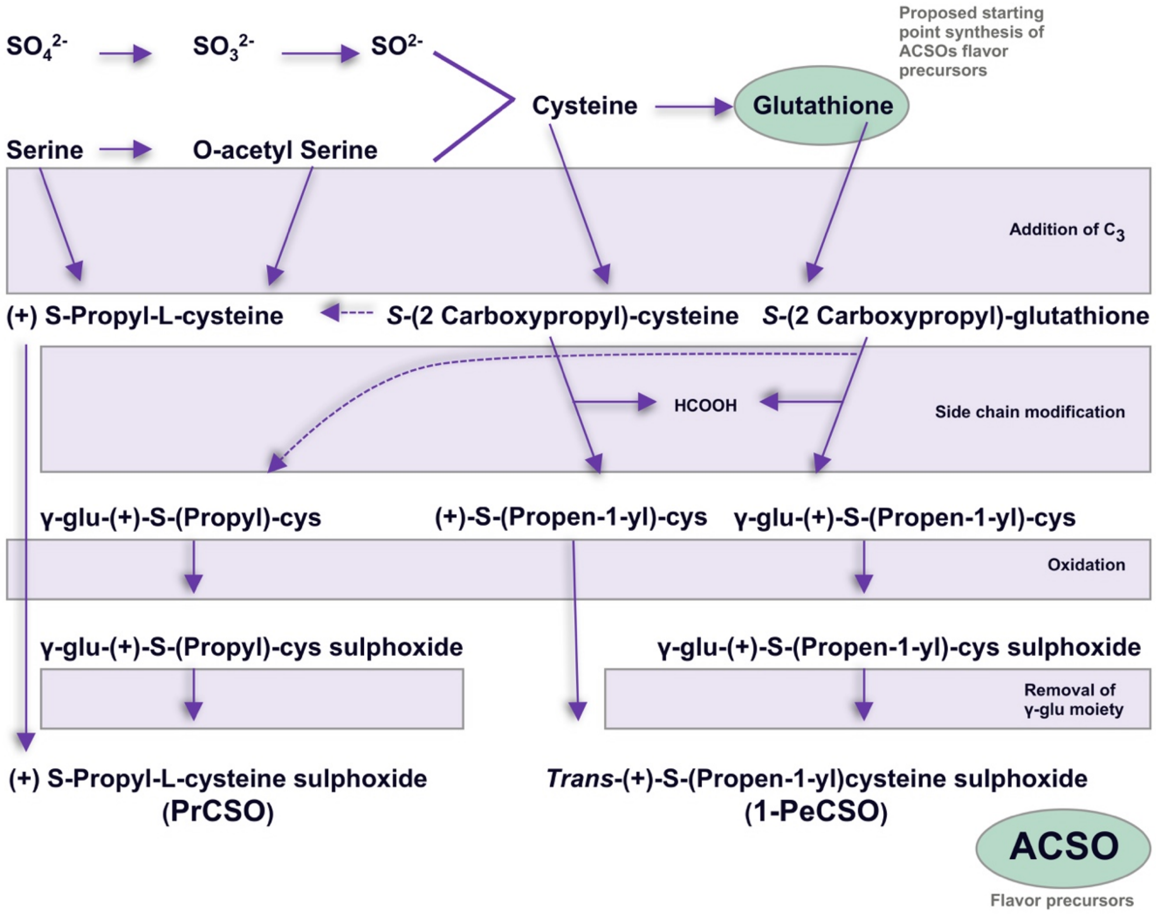

Figure 2 Schematic representation of the synthesis of flavor precursors, S-alk(en)yl-L-cysteine sulfoxides (ACSOs), in onions**.** Glutathione as key compound of the synthesis of flavor precursors, as well as the flavor precursors ACSOs are highlighted in green. The figure is modified after Griffiths et al. (2002).

1.5. Objectives

In order to provide new insights about quality aspects and storability of onion landraces from Western-Europe, a broad and complete analysis, including targeted and untargeted methods, after pot and field experiments is presented in this thesis. To determine the yield and quality potential of landraces, a comparison with other two well-known and established varieties from the region; “Red Baron” and “Sturon” is introduced and discussed. Moreover, the way in which plant physiology and metabolism are affected by increases Na^+ accumulation is analyzed in selected onion varieties. The results compiled by this study enable the answer to following questions:

(i) is there potential, with regard yield, quality aspects and storability, in local onion landraces? and is there potential for their use under organic farming conditions? (Chapter 2 and 3)

(ii) how does exposure to mild Na^+ concentrations affect the physiology, metabolism, pungency as well as the antioxidative defense system of onion plants? (Chapter 4)

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Chapter 2 – Quality aspects in open-pollinated onion varieties from Western Europe

Maria Romo-Pérez, Nikolaus Merkt, Sabine Zikeli, Christian Zörb

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[1]Department of Quality of Plant Products, Institute of Crop Science, University of Hohenheim, Stuttgart, Germany
[2]Coordination for Organic Farming and Consumer Protection, Institute of Crop Science, University of Hohenheim, Stuttgart

Quality aspects in open-pollinated onion varieties from Western Europe

Maria Romo Pérez[1], Nikolaus Merkt[1], Sabine Zikeli[2], Christian Zörb[1*]

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Summary

Commercial onion breeders limit their selection criteria by focusing almost exclusively on conventional farming. This raises the demand for certain well known varieties, but lowers the general diversity available on the mainstream market. A way to maintain biodiversity is to preserve old open-pollinated varieties. Through their distinct aroma and flavor, these plants are again drawing the interest of farmers and consumers alike, making them a viable alternative to commercial varieties. To assess yield and quality aspects of West-European open-pollinated onions, we have evaluated nine varieties and compared them against two of their commercial, well-established counterparts. The study included onion production on the field in South-West Germany, evaluation of the quality and flavor parameters, as well as a trained sensory taste panel. Results showed high diversity in yield and chemical properties of the studied onion varieties, where two varieties in particular, stood out significantly. Compared to the control, the variety "Birnförmige" performed best and demonstrated high concentrations of fructan and pyruvic acid, both known to have curative and medicinal properties. On the other end of the spectrum, the variety "Jaune des Cévennes" demonstrated low dry matter content, low concentration of enzymatically-produced pyruvic acid and a high bolting percentage. The study also confirmed the link between individual quality components in onion bulbs, including the significantly negative correlation between minerals (such as calcium and magnesium) and fructan.

Keywords: *Allium cepa* L., open-pollinated varieties, yield, quality compounds, sensory taste

Introduction

According to the Food and Agriculture Organization (FAO), onion (*Allium cepa* L.) is, after tomato and watermelon, the third most cultivated vegetable by production quantity in the world, with a total of 92 million tons produced in 2014 (FAOSTAT, 2017). Onions are highly valued for their unique flavor and their nutritional attributes including high amounts of vitamins, minerals and trace elements. These characteristics and in particular the following three compounds have been the focus of onion-related research worldwide: substances derived from flavor- and aroma-inducing sulfur compounds, powerful antioxidantive flavonoids, including quercetin and anthocyanin, as well as fructo-oligosaccharides and fructans. The last two act as prebiotics, since they cannot be digested in the upper intestine and serve in the caeco-colon as an important source of energy for the local microbiota (Brewster, 2008; Griffiths et al., 2002). On average, organic foods, *i.a.* onions, can contain higher levels of these healthy compounds, especially higher concentrations of antioxidants, aroma and flavor than conventionally produced foods as confirmed in many studies (Ren et al., 2017a; Ren et al., 2017b; Woese et al., 1997).

It is difficult to trace the exact origin of onions to a single source. Over thousands of years, onions have adapted to different climates, temperatures and photoperiods, creating a wide range of varieties and landraces (Brewster, 2008). Old open-pollinated varieties and landraces in particular have been valued for their yield stability and resistance against adverse conditions. Their production capacity, however, is less than that of modern cultivars (Zeven, 1998). With the modernization of agriculture and discovery of cytoplasmic male sterility (CMS) in the onion variety Italian Red, farmers have increasingly focused on growing hybrids and abandoned traditional landraces and open-pollinated varieties, leading to genetic erosion (Cramer, 2001; Petropoulos et al., 2015). In contrast to old varieties, modern onion hybrids are mainly bred to perform well in conventional systems that rely on chemical inputs. These varieties in turn do not match the standards of the organic sector where organic farmers do not apply synthetic pesticides and fertilizers, nor is the breeding methodology of CMS hybrids compatible with the principles set forth by the world umbrella organization for organic farming IFOAM (Osman et al., 2008). In order to optimize organic farming systems, new varieties are required that fit the growing conditions in organic agriculture (Lammerts van Bueren et al., 2002; Osman et al., 2008). Under unfavorable conditions landraces and old open-pollinated varieties may be able to compete and even outperform modern varieties, therefore offering organic farmers new opportunities to cope with plant biotic and abiotic stresses, as well as create base populations that can be used to generate new varieties (Zeven, 1998).

During the past few decades, many public and private initiatives started to collect, characterize and maintain genetic resources for different vegetable and cereal species around the world. In Europe, in particular, the Netherlands, Italy, Spain and Greece have already contributed with studies of genetic diversity and quality on their own old onion varieties (Liguori et al., 2017; Petropoulos et al., 2015; Rivera et al., 2016) but in Germany and many neighboring countries, such studies are still scarce. In order to provide new insights about the quality aspects of old onion varieties, the aim of this study was to characterize the morphological, agricultural and phytochemical characteristics of nine old open-pollinated varieties grown under organic conditions and compare them with two well establish open pollinating onion varieties marketed in West-Europe ("Red Baron" and "Sturon"), focusing on their nutritional compounds. The results compiled by this study should enable us to answer the following questions: (*i*) How big are the differences and potential of quality parameters among the varieties? (*ii*) Is there a link between the quality components of the studied onion varieties? (*iii*) Which varieties are better suited for organic farming in South-Germany?

Materials and methods

Plant material

A total of eleven onion varieties were used (Fig. 1, Tab. 1). Nine of these were West-European open-pollinated onion varieties provided by the organization Kultursaat e.V., whereas the two additional commercial open-pollinated cultivars "Red Baron" and "Sturon" that were used as control. They were selected based on their quality and because they are widely used in commercial organic farming in the study area.

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[*] Corresponding author

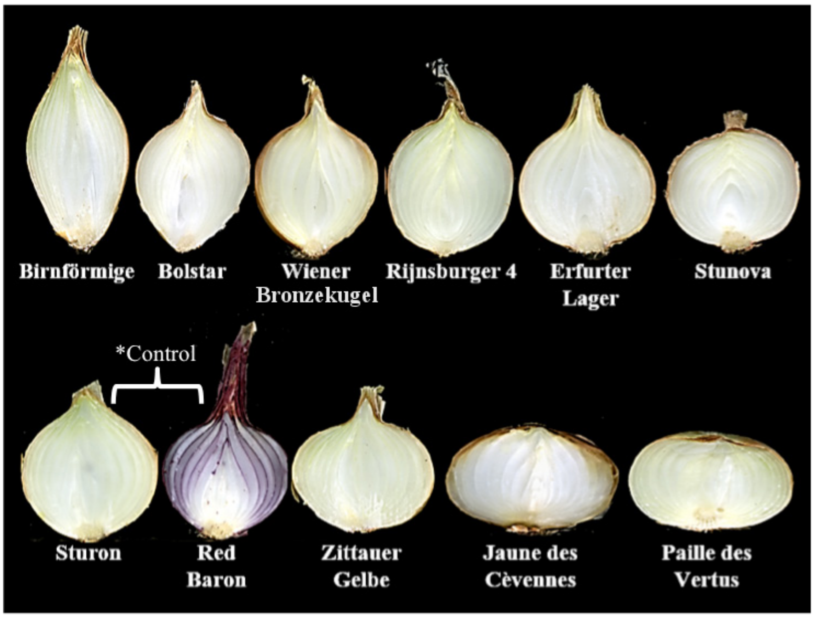


Fig. 1: Longitudinal section of open-pollinated onion varieties used in the field trial.

Tab. 1: Variety, skin color, country of origin or seed provider and market availability of analyzed open-pollinated onions.

Variety	Abbreviation	Skin color	Seed Provider / Origin	Availability
Jaune des Cévennes	JdC	Yellow	Kultursaat e.V. / France	Limited
Stunova	Stu	Yellow	Kultursaat e.V. / Germany	Limited
Sturon*	Str	Yellow	Bingheimer Saatgut / Germany	Common
Bolstar	Bol	Yellow	Kultursaat e.V. / The Netherlands	Limited
Wiener Bronzekugel	WBr	Yellow	Kultursaat e.V. / Austria	Limited
Rijnsburger 4	Rij4	Yellow	Kultursaat e.V. / The Netherlands	Limited
Birnförmige	Bif	Yellow	Kultursaat e.V. / Germany	Limited
Erfurter Lager	ErL	Yellow	Kultursaat e.V. / Germany	Limited
Zittauer Gelbe	ZtG	Yellow	Kultursaat e.V. / Germany	Limited
Paille des Vertus	PdV	Yellow	Kultursaat e.V. / France	Limited
Red Baron*	RB	Red	Bejo / The Netherlands	Common

* Control varieties

Field experiment

The field trial was conducted in 2015, with seeds of eleven onion varieties sown in a controlled environment in the greenhouse during the last week of February. After six weeks, seedlings were transplanted to the experimental station for organic farming "Kleinhohenheim", University of Hohenheim (48°43'54''N, 9°12'5''E Stuttgart, South-West Germany). Climate data can be found in Supplementary Fig. S1. The experiment was set up in a randomized block design with 4 replicates and an individual plot size of 3.2 × 1.5 m. Each experimental plot (one variety per plot) had three rows and a total of 90 plants per quadrat meter. Similar to the field experiment of Reents et al. (2007), a distance of 0.375 m was maintained between rows and 0.20 m between plants in the row. The rotation on the experimental field included the following precrops: 1st year grass-clover (high share of red clover), 2nd year cabbage, 3rd year summer wheat, 4th year onions (year of the experiment). N_{min} measurements were done before transplanting in April (0 - 60 cm 36 kg N/ha). During all plant growth stages, hand-weeding was carried out to combat weed. The onions were harvested when the foliage leaves senesced at the end of the first growing season. Each onions variety were lifted by hand after 60% of each plot top-down. For optimum storage quality, onions were cured in an indoor space for three weeks. After this, onions were stored for approximately four weeks until all samples were processed. During this period, observation of diseases and disorders in the onion bulbs was conducted. Yield and onion bulb size were determined prior to laboratory work. An onion size sorting machine was used to sort onion as < 30 mm, 30 - 45 mm, 45 - 55 mm, > 55 mm (Amazone BK3 sorting machine, AMAZONEN-Werke H. Dreyer GmbH & Co. KG, Hansberger, Germany).

Morphological features

After harvest, yield determination, and size sorting, nine individual onions (30 - 55 mm) from each plot were gathered for further analysis. Prior to phytochemical composition analysis, data of bulb length and diameter, dry weight, number and color of tunic, firmness and total soluble solids (TSS) of edible flesh were collected. Bulb diameter was measured using a caliper at the widest point of the bulbs and the statistical average of nine samples was used as variety diameter. Bulb length was obtained by measuring the average vertical length. Bulb firmness was determined in degree Shore with a durometer (Shore Instrument & Manufacturing Company, Inc., Jamaica, NY). Three single-point durometer readings were measured equatorially on each bulb to calculate the average durometer firmness.

Processing of samples

Four replicates of each variety composed of nine bulbs were used for sample processing. The outer dry scales were removed and the onions were cut longitudinally. One part of the sample was bulked and homogenized in a 1:1 vol (w/v) of double-distilled water (ddH$_2$O) using a mixer (BÜCHI Mixer B-400, BÜCHI Labortechnik AG, Flawil, Switzerland). The homogenates were then filtered using folded paper filter (520 A ½ 185 mm, Schleicher & Schuell, Dassel, Germany) and the clear supernatants were stored at -20 °C for further pyruvic acid analysis. A second part of the sample was chopped and freeze-dried. The resulting lyophilized onions were then ground into powder. The samples were stored at -20 °C prior to extraction of phenolic compounds. The third part of the sample was homogenized solely and stored for the analysis of sugar, soluble solids, and dry matter content.

Determination of dry matter content

20 g of homogenized samples were dried in an oven with air circulation, first at 70 °C for 42 h and then at 105 °C for 3 h. Every determination was made in triplicate.

Determination of soluble solids content (TSS)

The soluble solids content (in °Brix) was measured at 22 °C using the Bausch and Lomb Abbe 3L refractometer (Bausch and Lomb Incorporated, Rochester, NY) on the supernatant obtained from 1 g of raw homogenate by centrifugation for 10 min at 10000 rpm

Determination of non-structural carbohydrates

Non-structural carbohydrates were analyzed based on the Official Analytical Chemists (AOAC) method and the Megazyme fructan-assay kit using p-hydroxybenzoic acid hydrazine (PAHBAH) with some modifications (McCLEARLY et al., 2000). For the preparation of the standard curves, stock solutions containing 0, 0.1, 0.25, 0.5, 0.75 and 1 mg mL^{-1} of glucose were prepared. For reducing sugar analysis, samples of 50 µL of diluted extract (1 mL onion juice with 4 mL ddH$_2$O) were mixed with 200 µL ddH$_2$O and 2.5 mL of PAHBAH solution, heated for 4 min at 95 °C, diluted immediately with 5 mL ddH$_2$O and mixed. For sucrose determination, 50 µL of diluted extract were mixed with 100 µL sucrase solution (enzyme) and 100 µL sodium maleate buffer (100 mM, pH 6.5), heated at 40 °C for 30 min, diluted with 2.5 mL PAHBAH solution, heated again for 4 min at 95 °C and finally mixed with 5 mL ddH$_2$O. For hydrolysis and measurement of fructan concentration, samples of 1 mL of undiluted extract were mixed with 3 mL ddH$_2$O and 1 mL 1M HCl. The mixture was placed in a water bath at 95 °C for 15 min and then 1 mL of the hydrolysate was mixed with 1 mL of ddH$_2$O. For fructan measurement, 50 µL of the diluted hydrolysate was added to 200 µL ddH$_2$O and 2.5 mL PAHBAH solution. After 4 min at 95 °C this was mixed with 5 mL of ddH$_2$O. Absorbance was read against blank at 415 mm using spectrophotometer SPECORD 50 (Analytic Jena AG, Jena, Germany).

Determination of pyruvic acids

Due to the high correlation between both, volatile sulfur flavor components in onions are measured by a simple indirect method of determining enzymatically-produced pyruvic acid concentration in disrupted onion tissue (SCHWIMMER and WESTON, 1961). Enzymatically produced pyruvic acid concentration in the filtrated juice was measured using dinitrophenylhydrazine (DNPH) reagent according to the improved method of ANTHON and BARRETT (2003) and background pyruvic acid after deactivation of allinase by heating onion tissues in a microwave according to the method of YOO and PIKE (2001). A spectrophotometric assay was carried out using 25 µL of filtrate with 1 mL of ddH$_2$O and 1 mL of DNPH solution (12.5 mg DNPH / 50 mL 1 M HCl). The reaction mixture was placed in a water bath at 37 °C for 10 min and then 1 mL of 1.5 M NaOH was added. The absorbance was measured at 515 nm. The standard curve was prepared from a series of sodium pyruvate (0, 1, 2, 4, 6, 8 mM). The concentration of enzymatically produced pyruvic acid was calculated by subtracting the background concentration of pyruvic acid from the total pyruvic acid concentration. The results were expressed as µmol g^{-1} fresh weight (FW).

Selection of onion samples for the taste-panel assessment was assigned based on pyruvic acid results according the classification of CROWTHER et al. (2005). Onions with a pyruvic acid level below 4.0 µmol g^{-1} FW were classified as sweet, those with a level between 4.0 and 7.0 µmol g^{-1} FW as mild, and onions with levels more than 7.0 µmol g^{-1} FW were classified as pungent.

Determination of total phenolic content (TPC)

Phenolic compounds were extracted according to SANTAS et al. (2008), with some modifications. Aliquots of 0.15 g of freeze-dried onion powder were added to 1.5 mL ethanol:water (75:25 v/v). After 30 min with 900 rpm magnetic stirring at room temperature and 20 min sonification in an ultrasonic bath, the extract was centrifuged at 3000 rpm for 15 min. The whole extraction procedure was repeated twice with magnetic stirring for 45 and 90 min, respectively. The pooled supernatant fractions were stored at -20 °C in the dark for further analyses.

Total phenolic content was determined in triplicate using the Folin-Ciocalteau spectrophotometric method as follows: 200 µL of the diluted ethanolic onion extract (100 µL ethanol + 100 µL extract 1:2 v/v) was mixed with 1.5 mL diluted Folin-Ciocalteu reagent (dilution 1:10 reagent / water v/v). Samples were mixed properly and allowed to stand for 10 min at room temperature (20 °C). Then 1.5 mL of a 2% sodium carbonate solution was added. The mixture was incubated in the dark at room temperature for 120 min and the absorbance was measured at 765 nm against the blank, containing 75% ethanol instead of the sample extract. The standard curve was prepared from a series of gallic acid (GAE) standards (0, 20 50, 80, 100, 120, 200 mg/L) and the results were expressed as mg GAE g^{-1} dry weight (DW).

Determination of antioxidant activity

The radical scavenging activity of the extracts was carried out according to BRAND-WILLIAMS et al. (1995) using the '2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method. The DPPH reagent was prepared by sonication of 0.1 mM of DPPH in 75% ethanol for 1 h in an ultrasonic bath. 200 µL of the diluted ethanolic onion extract was mixed with 2 mL of DPPH reagent and incubated for 60 min in the dark at room temperature. Absorbance was read at 515 nm against the blank, containing 75% ethanol instead of the sample extract. The standard curve was prepared from a series of trolox standard solutions (0, 50, 100, 200, 300 µM) and the results were expressed as µmol Trolox Equivalent (TE) g^{-1} DW.

Determination of mineral elements and ash content

Mineral concentrations (K, Mg, and Ca) were determined using 300 mg of freeze-dried onion bulb material solubilized in 10 mL of 69% nitric acid by microwave digestion at 190 °C for 25 min (MARS 5; CEM Cooperation, Matthews, NC, USA). The solution was filtered and diluted to a final volume of 100 mL. Cations were measured using an atomic absorption spectrometer (3300 series; Thermo Fisher Scientific, Dreieich, Germany).

To determine the ash, approximately 100 mg of freeze-dried onion bulb material was placed into a ceramic crucible. Samples were placed into a muffle furnace for at least 7 h at 550 °C. After that, the samples were cooled in a desiccator and weighed to determine ash percentage.

Pungency taste-panel assessment

Taste-panel was conducted by twelve members of the Institute of Crop Science (Viticulture). Three samples were used for the triangle and ranking test. One sample was an onion variety classified as "sweet" with significant low levels of enzymatically produced pyruvic acid and the other two samples were the same variety, classified as "pungent" with very high levels of enzymatically produced pyruvic acid. For the triangle tests, panelists were asked to ingest a similar amount of the homogenized samples and then identify the deviating sample. Through the ranking test the panelists were then asked to assign scores to the samples by ranking them from lowest to highest for flavor intensity, with the rating: 1, mild; 2, slightly pungent; 3, pungent. Data was evaluated using the expanded tables for multiple comparison procedures in the analysis of ranked data from NEWELL and MACFARLANE (1987).

Statistical analysis

One-way ANOVA (Analysis Of Variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) were used for comparing multiple treatments. Differences were considered as significant when $p < 0.05$.

Correlation matrix and principal component analysis (PCA) were used to determine the relationship between the flavor/quality attributes and provide a graphical description of the varieties characteristics. PCA was applied to the centralized and standardized values of all eleven varieties. Standardization and centralization was necessary because of the different magnitudes and weight of the values. All analyses were performed using R (R DEVELOPMENT CORE TEAM, 2016).

Results

Onion Yield and Agronomic Traits

Mean yields of the best performing "Jaune des Cévennes" and the least performing "Red Baron" varieties were 33.3 and 5.52 t * ha^{-1} respectively (Fig. 2A). Jaune des Cévennes and the other studied varieties developed bulbs generally larger than 30 mm diameter, with an exception of Red Baron, which mostly yielded smaller onion bulbs. The highest yield was obtained for Jaune des Cévennes, but >14% was not marketable because of the high bolting percentage. The shortest time to achieve physiological maturity (158 days) was observed with Birnförmige, while Jaune des Cévennes, Red Baron, Bolstar, Erfurter Lager and Wiener Bronzekugel required more than 175 days from sowing to physiological maturity (Tab. 2).

Diseases and disorders were observed only in the variety Jaune des Cévennes. Symptoms of sour skin and neck rot were detected with a high number of bulbs in this variety (Fig. 2B).

Morphological Traits of the Bulbs

The morphology of the bulbs was significantly different between the varieties for the following traits: dry weight, diameter, height, number of tunics, firmness and total soluble solids.

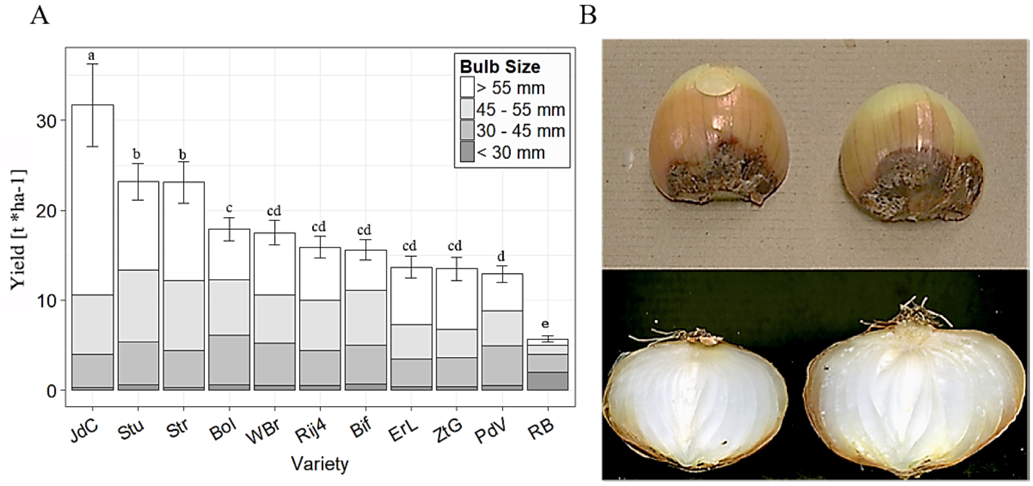


Fig. 2: **A:** Onion total yield and bulb size. Stacked bar graph in which the overall height of the bar indicates the total yield, data are mean ± SE, n = 4, letters indicate significant differences among the varieties determined by "Post-hoc" test ($p < 0.05$). The height of each segment (coded as shown in the figure) represents the amount of bulbs sorted by size in a variety. Jaune des cévennes (JdC), Stunova (Stu), Sturon (Str), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB). **B:** Diseases and disorders observed during curing and storage of onion bulbs (Jaune des Cévennes).

Significant differences were found among the onion varieties in their mean diameter and height. Birnförmige is an oval, pear-shaped variety (Fig. 1), and for that reason its height is with 66 cm significantly bigger than that of the other onion varieties. In contrast, because of their flat shape, both, Jaune des Cévennes with 61.8 cm and Paille des Vertus with 60.2 cm, have notably bigger diameters compared to other varieties (Tab. 3).

In terms of dry matter, total soluble solids, number of tunics, and firmness Jaune des Cévennes showed the lowest significant values compared to the other varieties. On the other side of the spectrum, Birnförmige and Paille des Vertus showed the highest content of dry matter, TSS, as well as good bulb firmness (Tab. 3).

Quality Traits

Bulb total sugar concentration ranged from 68.3 (Jaune des Cévennes) to 150.7 mg g^{-1} FW (Birnförmige). Total sugar (Fig. 3A) consisted mainly of fructans (Fig. 3B), where concentrations ranged between 7.8 (Jaune des Cévennes) and 135.4 mg g^{-1} FW (Birnförmige), sucrose concentrations (Fig. 3D) ranged between 1.9 (Jaune des Cévennes) and 13.5 mg g^{-1} FW (Sturon), reducing sugar concentrations (Fig. 3C) ranged between 8.7 (Birnförmige) and 58.3 mg g^{-1} FW (Jaune des Cévennes).

Fig. 3E shows the results of antioxidant activity by the commonly used method DPPH- in ethanol solvents. The variety "Red Baron" had the highest value of antioxidant activity (13.7 µmol TE g^{-1} DW) and "Sturon" the lowest activity (4.8 µmol TE g^{-1} DW).

Total phenolic concentrations (Fig. 3F) varied in the range of 2.8 mg GAE g^{-1} DW to 6.9 mg GAE g^{-1} DW for the variety "Jaune des Cévennes" and "Red Baron" respectively.

Enzymatically produced pyruvic acid of the entries (Fig. 4A) ranged between 2.9 (Jaune des Cévennes) and 7.4 µmoles g^{-1} FW (Birnförmige). Jaune des Cévennes was the only variety which could be categorized as "sweet", according to its pyruvic acid level results.

Tab. 2: Agronomic parameters of the varieties.

Variety	Number of Bulbs / Plot	Bolting (%)	Days to physiological maturity after sowing
JdC	184.2 ± 30.9 ab	14.1 ± 5.1 a	177.5 ± 1.7 a
Stu	187.0 ± 53.0 ab	0.11 ± 0.2 b	164.2 ± 3.9 bc
Str*	176.0 ± 17.6 ab	0.00 ± 0.0 b	161.0 ± 6.0 c
Bol	191.7 ± 38.3 a	0.11 ± 0.2 b	178.5 ± 6.8 a
WBr	158.0 ± 11.16 ab	1.28 ± 0.6 b	182.0 ± 3.5 a
Rij4	140.5 ± 45.2 abc	0.00 ± 0.0 b	175.2 ± 3.8 ab
Bif	148.7 ± 21.6 abc	0.00 ± 0.0 b	158.0 ± 0.0 c
ErL	113.7 ± 7.2 bc	0.69 ± 0.9 b	178.5 ± 6.5 a
ZtG	118.7 ± 24.4 abc	0.00 ± 0.0 b	161.0 ± 0.0 c
PdV	136.5 ± 39.1 abc	0.14 ± 0.3 b	165.5 ± 9.0 bc
RB*	76.75 ± 25.8 c	0.00 ± 0.0 b	185.2 ± 1.5 a

Data are mean ± SE, n = 4. Significant test by Tukey's HSD ($p < 0.05$) are indicated by different letters. Jaune des Cévennes (JdC), Stunova (Stu), Sturon (Str), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB). *Control varieties.

Tab. 3: Morphological parameters of onion bulbs.

Variety	Dry matter (%)	Diameter (mm)	Height (cm)	Number of tunics	Firmness (Shore)	TSS (°Brix)
JdC	9.2 ± 0.4 g	61.8 ± 1.5 a	48.6 ± 1.4 de	1.4 ± 0.1 d	84.4 ± 1.0 b	8.2 ± 0.3 g
Stu	14.3 ± 0.8 d	58.4 ± 1.6 abc	55.7 ± 1.4 bc	2.0 ± 0.1 ab	91.0 ± 1.7 a	12.7 ± 0.6 cd
Str*	15.7 ± 0.2 bc	58.5 ± 1.0 abc	55.6 ± 0.7 bc	2.0 ± 0.1 ab	90.3 ± 2.4 a	14.2 ± 0.3 b
Bol	11.9 ± 0.4 ef	57.8 ± 0.4 bc	53.6 ± 1.7 bc	2.0 ± 0.1 ab	93.3 ± 1.3 a	11.0 ± 0.1 ef
WBr	12.6 ± 0.1 e	55.4 ± 0.8 cd	57.0 ± 2.9 b	2.1 ± 0.2 ab	92.7 ± 0.7 a	12.0 ± 0.2 de
Rij4	12.5 ± 0.6 e	58.5 ± 1.4 abc	52.8 ± 0.8 c	2.1 ± 0.1 ab	90.0 ± 0.8 a	11.9 ± 0.7 de
Bif	18.1 ± 0.1 a	48.8 ± 1.1 e	66.0 ± 1.6 a	2.0 ± 0.0 ab	90.2 ± 1.2 a	16.4 ± 0.3 a
ErL	14.2 ± 0.3 d	59.0 ± 0.8 ab	52.7 ± 2.8 cd	1.8 ± 0.2 bc	93.8 ± 1.2 a	13.3 ± 0.3 bc
ZtG	14.9 ± 0.6 cd	58.0 ± 0.7 bc	46.3 ± 1.2 e	2.2 ± 0.2 a	91.9 ± 0.4 a	13.4 ± 0.4 bc
PdV	16.9 ± 0.8 ab	60.2 ± 1.1 ab	35.6 ± 0.7 f	1.7 ± 0.0 cd	93.1 ± 2.1 a	15.6 ± 0.6 a
RB*	11.1 ± 0.5 f	53.2 ± 3.2 d	54.0 ± 1.6 bc	2.1 ± 0.1 ab	90.8 ± 4.2 a	10.7 ± 0.6 f

Data are mean ± SE, n = 4. Significant test by Tukey's HSD ($p < 0.05$) are indicated by different letters. TSS: Total soluble solids. Jaune des Cévennes (JdC), Stunova (Stu), Sturon (Str), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB). *Control varieties.

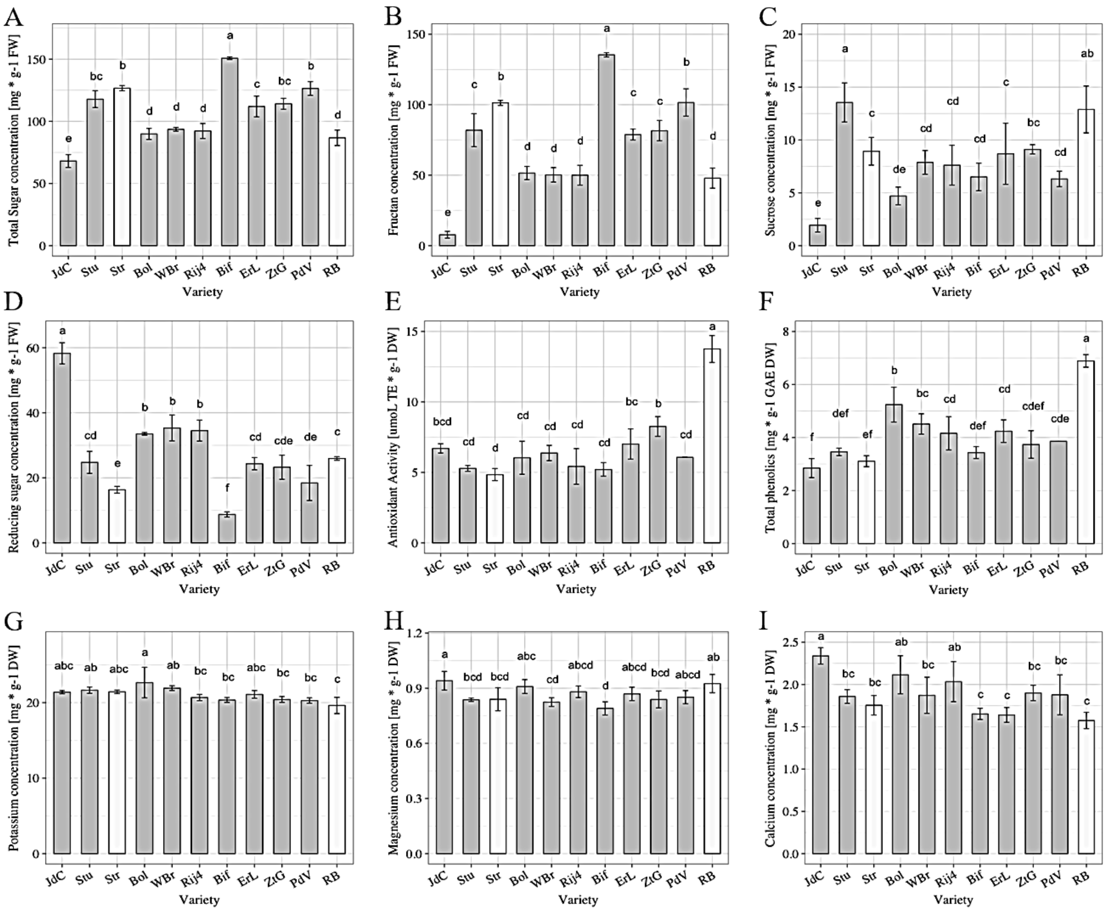


Fig. 3: Quality traits. **A:** Total sugar concentration, **B:** fructans, **C:** sucrose, **D:** reducing sugar as well as **E:** antioxidant activity, **F:** total phenolics, **G:** potassium, **H:** magnesium and, **I:** and calcium of the studied onion variaties. Data are mean ± SE, n = 4. Significant test by Tukey's HSD ($p < 0.05$) are indicated by different letters. Control varieties Sturon and Red Baron are highlighted as blank barplots. Jaune des Cévennes (JdC), Stunova (Stu), Sturon (Str), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB). DW = dry weight; FW = fresh weight; GAE = gallic acid equivalent; TE = Trolox Equivalent.

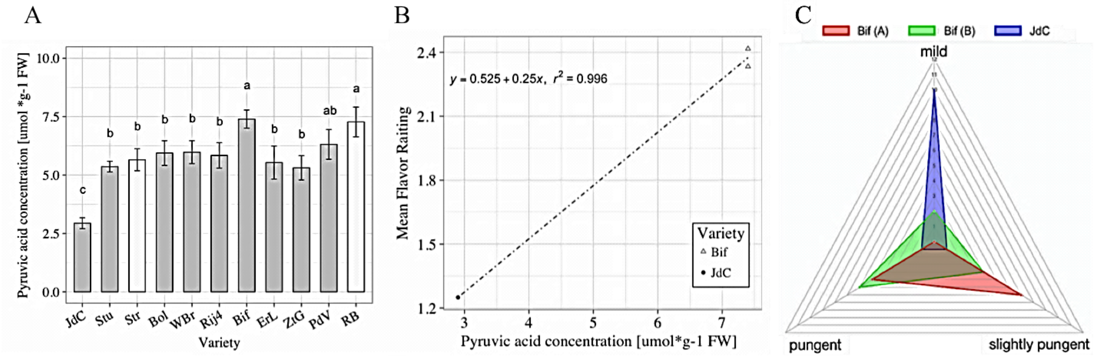


Fig. 4: Pungency of onions. **A:** Enzymatically produced pyruvic acid. Data are mean ± SE, n = 4. Significant test by Tukey's HSD ($p < 0.05$) are indicated by different letters. Control varieties Sturon and Red Baron are highlighted as blank barplots. Jaune des Cévennes (JdC), Stunova (Stu), Sturon (Str), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB). FW = fresh weight. **B:** Relationship between pungency perception and enzymatically produced pyruvic acid by two onion varieties Birnförmige (Bif), and Jaune des Cévennes (JdC). Regression coefficients are significantly different from zero ($p < 0.05$). Flavor rating scale: 1 = mild, 2 = slightly pungent, 3 = pungent. **C:** Radar chart of the sensory analysis, the samples of the onion varieties Birnförmige: Bif A (red), Bif B (green) and Jaune des Cévennes: JdC (blue), were categorized as mild, slightly pungent and pungent as described in the radar chart.

Most varieties fall into the category “mild”, except Birnförmige and Red Baron, which were categorized as “pungent”.

Depending on the enzymatically produced pyruvic acid concentrations, two varieties were selected for the pungency sensory taste panel. Fresh homogenates of the variety Jaune des Cévennes (sweet) and double samples of Birnförmige (pungent) were used for the triangle and ranking test. Results of the triangle test showed that nine of the twelve panelists identified the variety “Jaune des Cévennes” as the deviating sample. By averaging scores (1, mild; 2, slightly pungent; 3, pungent) given during the ranking test, Fig. 4B shows a significant high linear correlation (r = 0.99) between pyruvic acid levels and pungency taste-panel assessment. Fig. 4C shows the number of panelists (12) and their assignment of categories for flavor intensity during the ranking test. Ten from twelve members assessed the variety Jaune des Cévennes with the score 1, mild, while the double sample of the variety Birnförmige were assessed by the majority of the panelists either as 2, slightly pungent; or as 3, pungent.

Results of bulb mineral contents are shown in Fig. 3 G - I. Potassium, calcium and magnesium concentrations ranged between 19.6 (Red Baron) to 22.6 mg g^{-1} (Bolstar), 1.5 (Red Baron) to 2.3 mg g^{-1} (Jaune des Cévennes), and 0.79 (Birnförmige) to 0.94 mg g^{-1} DW (Jaune des Cévennes) respectively.

The principal component analysis (Fig. 5) summarizes information relating to all quality traits, *i.e.*, dry matter content, TSS, fructans, enzymatically produced pyruvic acid, total phenolics, antioxidant activity and reducing sugars. PC1 explained 61.3% of the total variance in the data set, while PC2 explained 25.0%. The correlation circle groups the most correlated varieties (Stunova, Sturon, Bolster, Wiener Bronzekugel, Rijnsburger 4, Erfurter Lager, Zittauer Gelbe, Paille des Vertus). Only three varieties, Birnförmige, Red Baron and Jaune des Cévennes, lie outside the circle, showing significantly different quality characteristics than the rest. The location of Birnförmige in the upper right-hand quadrant can be explained by its high values of dry matter, TSS, fructans, pyruvic acid and sucrose. In strong contrast, Jaune des Cévennes is on the negative side of PC1, which is a result of overall higher reducing sugar concentration and lower concentration of fructans, dry matter content, TSS and pyruvic acid. Red Baron, which is located in the lower quadrant of the plot is characterized by its high total phenolics and antioxidant activity levels.

The correlation matrix based on all quality trait data (Fig. 6) shows a high significant positive correlation (r > 0.95) between fructans, dry matter content and TSS. High significant negative correlation was observed between reducing sugar, fructans, dry matter content and TSS (r > 0.85). Pyruvic acid had a positive correlation with sugar, except reducing sugars, total phenolics and antioxidant activity. On the other hand, enzymatically produced pyruvic acid correlated negatively with potassium, magnesium, and calcium (r = -0.27, -0.41, and -0.55, respectively). A negative correlation could be also found by comparing fructans, dry matter content and TSS with potassium, magnesium, and calcium (r < -0.45). The element concentrations of calcium and magnesium showed a significantly positive correlation with reducing sugars (r > 0.52).

Discussion

Screening onion landraces and open-pollinated cultivars in Western Europe is an effective way of collecting material for future breeding programs. Onion varieties with high yield stability, high concentration of nutritional compounds and good flavor are in high demand. This study collected a comprehensive amount of data, from onion production on the field up to the evaluation of the quality and flavor parameters, including a sensory taste panel. Quality, as well as morphological and yield differences between the varieties indicate that the selected samples used in this study could represent a valuable source of information for breeding purposes. The characterization

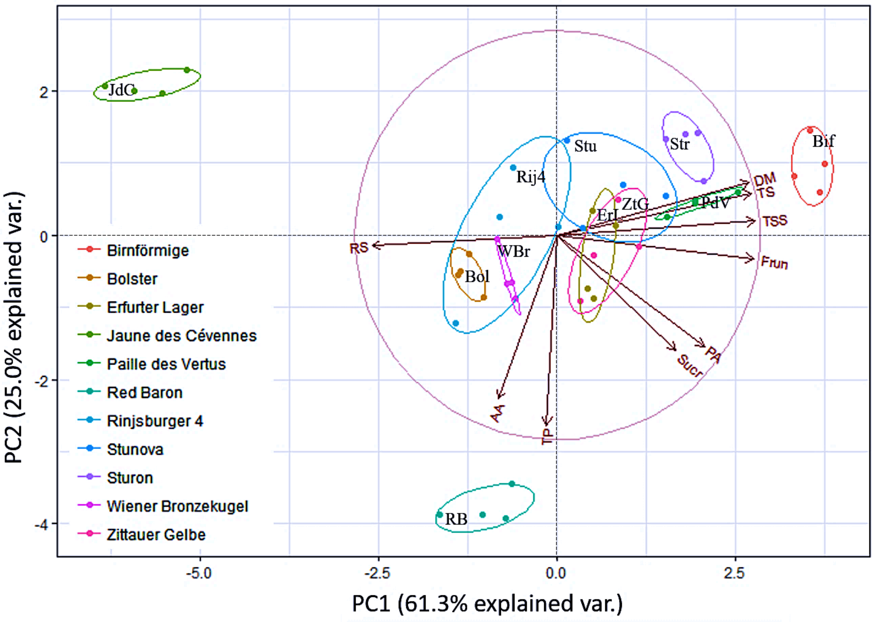
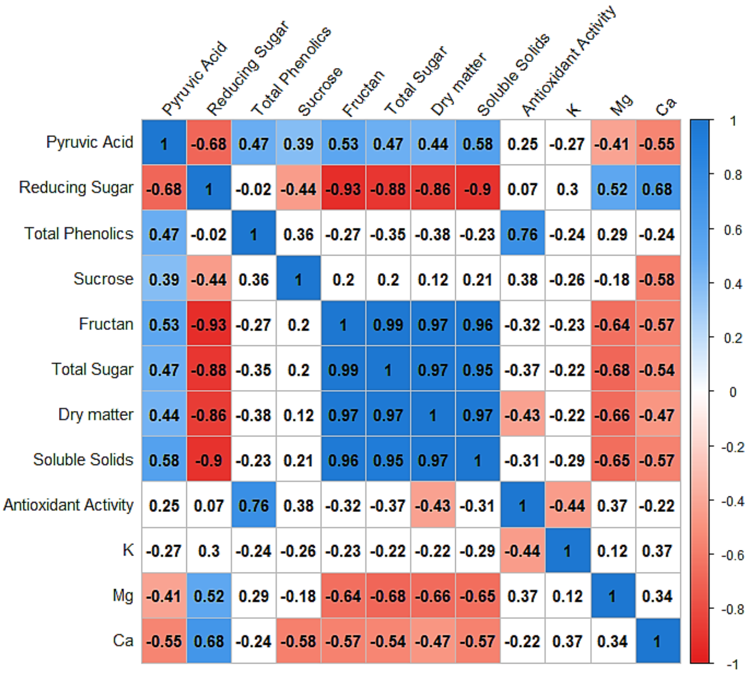


Fig. 5: Principal component analysis (PCA), describing the relationship among varieties depending on their quality traits. Quality traits: DM = Dry Matter, TS = Total Sugar, TSS = Total soluble solids, Frun = Fructan, PA = enzymatically produced pyruvic acid, Sucr = Sucrose, TP = Total Phenolics, AA = Antioxidant Activity, RS = Reducing Sugar. Onion varietiesare grouped by colors as shown in the figure. Points represent the biological replications of each variety n = 4. Jaune des cévennes (JdC), Sturon (Str), Stunova (Stu), Bolstar (Bol), Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij4), Birnförmige (Bif), Erfurter Lager (ErL), Zitauer Gelbe (ZtG), Paille des Vertus (PdV), Red Baron (RB).



	Pyruvic Acid	Reducing Sugar	Total Phenolics	Sucrose	Fructan	Total Sugar	Dry matter	Soluble Solids	Antioxidant Activity	K	Mg	Ca
Pyruvic Acid	1	-0.68	0.47	0.39	0.53	0.47	0.44	0.58	0.25	-0.27	-0.41	-0.55
Reducing Sugar	-0.68	1	-0.02	-0.44	-0.93	-0.88	-0.86	-0.9	0.07	0.3	0.52	0.68
Total Phenolics	0.47	-0.02	1	0.36	-0.27	-0.35	-0.38	-0.23	0.76	-0.24	0.29	-0.24
Sucrose	0.39	-0.44	0.36	1	0.2	0.2	0.12	0.21	0.38	-0.26	-0.18	-0.58
Fructan	0.53	-0.93	-0.27	0.2	1	0.99	0.97	0.96	-0.32	-0.23	-0.64	-0.57
Total Sugar	0.47	-0.88	-0.35	0.2	0.99	1	0.97	0.95	-0.37	-0.22	-0.68	-0.54
Dry matter	0.44	-0.86	-0.38	0.12	0.97	0.97	1	0.97	-0.43	-0.22	-0.66	-0.47
Soluble Solids	0.58	-0.9	-0.23	0.21	0.96	0.95	0.97	1	-0.31	-0.29	-0.65	-0.57
Antioxidant Activity	0.25	0.07	0.76	0.38	-0.32	-0.37	-0.43	-0.31	1	-0.44	0.37	-0.22
K	-0.27	0.3	-0.24	-0.26	-0.23	-0.22	-0.22	-0.29	-0.44	1	0.12	0.37
Mg	-0.41	0.52	0.29	-0.18	-0.64	-0.68	-0.66	-0.65	0.37	0.12	1	0.34
Ca	-0.55	0.68	-0.24	-0.58	-0.57	-0.54	-0.47	-0.57	-0.22	0.37	0.34	1

Fig. 6: Correlation matrix of the most important quality traits in the studied onion varieties. Correlations with p-value < 0.01 are considered as significant and are highlighted: Red = negative correlation, Blue = positive correlation. Non significant coefficient values are not highlighted.

of "traditional varieties" provides valuable information in order to preserve and maintain the local genetic variability and therefore act against genetic erosion.

How big are the differences and potential of quality parameters among the varieties?

The study places particular emphasis on differences in distinct quality parameters of open-pollinated varieties by analyzing the dry matter content, total soluble solids (TTS), non-structural carbohydrates, enzymatically produced pyruvic acid and total phenolics. The resulting data showed significant differences between all onion varieties, but the largest deviation was identified with three varieties, in particular, Red Baron, Birnförmige and Jaune des Cévennes, where results varied significantly outside of the correlation circle as shown in Fig. 5.

Flavor and quality of onions are determined by the concentration and interaction between sulfur compounds and carbohydrates (McCallum et al., 2010; Vagen and Slimestad, 2008). Both of these compounds were responsible for the significant differences in quality found among the eleven varieties. In the variety "Birnförmige", high concentration of pyruvic acid and fructan, as well as high dry matter content were observed, meeting the "dehydrated onion" criteria. By contrast, Jaune des Cévennes showed lower pungency, lower dry matter content and higher concentration of reducing sugars, which are distinctive characteristics of a "sweet onion" (Mallor et al., 2011). "Sweet onions" are also well-known for their lower storability due to higher water content (Suzuki and Cutcliffe, 1989), which is also a primary reason for their rapid degradation. Moreover, pathogen symptoms were found solely in Jaune des Cévennes after a few weeks of postharvest storage.

Early studies already confirmed the positive high correlation between mean pungency rating and enzymatically produced pyruvic acid (Crowther et al., 2005; Wall and Corgan, 1992). Differences in the pyruvic acid concentration among the varieties were also detected through our pungency taste panel. These were determined by tasting the two opposing onion varieties. The mildness of Jaune des Cévennes against the pungency of Birnförmige was clearly perceived by the test persons, giving a high correlation between enzymatically produced pyruvic acid and taste pungency ($r^2 = 0.99$).

Within the eleven studied onion varieties, Red Baron stands out significantly due to elevated total phenolics concentration and almost 3-fold higher difference of antioxidant activity levels *e.g.* compared to the yellow variety "Sturon". Similar studies with various onion varieties have demonstrated that red onions had higher total phenolics concentrations and antioxidant activity levels than yellow and white varieties, because of anthocyanins present only in red onions (Lee et al., 2015; Lisanti et al., 2016; Sharma et al., 2015). At least 25 different anthocyanins have been reported for red onions, representing approximately 10% of the total flavonoid content (Fuleki, 1971; Slimestad et al., 2007).

Is there a link between the quality components of the studied onion varieties?

A study of correlation between all analyzed quality parameters was carried out to discover the link between individual quality compounds in fresh onion bulbs. The high positive correlations between fructans, total sugar and TSS revealed that the non-digestible carbohydrates are the main carbohydrate fraction, contributing to more than 60% of the total sugar concentration, in almost all onion varieties, except for Jaune des Cévennes. This variety demonstrated an opposite behavior with high levels of reducing sugar (glucose, fructose) and lower levels of fructans and dry matter. This positive correlation confirms the results of a study of Jaime et al. (2001), showing a very high positive correlation between fructans, TSS, total sugar and dry matter (r = 0.97) and a high negative correlation between reducing sugar, TSS, total sugar, and dry matter (r = 0.86).

It has been speculated that the more pungent the onion, the higher the potential of health benefits, because of the strong concentration of the volatile sulfur compounds in these varieties (LEE et al., 2015). In our results, however, we found that the most pungent onion “Birnförmige” did not have the highest antioxidant levels. This confirms the results of LEE et al. (2015), who observed only a low positive correlation between antioxidant activity levels and enzymatically produced pyruvic acid (r = 0.25). This could be due to phenolic compounds, which were found to be the primarily responsible for the antioxidant activity. The positive correlation between total phenolics and antioxidant activity is already known from previous studies (ASAMI et al., 2003; CHENG et al., 2013; CHU et al., 2002). In our study we were also able to confirm that phenolics represent a considerable part of the total antioxidant activity (r = 0.76).

The elemental composition of the edible onion parts has not been thoroughly studied so far. Nevertheless, there are studies demonstrating a correlation among minerals and trace elements (RODRÍGUEZ GALDÓN et al., 2008) but no information is available regarding the correlation between minerals and other quality compounds in onions. Interestingly, significant positive correlations were found between calcium, magnesium and reducing sugar (r = 0.68, 0.52 respectively) and significant negative correlations between calcium, magnesium and fructans (r = -0.57, -0.64 respectively). A significant negative correlation could also be confirmed between calcium, magnesium and pyruvic acid, which would mean that obtaining sweeter onion through calcium or magnesium fertilization might be a possibility.

Which varieties are better suited for organic farming in South-Germany?

Our results demonstrated that, with the exception of the variety “Jaune des Cévennes”, most of the researched varieties adapt well to the climate conditions of South West Germany. For example, the variety “Birnförmige” produced an acceptable yield with onions free from bolting and diseases, good firmness, high concentration of fructan, pyruvic acid, dry matter and sucrose in less than 160 days.

“Jaune des Cévennes”, despite the high yields, exhibited high bolting percentages and a large number of unmarketable onions, with high risk of diseases and disorders. As ZEVEN (1998) mentioned in his article: “Landraces are a population which naturally developed in a certain region under the influence of regionally prevailing conditions of climate, soil management, without or with little mass selection”. This allows us to speculate why the old French variety “Jaune des Cévennes”, which comes from the mountainous region of The Cévennes (elevation 1700 m) and different climate conditions, may be better suited for planting on higher altitude terraces than the region of Stuttgart (elevation 245 m) in the South-West of Germany.

Through the study a clear trend was established where most of the varieties and old open-pollinated cultivars from South-Germany performed similar or better compared to the commercial cultivars (Sturon and Red Baron) in both quality parameters and yield, therefore demonstrating the potential to provide an important contribution to the regional organic farming system.

Conclusions

The focus of this study is the analytical characterization of local open-pollinated onion types, while comparing them against commercial, well-established varieties and examining their agricultural, morphological and nutritional traits.

Qualitative and quantitative analytical differences that were detected among the varieties allowed a clear discrimination and identifications of the cultivars particularly suitable for organic farming based on their agricultural and quality performances. High variability and correlations found in the examined traits point out that these onion varieties could be well-suited candidates for future studies. Yield value from the old open-pollinated varieties were significantly higher than control varieties Sturon and Red Baron, indicating that the maximum yield potential of the control varieties has not yet been reached and an optimization of their yield could be attained though breeding programs. Among the 11 varieties, Birnförmige is the most promising in terms of quality, aroma and flavor. In contrast, the cultivar Jaune de Cévennes showed poor quality and small concentrations of sugar and pyruvic acid.

Nonetheless, further study of molecular and metabolome characterization should be conducted. Morphological, analytical and agricultural research accompanied with molecular and metabolome characterization will result in broader dataset, able to clarify the genetic variability within and among the varieties.

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Address of the authors:
Maria Romo Pérez, Nikolaus Merkt, Christian Zörb, Department of Quality of Plant Products, Institute of Crop Science, University of Hohenheim, Schloss Hohenheim 1, 70599 Stuttgart, Germany
E-Mail: christian.zoerb@uni-hohenheim.de
Sabine Zikeli, Coordination for Organic Farming and Consumer Protection, Institute of Crop Science, University of Hohenheim, Fruwirthstrasse 14, 70599 Stuttgart

Supplementary material

Supplementary material

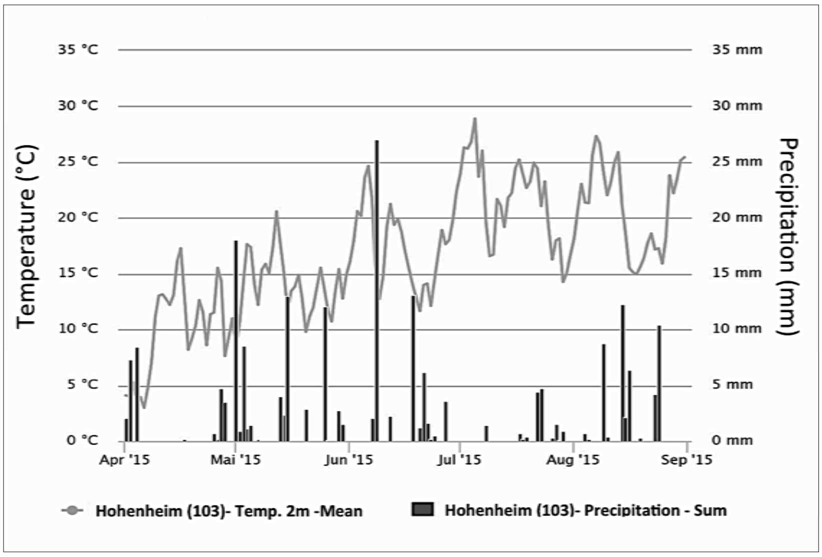

Fig. S1: Daily mean precipitation and temperature over the main onion growing season in Kleinhohenheim, Stuttgart, Germany. Source: Agrarmeterologie Baden-Württemberg.

Chapter 3 – Metabolite profiling of onion landraces and the cold storage effect

M.L. Romo-Pérez, C.H. Weinert, M. Häußler, B. Egert, M.A. Frechen, B. Trierweiler, S.E. Kulling, C. Zörb

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Research article

Metabolite profiling of onion landraces and the cold storage effect

M.L. Romo-Pérez[a], C.H. Weinert[b,*], M. Häußler[b], B. Egert[b], M.A. Frechen[b], B. Trierweiler[b], S.E. Kulling[b], C. Zörb[a]

[a] University of Hohenheim, Institute of Crop Science, Quality of Plant Products 340e, Schloss Westflügel, 70599, Stuttgart, Germany
[b] Max Rubner-Institut, Department of Safety and Quality of Fruit and Vegetables, Haid-und-Neu-Straße 9, 76131, Karlsruhe, Germany

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ABSTRACT

Today, commercial onion breeders focus almost entirely on conventional farming which reduces diversity in the market and leads to loss of desirable traits such as those that impact nutritional and sensory aspects of onions. A way to preserve phenotypic and genetic diversity is to re-evaluate traditional landraces to introduce their benefits to the broader public. Common onion genotypes vary greatly in their storability. In particular, temperature and relative humidity during storage have significant impact on the metabolites in onions after storage. The aim of this study was to assess changes in the metabolite profile of ten onion genotypes after five months of cold storage. In addition, a characterization of onion landraces in their fresh state was also conducted in order to compare their properties against a commercial genotype. Onion genotypes were grown under organic farming conditions. After harvest and curing, bulbs were stored for up to 22 weeks. Before and after storage, bulb samples were analyzed through targeted and untargeted methods. Out of 189 identified metabolites, 128 showed a storage effect. Mainly fructans decreased because of respiration and energy demand, while monosaccharides increased. Further, amino acids were altered in their concentration after storage with an effect on aroma precursors. Eight of the nine landraces had good storability without critical losses. In their fresh state, the onion genotypes clustered into three major groups. For instance, landraces of group III showed consistently and substantially higher levels of amino acids and certain sugars, indicating a high potential of aromatic properties in those onion landraces.

1. Introduction

Onion (*Allium cepa* L.) is the second most important vegetable crop after tomato, with a total world production of 98 million tons of dry bulbs in 2017, a figure that doubled since the year 2000 (FAOSTAT, 2019). It is difficult to trace the exact origin of onions to a single source. Over time, they have adapted to different climates, temperatures and photoperiods, creating a wide range of genotypes and landraces, as well as establishing the species as one of the few with likely worldwide domestication. With the modernization of agriculture, farmers have increasingly focused on growing hybrids and abandoned open-pollinated genotypes and landraces, leading to genetic erosion. A way to maintain biodiversity as well as genetic variability and metabolite diversity is to preserve landraces. Through their distinct properties, these genotypes are drawing the interest of farmers and consumers alike, making them a viable alternative to mainstream genotypes and hybrids (Petropoulos et al., 2015; Baldina et al., 2016; Klee and Tieman, 2013).

Beside genetic predisposition, multiple pre- and post-harvest factors are linked to long-term storage of onions and their possible change of metabolite concentrations during curing and storage of the bulbs (Petropoulos et al., 2017). Development of onions after harvest is typically divided into three phases: rest, dormancy and regrowth. Prolonging bulb dormancy in particular is the aim of post-harvest technologies such as cold storage, which seeks to extend durability of onion during lengthy product-marketing periods. The main principle of cold storage is to maintain the bulbs between 2 and 5 °C temperature and medium to high relative humidity (RH 65–70 %), preventing early sprouting and rooting. However, depending on the genotype, cold storage does also significantly affect quality such as metabolite profile and aroma, as well as water content of onion bulbs (Petropoulos et al., 2016; Brewster, 2008).

The unique flavor and the extraordinary nutritional attributes of onions have recently peaked interest in their chemical composition. Over the past few years, new methods were developed to identify and quantify large numbers of metabolites in onion bulbs (Soininen et al., 2014; Pöhnl et al., 2017; Böttcher et al., 2017). In particular, the application of metabolomics techniques enables a comprehensive compositional analysis and the detection of changes in the onion metabolite

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[*] Corresponding author.
E-mail address: christoph.weinert@mri.bund.de (C.H. Weinert).

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profile caused by different conditions or treatments. Gas chromatography mass spectrometry (GC-MS) is especially suitable for the analysis of primary metabolites and thus study of general metabolic state of a biological system (Papadimitropoulos et al., 2018). Compared to one-dimensional GC-MS, comprehensive two-dimensional gas chromatography mass spectrometry (GC × GC-MS) possesses higher sensitivity and an improved chromatographic separation performance, making it very useful for the analysis of complex samples (Weinert et al., 2015) as previously described in similar studies on tomato genotypes and kiwi fruits (Wojciechowska et al., 2014; Mack et al., 2017). Onions contain a considerable number of primary and secondary metabolites, some of them having a significant impact on human health. They are rich in flavonoids, sulfur-compounds and non-structural carbohydrates. The latter in particular accou for up to 80 % of the bulbs dry weight and, because of a lack of starch, they serve as a major energy reserve. Transformation of onion carbohydrate and sulfur metabolism during storage has been widely studied, but little is known about the changes in free amino acid and organic acid profiles during mid- or long-term storage (Hansen, 2001; SalamalHicks and Nock, 1990).

In this study, for the first time, an established GC × GC-MS workflow (Weinert et al., 2015) and targeted methods were used to explore: (*i*) the impact of cold storage on the metabolite profile of onions; (*ii*) the storability of onion landraces; (*iii*) bulb composition of onion landraces in their fresh state, compared with a reference cultivar.

2. Materials and methods

2.1. Plant material and field experiment

The following ten onion genotypes were field grown; Birnförmige (Bif), Paille des Vertus (PdV), Sturon (StR), Erfurter Lager (ErL), Stunova (Stu), Zittauer Gelbe (ZiG), Bolstar (Bol), Rijnsburger 4 (Rij), Wiener Bronzekugel (WBr), Jaune des Cévennes (JdC). Nine of these were European onion landraces provided by Kultursaat e.V. (Echzell, Germany), whereas the commercial cultivar StR was used as reference. StR was selected as reference based on its quality properties and because it is widely used in commercial organic farming within the geographical area of the study.

The field trial was conducted in 2015. Seeds of ten onion genotypes were sown in a controlled environment in the greenhouse during the last week of February. After six weeks, seedlings were transplanted to the experimental station for organic farming at ‘Kleinhohenheim’, University of Hohenheim (48°43′54″N, 9°12′5″E Stuttgart, South-West Germany). The experiment was set up in a randomized block design with four replicates for each genotype and an individual plot size of 3.2 × 1.5 m. Each experimental plot (one genotype per plot) had three rows and a plant density of 90 plants per square meter as described by Romo Pérez et al., 2018. The rotation on the experimental field included the following precrops: 1st year grass-clover (high share of red clover), 2nd year cabbage, 3rd year summer wheat, 4th year onions (year of the experiment). N_{min} measurements were done before transplanting in April (0–60 cm 36 kg N/ha). During all plant growth stages, hand-weeding was carried out to combat weed. The onions were lifted by hand after 60 % of their leaves topped-down at the end of the growing season. For optimum storage quality, onions were cured in an indoor space by temperatures between 25 °C–30 °C before cold storage.

2.2. Cold storage of onions

After transport to the storage facility, onions were first stored in a cold storage room at 6–7 °C and approximately 65 % relative humidity. After two weeks, in order to reduce microbial risks, storage was continued at 2–3 °C and < 60% relative humidity due to the use of an additional air dehumidifier in the storage chamber. Bulbs of the same genotype (n = 4) were stored together in open stacked plastic boxes (one box per plot containing approximately 25 bulbs). Bulbs were assessed visually on a regular basis to detect any signs of spoilage caused by bacteria or fungi. After approximately 6 weeks of storage, an increasing number of bulbs of the genotype JdC exhibited signs of fungal infections, most probably caused by *Botrytis* and *Aspergillus* species. In order to prevent a complete loss of the bulbs, post-storage sampling of JdC was done after 8 weeks of storage (Table S2, supplemental material). The bulbs of the other nine genotypes were stored for another 3 months, with only minimal losses due to spoilage and sprouting. The extent of sprouting was evaluated after 5 months of storage directly before onions were sampled for metabolome analysis. For this, onions were cut longitudinally and sprouting was classified to be < 1 cm, 1.0–3.5 cm or > 3.5 cm (see supplementary Table S2).

2.3. Targeted analyses

Four replicates of each genotype composed of nine bulbs before the storage experiment were used for sample processing. The outer dry scales were removed and the onions were cut longitudinally. One part of the sample was bulked and homogenized in a 1:1 vol (w/v) of double-distilled water (ddH_2O) using a mixer (BÜCHI Mixer B-400, BÜCHI Labortechnik AG, Flawil, Switzerland). The homogenates were then filtered using a folded paper filter (520 A ½ 185 mm, Schleicher & Schuell, Dassel, Germany) and the clear supernatants were stored at − 20 °C for further pyruvic acid analysis. The second part of the sample was homogenized solely and stored for the analysis of sugar, soluble solids, and dry matter content. The whole procedure was repeated with the samples obtained post-storage.

For the determination of dry matter content, 20 g of homogenized samples were dried in a ventilated oven until dry matter content was stable, firstly at 70 °C for 42 h and then at 105 °C for 3 h.

For the determination of soluble solids content (TSS), the supernatant obtained from 1 g of raw homogenate after centrifugation for 10 min at 10000 × *g* was measured at 22 °C using the Bausch and Lomb Abbe 3L refractometer (Bausch and Lomb Incorporated, Rochester, NY).

Non-structural carbohydrates were determined based on the Official Analytical Chemists (AOAC) method and the Megazyme fructan-assay kit using p-hydroxybenzoic acid hydrazine (McCleary et al., 2000) with some modifications described by Romo Pérez et al., 2018.

As an indicator of onion pungency, total pyruvic acid concentration in the filtrated juice was measured spectrophotometrically using dinitrophenyl hydrazine (DNPH) reagent according to the improved method of Anthon and Barrett (2003), and background pyruvic acid after deactivation of alliinase by heating onion tissues in a microwave according to the method of Yoo and Pike (2001). Enzymatically produced pyruvic acid (EPPA) was calculated by subtracting the background pyruvic acid from the total pyruvic acid concentration.

2.4. Untargeted metabolome analysis

For each cultivar and each stage (fresh/stored), onions were cut and quickly frozen in liquid nitrogen, thereafter crushed with a mortar. The frozen tissue powder was freeze-dried and stored at − 80 °C.

For metabolite analysis, fresh onion samples were first milled with a ball mill (model MM 200, Retsch, Haan, Germany) for 60 s at 25 Hz. To achieve a lower particle size, the samples were further milled with another ball mill (model MM 400, Retsch, Haan, Germany) for 5 s at 30 Hz. The stored samples were only milled with the modell MM 400 for 20 s at 30 Hz. Both procedures led to the same milling result. On the day of analysis, samples (20 ± 0.1 mg) were extracted twice with methanol and an aliquot of each supernatant was evaporated in a rotary vacuum concentrator. Subsequently, dried samples were derivatized using a common two-step procedure involving methoximation and trimethylsilylation. A detailed protocol can be found in Section 1.1 of the supplemental material.

The GC × GC-MS analysis was performed with a system described recently (Weinert et al., 2015). In brief, separation was performed with a common non-polar × medium-polar column setup. A combined injector temperature/split ratio program enabled the chromatographic separation of a wide range of compounds (from small amino/organic acids up to tetrasaccharides) while avoiding carry-over of higher oligosaccharides. Further analytical details can be found in Section 1.2-1.5 of the supplemental material.

The analysis of the 160 samples was performed in 18 batches. Per day, one reagent blank, six quality control (QC) samples and nine study samples were injected. The study samples were analyzed in completely randomized order. Liner exchange and tuning was performed at the beginning of each week, followed by six equilibration runs with QC samples. The injector septum was exchanged after approx. 100 runs.

GC × GC-MS data were processed as described recently (Egert et al., 2015) with minor modifications. Raw data extraction, *i.e.* peak integration and compound annotation was performed using the Postrun Analysis module of the software GCMS Solution (version 4.11) leading to one peak list per run. All further steps comprising the import of the peak lists, data aggregation, de-noising, alignment, peak merging and QC-based drift correction were done using the SquareDance approach implemented in R (R Development Core Team, 2016).

After automatic data processing, 319 drift-corrected raw analytes were closely inspected in order to exclude artefacts, not automatically removed sections of noise bands, internal standards as well as co-eluting or irreproducible analytes (mean intra-day repeatability RSD ≤ 30 %). A total of 189 sound analytes were finally considered for statistical testing.

2.5. Statistical analysis

For the statistics of the targeted analysis, one-way ANOVA (Analysis Of Variance) followed by post-hoc Tukey HSD (Honestly Significant Difference) were used to display the storage effect per genotype. Differences were considered as significant when $p < 0.05$. Principal component analysis (PCA) were used to determine the effect of “storage” and the “genotype” on the metabolite profile of all 10 onion genotypes, all values were centralized and standardized. Analyses were performed using R (R Development Core Team, 2016).

The statistical analyses of the untargeted metabolomics were done with the software JMP (Version 13.2.1; SAS Institute, Cary, USA). First, a principal component analysis (PCA) was performed to assess the overall impact of the factors “storage” and “genotype”. For this, 176 out of the 189 reliable analytes, which exhibited less than 30 % undetectable metabolites, were considered after replacing missing values with half of the minimum.

2.5.1. Statistical analysis of fresh vs. stored onions

To analyze the metabolic changes after cold storage, a comparison of all fresh with all stored onion samples independently of the genotype was done. One of the 189 reliable analytes had to be excluded because it was almost only detectable in the stored samples. The remaining 188 analytes were then analyzed using a one-factorial ANOVA screening approach with FDR correction (‘Response Screening’ platform) without prior replacement of the non-detects. 120 metabolites showed a significant storage-dependent difference in the ANOVA analysis (FDR *p* value < 0.01) and passed also the subsequent Wilcoxon test (*p* value < 0.05). 105 out of the 120 metabolites were judged as relevant based on their fold changes (> 1.2 or < 0.80).

2.5.2. Statistical analysis of metabolite profiles of the single genotypes in the fresh state

The metabolite profiles of the onions in the fresh state were compared. After exclusion of four metabolites which had an overall missing rate of > 50 %, another one-factorial ANOVA screening analysis was performed using the remaining 185 analytes without replacement of the non-detects. 147 significant analytes were selected in the first stage and further analyzed as described recently (Weinert et al., 2017). Deviating from the reported workflow, the parametric ‘Dunnett with Control’ or the non-parametric ‘Steel with Control’ post-hoc tests were used to compare all genotypes with the reference cultivar StR.

2.5.3. Statistical analysis of metabolite profiles of the genotype groups I-III in the fresh state

Apart from the genotype JdC, the other nine genotypes had more comparable metabolite profiles but formed three groups, each consisting of three genotypes with apparently similar profiles. The genotype groups I-III were mostly separated along PC1. To find out whether the differences between these groups were due to unequal times the bulbs of the different genotypes needed to achieve physiological maturity after sowing (Table S1) or due to genuine genotype-specific profiles, a one-factorial ANOVA screening analysis was performed again. The same selection of metabolites was used as for the previous analysis but this time the metabolites profiles of the genotype groups instead of the single cultivars were compared. Apart from this, the analysis was exactly performed as reported Weinert et al. (2017). In total, 106 metabolites were found to be significant.

3. Results

3.1. Targeted analysis: concentration changes of non-structural carbohydrates, TSS, EPPA, and dry matter content

In order to identify quality parameters that were affected by storage and to determine the differences between onion genotypes, a principal component analysis (PCA) was performed for both fresh and stored

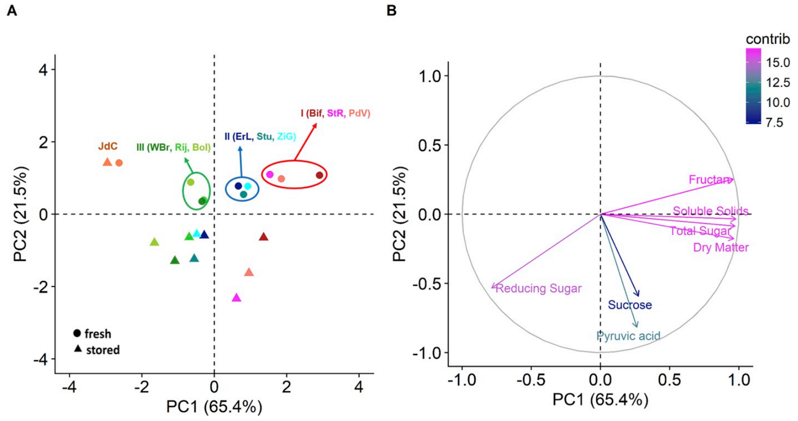

Fig. 1. A: Scores plot showing the impact of the factors “genotype” and “storage” on the metabolome profile (targeted analysis) of stored (▲) and fresh (•) onions. Group I (Birnförmige (Bif), Sturon (StR), Paille des Vertus (PdV)). Group II (Erfurter Lager (ErL), Stunova (Stu), Zittauer Gelbe (ZiG)). Group III (Wiener Bronzekugel (WBr), Rijnsburger 4 (Rij), Bolstar (Bol)). Jaune des Cévennes (JdC). Cultivar groups are visually highlighted by coloured ellipses. B: Loadings plot indicating the total contribution of variables to the first two principle components. Variables are colored based on their weight of contribution to the two axes shown.

onion bulbs. The PCA scores plot (Fig. 1A) shows that > 85 % of the variation is explained by the first two components, of which 65.4 % belongs to the first principal component (genotype differences) and 21.5 % to the second one (storage effect). Regarding the genotype differences, individual PCA allows to distinguish three groups based on their similarities among the fresh onions; Group I (Bif, PdV, StR), Group II (ErL, Stu, ZiG), and Group III (WBr, Rij, Bol). Only the genotype JdC, both in fresh and stored form, showed a particular different profile apart from all others (Fig. 1A). The PCA loadings plot (Fig. 1B) highlights the contributions of the following parameters: fructans, soluble solids (TSS), pyruvic acid (EPPA), and reducing sugars, as well as dry matter of all genotypes at both fresh and stored conditions. Within this PCA, variables that are closer together are considered highly positively correlated, for example, dry matter and total sugars, whereas those that are on opposite sides of the circle, are negatively correlated such as fructan and reducing sugars. The x-axis (PC1) is mainly described by the total sugar concentration, whereas the y-axis (PC2) is related to EPPA concentration, demonstrating that the changes in the concentration of EPPA are strongly associated with the effect of storage.

Fig. 2A–F highlights storage-related changes in the concentration of fructans, soluble solids, EPPA, and soluble sugars, as well as dry matter depending on the genotype. After storage, EPPA concentrations (Fig. 2A) increased between 20 and 35 % in all genotypes, except for the genotype JdC where little or no increase was observed. Total sugar (Fig. 2F) and dry matter (Fig. 2C) showed a similar pattern compared to each other, with only little decrease post-storage. Among the non-structural carbohydrates (Fig. 2D and E), reducing sugars increased significantly and fructan decreased up to five times in almost all genotypes.

3.2. Untargeted metabolome analysis

To get an overview of the impact of genotype-differences and the storage on the onion metabolite profile, another principle component analysis was performed (Fig. 3). Similar to the results of PCA performed using the data of the targeted analysis (Fig. 1A), a clear separation of fresh/stored onions along PC1 was observed in addition to the formation of three distinct genotype groups. Analogously, a hierarchical clustering analysis resulted in a separation of the landrace JdC and the genotypes of group III (WBr, Rij, Bol), while the groups I (Bif, PdV, StR) and II (ErL, Stu, ZiG) remained mingled (data not shown). Among the fresh onions (filled circles in Fig. 3), differences between the ten genotypes led to a partial separation along PC1 and PC2. The samples of the genotype JdC (orange triangles and circles) exhibited a specific metabolite pattern which changed not much during the short storage period of eight weeks. The samples of the other nine genotypes after storage (triangles in Fig. 3) cluster at the right side of the PCA. In analogy to the results of Fig. 1A, the distinction of the genotype groups was less clear after storage.

3.2.1. Storage effect

The effect of cold storage on the metabolite profile of the different onion genotypes was analyzed using an ANOVA approach (see section 2.5) and the results are summarized in Fig. 4 and in Supplemental Table S3. Among the 120 metabolites exhibiting a significant storage effect, 56 could be identified with high confidence (level 1) or most likely (level 2) while further 43 metabolites could be assigned to a compound class (level 3) and 21 metabolites remained unidentified (level 4). The concentrations of 88 metabolites increased, and other 32 decreased after storage (Table S3). Among the group of amino acids, increases after storage were observed for those amino acids with acidic or basic side chains and for some of the putative sulfur-containing amino acid derivatives (Fig. 4). To some extent, this effect could also be observed for closely related substances such as methiin, methionine, *S*-methylcysteine, alliin, and cysteine. On the other hand, leucine, isoleucine, 2-aminobutyric acid, ethanolamine and citrulline were found to be significantly decreased after storage. Concerning the organic acids, storage resulted in substantially higher levels of free phosphoric acid, linoleic, pyruvic, dehydroascorbic, malic acid and maleic acid. In contrast, concentrations of citric acid were strongly reduced, followed by oxalic, and fumaric acid which also decreased. A total of 25 sugars and sugar-derived compounds comprising monomers up to trisaccharides were detected in higher amounts after storage, with relative fold changes often being larger than 2 (Table S3). High relative fold changes were observed for fructose, xylose, trehalose and fucose, while glucose levels remained unaffected by storage consistently in all cultivars. In contrast, some trisaccharides and all tetrasaccharides including nystose markedly decreased after storage.

In addition to the general storage effect on sugars and sugar-related compounds, raffinose was one of the few metabolites only detected in stored onions.

Further, 21 unidentifiable compounds also exhibited a relevant storage effect. Interestingly, while an overall storage effect could be determined for these 120 metabolites, it has to be mentioned that there was often no or only a minor storage effect in case of the genotype JdC.

3.2.2. Bulb composition of fresh onions

The specific metabolite profiles of the fresh onion genotypes observed in the PCA scores plot (Fig. 3) were investigated in more detail using a ANOVA (see Fig. 5 and Supplemental Table S4). Predominantly, concentration differences of amino acids and sugars or sugar-related compounds separated the fresh samples of the different genotypes.

The comparison of the genotype groups I-III highlighted a general pattern: Many metabolites concentrations were higher in the samples of genotype group III when compared to group I, with samples of group II usually having intermediate concentrations. Within group III, the genotype Rij often reached the highest concentrations, especially with respect to the group of amino acids.

The peculiarity of the genotype JdC was confirmed again: While the amino acid profile of JdC was similar to that of the reference genotype StR, the sugar profile was more similar to the samples of group III. However, especially in case of several monomeric sugars such as fructose, ribose, xylose or several unknown sugars as well as the (uncharacterized) disaccharide A0536, by far the highest levels were found in the bulbs of the genotype JdC. Moreover, levels of most di, tri- and tetrasaccharides were lowest (or even not detectable) in this landrace.

Compared to amino acids and sugars or sugar-related compounds, the levels of aliphatic organic acids were rather comparable. Higher relative fold changes were observed only in case of low concentrated compounds such as fumaric acid, pyruvic acid and erythronic acid. Further, the genotypes ErL and WBr showed higher concentrations of two putative phytosterols.

4. Discussion

4.1. Exploring the onion metabolite profile

Overall, the results of the targeted analysis and the untargeted metabolome analysis were highly comparable and complementary, highlighting: a) a substantial, but time-dependent effect of cold storage, b) genotype-specific metabolite profiles, enabling the distinction of three genotype groups (I, II, III) and c) the particularity of the landrace Jaune des Cévennes (Figs. 1A and 3). In recent years, metabolite profiles of other onion genotypes have been compared but these studies focused on modern conventional genotypes (Böttcher et al., 2017, 2018) or local genotypes of India (Das et al., 2015), Japan (Kimura et al., 2014) or Spain (Pérez-Gregorio et al., 2010). In many of these cases, a separation of different genotypes based on their metabolite profiles was also possible, although other factors such as harvest year and growing region were also shown to have an impact on bulb composition (Böttcher et al., 2018; Kimura et al., 2014). Moreover, the effect of storage or different storage conditions have been investigated but mostly focusing on specific metabolites, *e.g.,* sulfur-containing

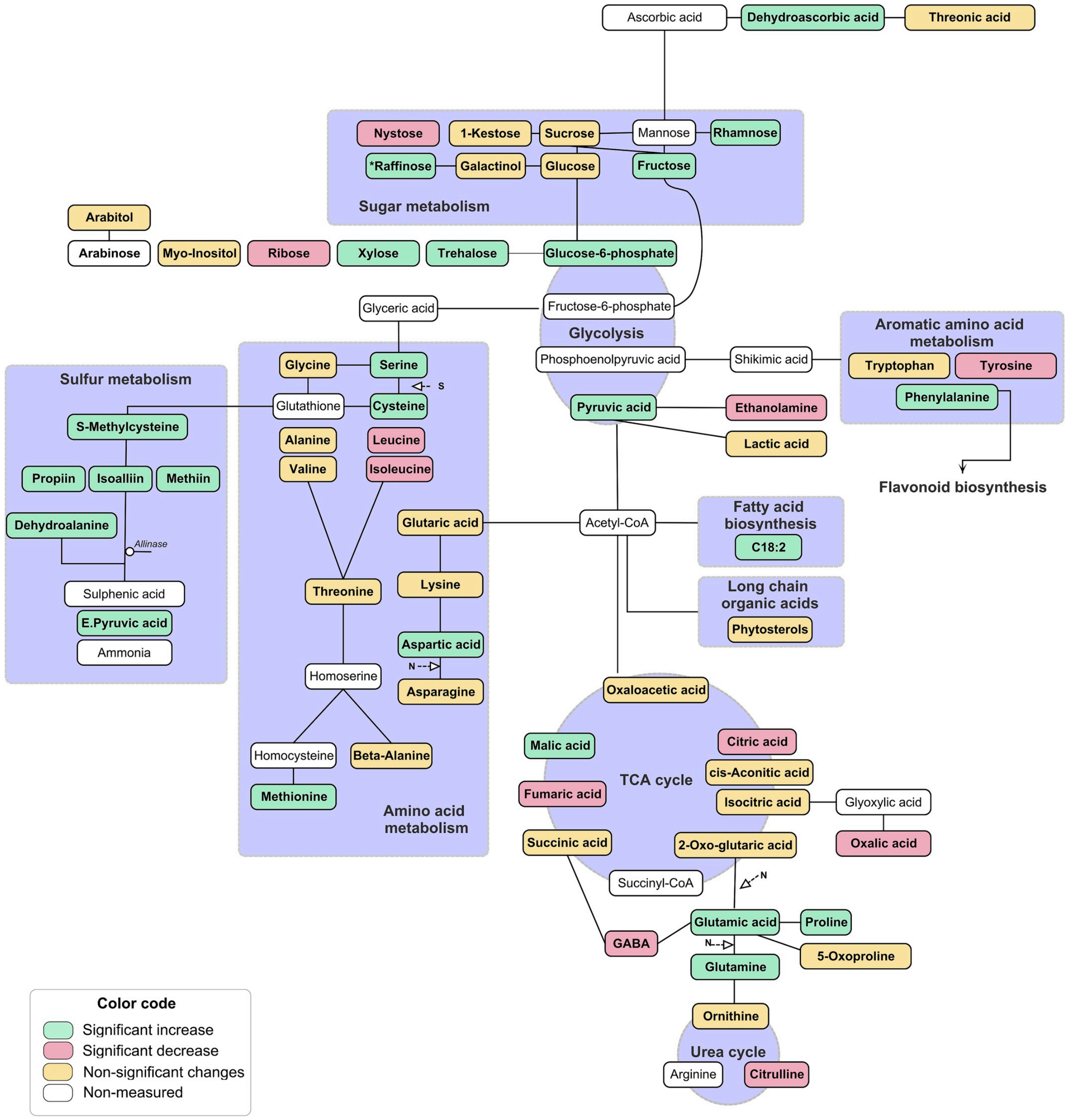

Fig. 4. Mapping of measured metabolites onto *Allium Cepa* L. biosynthetic pathways, with those showing relative significant (fold change FC > 1.2 or < 0.8) increased levels after storage in green, decreased levels in red and non-significant changes in yellow. Non-measured metabolites are not highlighted. *Raffinose was only detected in stored onion bulbs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

indicate that the initial genotype differences gradually disappear due to post-harvest changes, as onions are preparing to sprout. Therefore, authenticity studies should preferably be carried out on fresh onion bulbs.

4.3. *Storability of onion landraces (ii)*

Bulb dry matter is one of the most important onion quality parameters. Multiple research papers have already correlated dry matter to onion storability (Hansen, 1999) and about 80 % of bulb dry matter consists of non-structural carbohydrates (Darbyshire and Henry, 1979; Suzuki and Cutcliffe, 1989). In this study, both targeted and untargeted analysis exhibited significant storage-related changes in the non-structural carbohydrate levels of all genotypes, with exception of the genotype JdC. Because of rapid spoilage, JdC was sampled 14 weeks earlier than the other genotypes. Post-storage, fructan levels of JdC decreased from low concentrations to almost zero (Fig. 2) and this genotype failed to maintain its quality or began sprouting. All other genotypes physiologically responded with a decay of fructans and a subsequent significant increase of monosaccharides. Generally, genotypes with a high

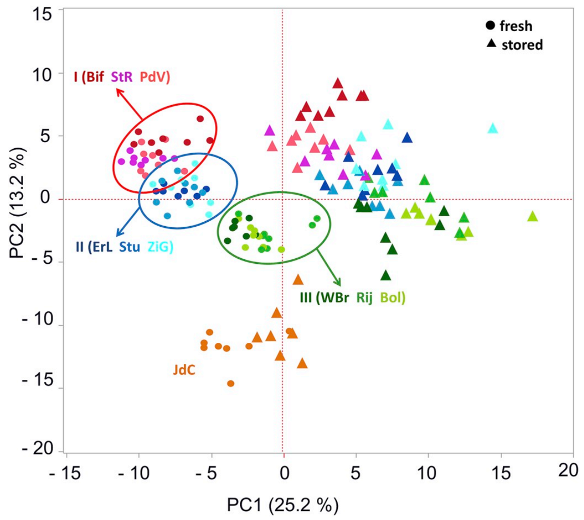

Fig. 3. Principal component analysis (PCA) with the impact of the factors "genotype" and "storage" on the metabolite profiles (untargeted analysis) of stored (▲) and fresh (•) onions. Group I (Birnförmige (Bif), Sturon (Str), Paille des Vertus (PdV)). Group II (Erfurter Lager (ErL), Stunova (Stu), Zittauer Gelbe (ZiG)). Group III (Wiener Bronzekugel (WBr), Rinjsburger 4 (Rij), Bolstar (Bol)). Jaune des Cévennes (JdC). Cultivar groups are visually highlighted by coloured ellipses, as in Fig. 1A.

compounds. Therefore, some of the detected complex sugar compounds were designated as trisaccharides but could not be completely identified.

Nevertheless, with the help of targeted and untargeted methods, it was possible to explore in a complementary and consistent manner; *(i)* the impact of cold storage on the metabolite profile of onions, *(ii)* the storability of onion landraces, and *(iii)* bulb composition of onion landraces in their fresh state, compared with a reference cultivar.

4.2. *Impact of cold storage on the metabolite profile of onions (i)*

Through cold storage, metabolic processes and respiration can be slowed down but not completely stopped. During storage, when the onion breaks dormancy, the bulb undergoes a source (scales) to sink (base plate) transition, which is reflected in the metabolic changes of onions (Nile et al., 2017). Table S2 demonstrates that non-visible, i.e. internal sprouting was slightly initiated in all onion genotypes (sprout leaf length of most genotypes was below 1 cm with some others up to 3.5 cm), indicating an elongation of the sprout leaf and start of dormancy breaking. The results of the targeted and untargeted analysis showed that primarily non-structural carbohydrates, some amino acids (especially those with an acidic or basic side chain or sulfur-rich ones), some organic acids and some unknown metabolites were significantly affected during storage (Fig. 4). Onions redistributed non-structural carbohydrates but in contrast, total sugar concentration in dry matter remained almost unchanged. Where levels of fructose increased significantly, sucrose increased slightly, glucose remained constant and total fructans decreased up to five times compared to their initial concentrations (Figs. 2 and 4). These results are consistent with the results of Salamal,Hicks and Nock (1990) and Ohanenye et al. (2019). Since 1-kestose (a DP3 fructan) is highly positively correlated with levels of glucose (Ohanenye et al., 2019), relative levels of 1-kestose also did not change significantly. On the other hand, nystose (a DP4 fructan) and other major tetrasaccharides decreased after five months cold storage. Another carbohydrate, raffinose was only detectable in stored onions (Fig. 4). This might be due to the effect of low temperatures during storage and may serve as a compatible solute under stress situations (Kashash et al., 2018; ElSayed et al., 2014; Gilmour et al., 2000).

Previous storage studies focused on changes in S-containing amino acids and the aroma precursors S-alkenyl cysteine sulfoxides (ACSOs), but changes in other amino acids that are linked to the onion aroma are scarce. Due to the respiration process in onions during storage, in our experiment, the carbohydrate concentration was severely altered. As fructans form a considerable part of the onion metabolome (65 % or more of the dry weight), it is expected that fructans and other non-structural carbohydrates provide the carbon skeleton and energy supply for the synthesis of amino acids. The decrease of fructans (e.g. nystose) may have led to an overall increase of amino acids, since carbohydrate biosynthesis and N-assimilation into amino acids compete for input of energy and reduced carbon (Druege et al., 2000; Cheng et al., 1998). The increase in amino acids comes at the expense of nonstructural carbohydrates rather than an increased *de novo* synthesis of amino acids based on intermediates of the central metabolism.

In terms of S-containing amino acids, changes in flavor precursors are usually strongly linked to temperature and atmosphere composition. Consistent with the results of Yoo et al. (2012), this study also demonstrated an overall increase of the ACSOs and enzymatically-produced pyruvic acid (EPPA) after cold storage. EPPA was measured as an indicator of pungency, since this substance highly correlates with the volatile products responsible for the flavor of onions (Lee et al., 2009). Some reports (Yoo et al., 2012; Lancaster and Shaw, 1991) suggest that the increase in pungency during storage is due to the release of (1-propenyl)-L-cysteine sulfoxide (1-PeCSO) from γ-glutamyl-cysteine sulfoxide by γ-glutamyl peptidase, which increases its activity up to five-fold just before the onion starts sprouting. Moreover, changes in levels of amino acids, which were also involved in the biosynthesis of ACSOs, may also contribute to the overall increase of pungency (Prince et al., 1997). Our data demonstrated that amino acids involved in the ACSOs pathway such as Ser, Cys, Glu and Met substantially increased during storage (Fig. 4, Table S3), Val, another amino acid also involved in the biosynthesis of the sulfur-containing compounds in onions, did not increase significantly. In addition to the increase of sulfur-containing metabolites which indicates an enhancement of onion pungency, a significant increase of glutamic acid and aspartic acid, also called "umami amino acids" (Gong et al., 2016), result in development of onion sensory properties during cold storage.

The amino acid phenylalanine (Phe) acts as a biosynthetic flavonoid precursor through the phenylpropanoid pathway. Changes in Phe concentrations may lead to changes in the flavonoid profile. Hansen (2001) reported an overall increase of Phe during five months cold storage but the concentration of quercetin was not measured in this study. Recently, Sharma et al. (2014, 2015) reported an increase in Phe concentration, which correlated to an increase in quercetin levels after cold storage. Our results are consistent with a significant relative increase (up to 54 %) in phenylalanine levels after 5 months of cold storage. This suggests that flavonoid levels, mainly quercetin, can be elevated by long-term storage and previously unmarketable onions might be used as source for quercetin production.

Little is known about changes in organic acid concentration in onions during mid- or long-term storage. Recent reports only highlighted that malic and citric acid are the major organic acids in onion bulbs (Petropoulos et al., 2016; SalamalHicks and Nock, 1990). We found increased concentrations of malic acid after cold storage while, at the same time, citric acid decreased up to 44 % compared to its initial level. This might indicate a link between organic acid concentration changes and the breakdown of dormancy in onions. However, further studies are required to shed more light on this phenomenon.

The pattern of metabolite profile of fresh onions observed in the PCA scores plots (Figs. 1A and 3), enables a categorization into three genotype groups (and JdC). Post-storage, these groups were also present but not as clear as with fresh onions. This phenomenon may

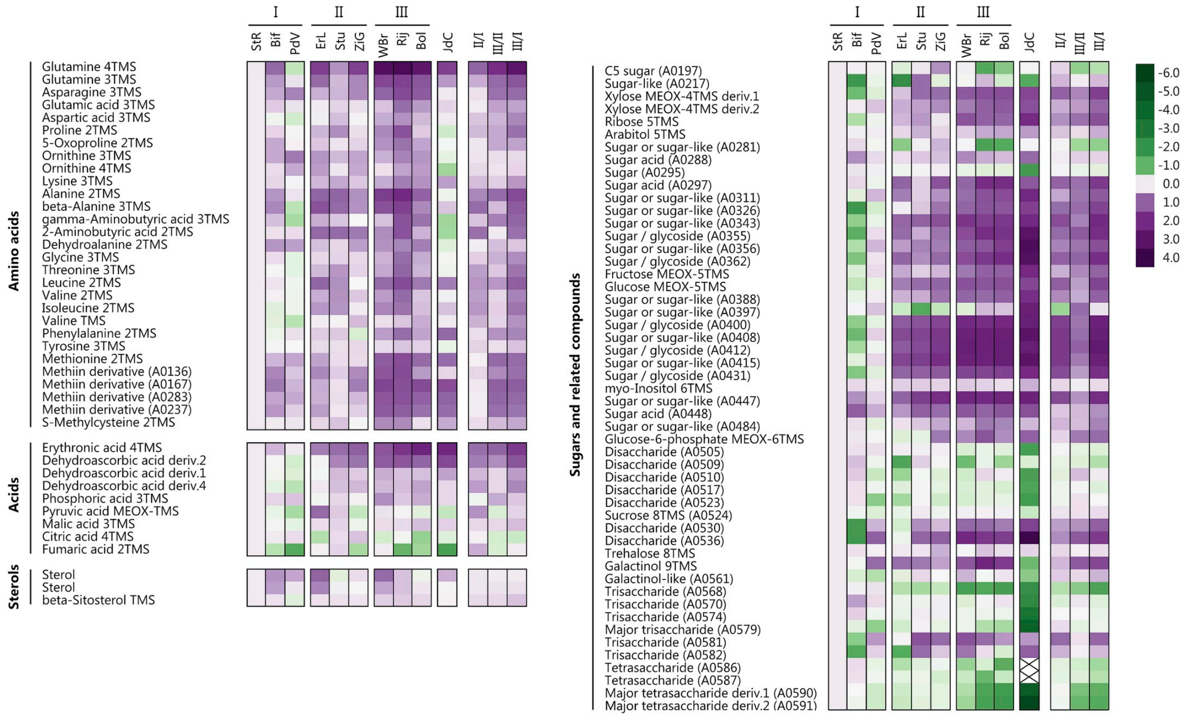

Fig. 5. Comparison of the metabolite profiles of the onion genotypes and genotype groups in fresh form. The heatmap highlights selected metabolites showing significant genotype differences (high = violet or low = green) against the control genotype Sturon (StR) and significant differences between the genotype groups I-III on the basis of log2-transformed relative fold changes. Crossed-out boxes: analyte not detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

initial dry matter content and high fructan concentrations such as Bif, StR and PdV had higher storability which is in accordance to the results of Jaime et al. (2001). In addition, slower hydrolysis of nystose and tetrasaccharides during cold storage was observed only in the genotype Bif, what also confirms the good storability of this genotype. In contrast, JdC, with low dry matter content (7–8 %) and high monosaccharide concentrations, demonstrated reduced quality as well as glassiness and rot symptoms, making it unsuitable for mid- or long-term storage.

4.4. *Bulb composition of onion landraces in their fresh state, compared with a reference cultivar (iii)*

In a previous study (Romo Pérez et al., 2018), some targeted agronomic and biochemical quality aspects of the studied landraces have been identified, allowing us to determine general differences between the European onion landraces and the reference genotype StR. In this study, employing more comprehensive and non-targeted metabolome methods, the differences between these genotypes could be clearly visualized (Fig. 5). Based on the data of targeted and untargeted analysis, a clear separation of three genotype groups and the landrace JdC was observed. Compared to the groups I and II, group III (in particular Rij) was characterized by very high concentrations of amino acids (up to 11 times) and sugars (up to 5 times). Some of these metabolites are precursors of the aromatic properties in onions. This indicates the potential of Rij to provide aromatic and flavoring substances. Phytosterols are another source of *koku* attributes which enhance aroma persistence in foods (Nishimura et al., 2016). Besides beta-sitosterol, a major phytosterol detected in raw onions, another two phytosterols were identified in landraces of our study (Fig. 5). Largest concentrations of phytosterols were detected in the genotype ErL, potentially contributing to its *koku* aroma attributes. Differences between the landraces and StR may indicate that part of the aromatic properties found in traditional onions are lost in modern commercial genotypes.

In conclusion, targeted and untargeted analysis of onions demonstrated (*i*) the impact of mid-term storage on the metabolite profile of onions. Out of 189 relatively quantified analytes, 120 exhibited significant changes after five-months storage. Mainly monosaccharides, fructans, EPPA, amino acids (with basic or acid side chain or sulfur-containing ones) changed during cold storage (Fig. 6). In addition, this study demonstrated, (*ii*) the storability of onion landraces. All studied landraces presented good storability, except of JdC which is only suitable for fresh consumption. The study of fresh onions bulbs demonstrated (*iii*) genotype and group differences. Group III had the highest levels of amino acids suitable as precursors for aromatic substances, as well as high levels of sugars. In particular genotype Rij reached up to 11 times higher amino acid levels and 5 times higher sugars levels compared to StR. This group of landraces may serve as a genetic pool for future breeding of aromatic onions.

Declaration of competing interest

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

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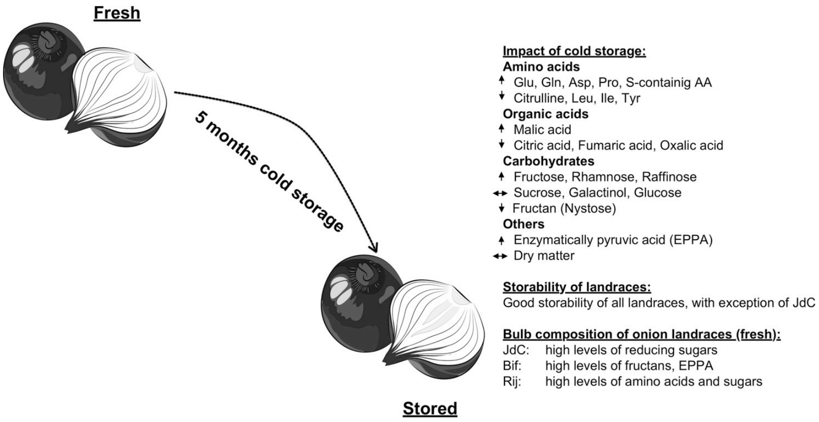

Fig. 6. Schematic summary of the general effect of storage on onion bulbs after 5 months storage under cold conditions. The orientation of the arrows implies the effect after storage: ↑, increase; ↔, non-change; ↓ decrease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.11.007.

Contribution

- **Maria Luisa Romo-Pérez, Universität Hohenheim**
  Conception of study design, field trial, curing of onion bulbs.
  Analysis of fresh and stored material using targeted methods.
  Data analysis and biological interpretation.
  Manuscript elaboration.
- **Christoph H. Weinert, Max-Rubner Institut (MRI)**
  Storage trial.
  Development of the untargeted GC × GC-MS method for the analysis of onions.
  Supervision of untargeted GC × GC-MS measurements.
  Data quality control and data analysis.
  Manuscript elaboration.
- **Marina Häussler, Max-Rubner Institut (MRI)**
  Storage trial.
  Development of the untargeted GC × GC-MS method for the analysis of onions.
  Analysis of fresh and stored onion samples using the untargeted GC × GC-MS method.
  Data quality control.
- **Björn Egert, Max-Rubner Institut (MRI)**
  Bioinformatic processing of untargeted GC × GC-MS metabolomics data.
  Data analysis and visualization.
- **Matthias Frechen, Max-Rubner Institut (MRI)**
  Optimization and control of the storage conditions.
- **Bernhard Trierweiler, Max-Rubner Institut (MRI)**
  Supervision of the storage trial.
- **Sabine Kulling, Max-Rubner Institut (MRI)**
  Head of the department of safety and quality of fruit and vegetable at MRI.
- **Christian Zörb, Universität Hohenheim**
  Head of the department of quality of plant products at Universität Hohenheim.
  Conception of study design, supervision of field trial.
  Critical review of the manuscript.

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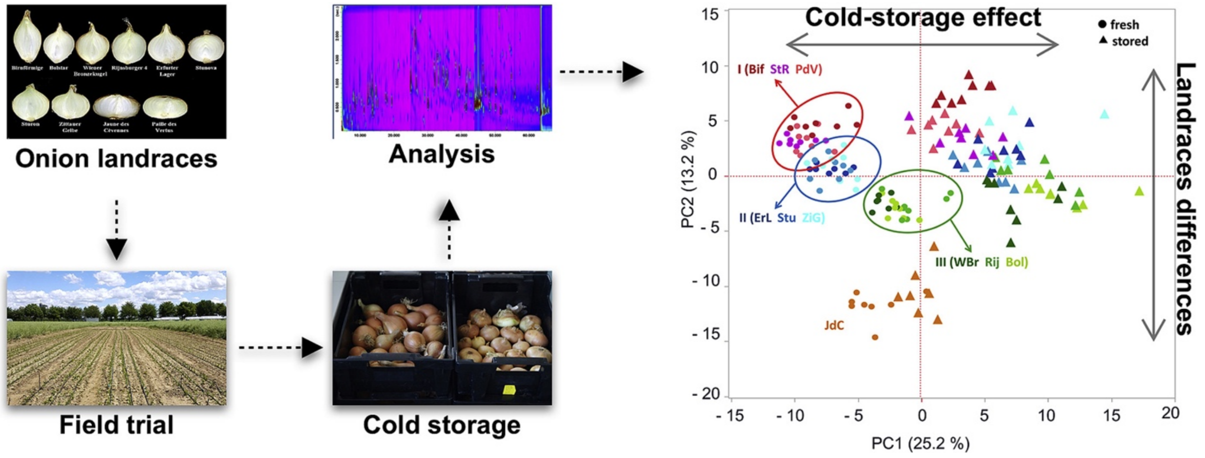
Supplementary material

Graphical abstract



Untargeted GC×GC-MS metabolome analysis

Sample extraction, evaporation and derivatization

Work step	Action
	Extraction
1	Thaw study samples and QC sample aliquots on ice
2	Add 750 µL of methanol
3	Add 20 µL of a mixture of internal standards containing hexylamine (5 mM), 1-*O*-methyl-2-desoxy-D-ribose (5 mM), 2-chlorophenylacetic acid (3 mM), *p*-chloro-L-phenylalanine (2.5 mM), D –pinitol (5 mM), 3-*O*-methyl-D –glucopyranose (1 mM), 5-bromo-2,4-dihydroxybenzoic acid (1 mM), and phenyl-β-D-glucopyranoside (1 mM) in ethanol/water
4	Mix samples vigorously
5	Incubate samples for 10 min at 35 °C and 1,400 rpm in a shaker
6	Remove insoluble matter by centrifugation using a mini centrifuge
7	Transfer 700 µL of the supernatants into new 2 mL reaction tubes
8	Re-extract the samples (repeat steps 2, 4, and 5)
9	Combine and mix both supernatants
10	Sediment insoluble matter by centrifugation for 5 min at 4 °C and 16100 × g
11	Transfer 30 µL of the combined supernants to screw-capped amber glass vials with 200 µL micro inserts
	Evaporation
12	Dry samples in a rotary vacuum concentrator for 1 h and 40 °C and p< 1 mbar
13	Re-dissolve samples again in 20 µL of methanol
14	Dry samples again to remove traces of water (30 min, 40 °C, p< 1 mbar)
	Derivatization
15	Add 25 µL of methoxylamine-hydrochloride in pyridine (20 mg/mL)
16	Incubate for 1 h at 40 °C and 1,400 rpm in a shaker
17	Add 70 µL of MSTFA with 1 % TMCS
18	Incubate for 1 h at 65 °C without shaking
19	Add 8 µL of retention index marker mixture only to daily blank

GC×GC-MS system

Instrument component	**Name**	**Manufacturer**
Gas chromatograph (single oven)	GC-2010	Shimadzu Corp, Kyoto, Japan
Single quadrupol mass spectrometer	QP2010 Ultra	Shimadzu Corp, Kyoto, Japan
Autosampler	AOC-5000	Shimadzu Corp, Kyoto, Japan
PTV Injector	OPTIC-4	GL Sciences, Eindhoven, The Netherlands
Loop-type cryogenic modulator	ZX2	ZOEX Corp., Houston, USA
Roughing pump	RV-3	Edwards, Crawley, UK

GC×GC method and consumables

Carrier gas	Helium 5.0
GC mode	Constant velocity
Purge flow	3 mL/min
Initial column head pressure	198 kPa
Liner type	Deactivated, fritted split liner with quartz wool
Primary column	Rxi-5SilMS, ^{1}L = 30 m plus 5 m of an integrated pre-column, $^{1}d_c$ = 0.25 mm, $^{1}d_f$ = 0.25 µm (Restek, Bellefont, USA)
Secondary column	BPX50, $^{2}L_{total}$ = 2.4 m, including a “separation segment” of $^{2}L_{sep}$ = 0.7 m, $^{2}d_c$ = 0.15 mm, $^{2}d_f$ = 0.15 µm (Trajan Scientific, Ringwood Victoria, Australia)
Column connector	SilTite MiniUnion (Trajan Scientific, Ringwood Victoria, Australia)
GC temperature ramp	90 °C → 2 °C/min → 150 °C → 2.5 °C/min → 190 °C → 4 °C/min → 220 °C → 5 °C/min → 250 °C → 3.5 °C/min → 270 °C→ 5 °C/min → 330 °C → 40 °C/mi → 340 °C (hold 10 min)
Run time	87.5 min.
Injection mode	Cold split
PTV temperature program	90 °C → 60 °C/s → 220 °C (hold 1 min) → 60 °C/s→ 280 °C (hold until end of run)
Split ratio program	1:5 (hold 1 min) → 1:100 (hold until end of run)
Injection volume	1.2 µL
Injector syringe	10 µL, with PTFE-tipped plunger (Trajan Scientific, Ringwood Victoria, Australi
Syringe wash solvent 1	Acetone
Syringe wash solvent 2	Heptane
Interface temperature	320 °C

Modulator settings

Modulation period (P_M)	2.7 s
Cold jet temperature	-90 °C
Hot jet temperature program	200 °C (hold until 25 min) → 250 °C (hold until 48 min) → 300 °C (hold unti 60 min) → 360 °C (hold until 85 min) → 200 °C (hold until end of run)
Hot jet pulse duration	375 ms

MS settings

Interface temperature	320 °C
Ion source temperature	200 °C
Ionization mode	EI (70 eV)
MS Mode	Scan
Scan speed	20.000 u/s
Scan range	m/z 60-550
Event time	30 ms
Data acquisition frequency	33 s^{-1}
Data acquisition period	4.5 -85 min
Detector voltage windows	4.50 – 22.21 min: 1.34 kV 22.21 – 22.65 min: 1.08 kV 22.65 – 41.31 min: 1.34 kV 41.31 – 43.55 min: 1.03 kV 43.55 – 61.21 min: 1.34 kV 61.21 – 64.13 min: 1.08 kV 64.13 – 72.94 min: 1.34 kV 72.94 – 85.00 min: 1.20 kV

Results

Agronomic data

Table S1. Variety, storage time

Variety	Abbreviation	Time to physiological maturity (weeks)	Storage time (weeks)
Birnförmige	Bif	23	22
Paille des Vertus	PdV	24	22
Sturon*	Str	23	22
Erfurter Lager	ErL	26	22
Stunova	Stu	23	22
Zittauer Gelbe	ZiG	23	22
Bolstar	Bol	26	22
Rijnsburger 4	Rij	25	22
Wiener Bronzekugel	WBr	26	22
Jaune des Cévennes	JdC	25	8

*Control variety

Table S2. Percentage sprouting after 5 months of storage, rot and glassiness of onion varieties during storage

Variety	Storage time (weeks)	Percentage sprouting. Sprout leaf length (SL)			Percentage rot and glassiness
		< 1.0 cm	1.0 - 3.5 cm	> 3.5 cm	
Bif	**22**	49%	49%	0%	1%
PdV	**22**	43%	57%	0%	1%
StR	**22**	76%	23%	0%	1%
ErL	**22**	78%	22%	0%	0%
Stu	**22**	86%	14%	0%	1%
ZiG	**22**	31%	68%	0%	1%
Bol	**22**	37%	63%	0%	0%
Rij	**22**	63%	37%	0%	0%
WBr	**22**	81%	16%	0%	3%
JdC	**8**	68%	23%	0%	5%

Untargeted analysis

Chromatographic separation

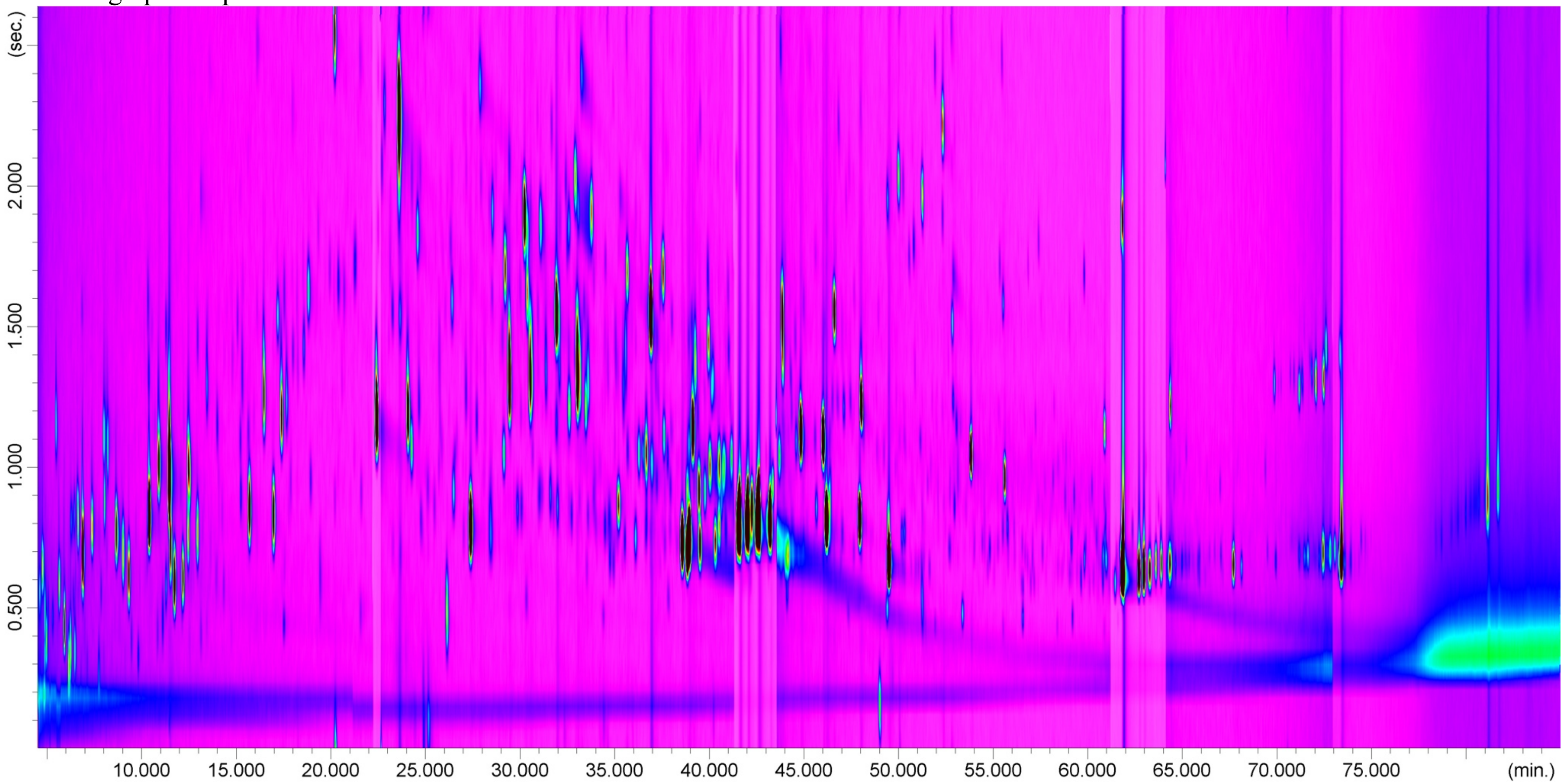


Figure S 1 2D chromatogram of onion bulbs after optimization of GC-temperature program.More than 189 analytes were detected after exclusion of known aterfacts, noisy bands, internal standards and irreproducible analytes.

Storage effect

Table S3. Metabolites showing a significant storage effect. - The names of known compounds are highlighted in bold. Within each compound class, metabolites are sorted by decreasing percentage relative fold changes Significant metabolites (relative fold change > 1.20 or < 0.80) are highlighted in colors.

cID	Identification		Storage	
	Name	MSI^1	FDR LogWorth^2	FC^3
	Amino acids and amines			
A0277	**Glutamine 4TMS**	2	6.7	4.14
A0274	Amino acid derivative	3	10.9	2.24
A0189	**Alliin derivative**	1	11.3	2.17
A0103	**Serine 3TMS**	1	14.4	1.91
A0121	***S*-Methylcysteine 2TMS**	1	24.9	1.91
A0280	Amino acid derivative	3	18.1	1.89
A0155	**Methionine 2TMS**	1	16.7	1.89
A0299	**Glutamine 3TMS**	1	17.2	1.87
A0136	**Methiin derivative**	1	8.7	1.86
A0423	Amino acid derivative	3	16.2	1.85
A0223	Amino acid derivative	3	25.6	1.84
A0428	Amino acid derivative	3	8.6	1.84
A0190	Amino acid derivative	3	29.8	1.81
A0254	Amino acid derivative	3	23.7	1.80
A0243	Amino acid derivative	3	22.2	1.80
A0232	Amino acid derivative	3	26.5	1.79
A0211	**Glutamic acid 3TMS**	1	27.6	1.78
A0251	Amino acid derivative	3	27.2	1.77
A0261	Amino acid derivative	3	27.7	1.76
A0204	Amino acid derivative	3	23.3	1.76
A0212	Amino acid derivative	3	10.6	1.75
A0253	Amino acid derivative	3	28.0	1.75
A0180	**Cysteine 3TMS**	1	4.9	1.72
A0161	**Aspartic acid 3TMS**	1	17.7	1.68
A0060	**Serine 2TMS**	1	8.8	1.56
A0209	**Phenylalanine 2TMS**	1	13.8	1.54
A0042	**Dehydroalanine 2TMS**	1	20.2	1.51
A0166	**Glutamic acid 2TMS**	1	9.9	1.47
A0167	**Methiin derivative**	1	2.6	1.31
A0080	**Proline 2TMS**	2	2.2	1.29
A0033	**Proline TMS**	2	2.1	1.28
A0283	**Methiin derivative**	1	2.3	1.22
A0122	**Aspartic acid 2TMS**	2	2.2	1.19
A0153	**5-Oxoproline 2TMS**	2	2.4	1.13
A0383	**Lysine 4TMS**	2	2.5	0.89
A0465	**Tryptophan 2TMS**	2	2.6	0.88
A0344	**Lysine 3TMS**	2	2.1	0.88
A0199	**Asparagine 2TMS**	2	3.0	0.84
A0014	**Valine TMS**	2	2.6	0.81
A0389	**Tyrosine 3TMS**	2	8.2	0.80
A0029	**Leucine TMS**	2	3.3	0.78
A0034	**Isoleucin TMS**	2	3.8	0.77
A0032	**2-Aminobutyric acid 2TMS**	2	2.5	0.75
A0064	**Ethanolamine 3TMS**	1	9.0	0.61
A0279	**Citrulline 2TMS**	2	12.9	0.59

cID	Identification		Storage	
	Name	MSI^1	FDR LogWorth^2	FC^3
	Sugars and sugar-related compounds			
A0566	Sugar (>disaccharide)	3	19.2	7.17
A0468	Sugar / glycoside	3	10.2	3.50
A0397	Sugar or sugar-like	3	21.4	3.40
A0168	Sugar (C4/C5)	3	36.1	3.01
A0509	Disaccharide	3	28.5	2.71
A0581	Trisaccharide	3	11.4	2.56
A0311	Sugar or sugar-like	3	28.4	2.45
A0582	Trisaccharide	3	7.6	2.38
A0362	Sugar / glycoside	3	19.1	2.36
A0356	Sugar or sugar-like	3	11.5	2.30
A0530	Disaccharide	3	29.3	2.29
A0355	Sugar / glycoside	3	15.5	2.22
A0475	Sugar (phosphorylated)	3	16.1	2.16
A0561	Galactinol-like	3	17.5	1.98
A0233	**Xylose MEOX-4TMS deriv.2**	1	13.7	1.95
A0365	**Fructose MEOX-5TMS**	1	22.3	1.87
A0536	Disaccharide	3	5.9	1.86
A0539	**Trehalose 8TMS**	1	43.1	1.84
A0288	Sugar acid	3	30.8	1.69
A0227	**Xylose MEOX-4TMS deriv.1**	1	9.5	1.66
A0491	**Glucose-6-phosphate MEOX-6TMS**	1	8.9	1.54
A0523	Disaccharide	3	3.9	1.32
A0268	**Fucose/Rhamnose MEOX-4TMS**	2	6.1	1.27
A0281	Sugar or sugar-like	3	2.4	1.23
A0135	Dialdose derivative	3	2.1	1.22
A0441	*myo*-Inositol 6TMS	2	14.7	1.17
A0524	**Sucrose 8TMS**	2	9.0	1.16
A0271	**Arabitol 5TMS**	1	3.3	0.85
A0505	Sugar / disaccharide	3	5.9	0.77
A0248	**Ribose MEOX-4TMS**	1	2.1	0.76
A0517	Disaccharide	3	7.0	0.75
A0586	Tetrasaccharide	3	2.5	0.72
A0510	Disaccharide	3	5.7	0.68
A0587	Tetrasaccharide	3	6.8	0.64
A0568	Trisaccharide	3	3.6	0.64
A0574	Trisaccharide	3	11.3	0.63
A0584	Trisaccharide	3	9.0	0.62
A0570	Trisaccharide	3	12.0	0.57
A0591	Major tetrasaccharide deriv.2	3	7.6	0.52
A0590	**Nystose 14TMS**	1	9.4	0.51

Table S3. Continued.

cID	Identification		Storage	
	Name	MSI[1]	FDR LogWorth[2]	FC[3]
	Acids			
A0472	**Linoleic acid TMS**	1	12.6	1.80
A0360	**Dehydroascorbic acid deriv.2**	2	7.0	1.50
A0003	**Pyruvat MEOX-TMS**	2	4.5	1.47
A0082	**Maleic acid TMS**	1	8.9	1.32
A0068	**Phosphoric acid 3TMS**	2	12.7	1.30
A0142	**Malic acid 3TMS**	2	24.6	1.26
A0390	**Dehydroascorbic acid deriv.4**	2	5.2	1.22
A0341	**Dehydroascorbic acid deriv.1**	2	6.0	1.21
A0006	**Lactic acid 2TMS**	2	2.0	0.85
A0097	**Fumaric acid 2TMS**	2	2.6	0.78
A0024	**Oxalic acid 2TMS**	2	20.5	0.73
A0327	**Citric acid 4TMS**	1	15.5	0.56
	Miscellaneous			
A0002	**2-Hydroxypyridine TMS**	2	4.5	1.21
A0576	**β-Sitosterol TMS**	1	3.9	1.15

cID	Identification		Storage	
	Name	MSI[1]	FDR LogWorth[2]	FC[3]
	Unknowns			
A0186	Unknown	4	16.6	2.34
A0270	Unknown	4	11.5	2.01
A0216	Unknown	4	15.1	1.99
A0257	Unknown	4	11.0	1.84
A0220	Unknown	4	29.1	1.81
A0282	Unknown	4	27.6	1.80
A0444	Unknown	4	27.3	1.77
A0222	Unknown	4	27.0	1.74
A0466	Unknown	4	18.5	1.72
A0457	Unknown	4	25.0	1.69
A0236	Unknown	4	8.2	1.69
A0054	Unknown	4	8.3	1.55
A0255	Unknown	4	12.5	1.50
A0052	Unknown	4	12.0	1.39
A0229	Unknown	4	8.4	1.37
A0125	Unknown	4	8.7	1.30
A0111	Unknown	4	2.9	1.04
A0175	Unknown	4	3.6	0.85
A0098	Unknown	4	29.7	0.40
A0019	Unknown	4	42.5	0.25
A0443	Unknown	4	10.5	0.24

[1] Metabolomics Standards Initiative identification levels: Identity confirmed by spiking with reference compound (1), compound probably identified by matching against internal or external spectral libraries, including retention indices (2), compound class tentatively identified by matching against spectral libraries or due to the presence of marker fragments (3), no assignment to compound class possible due to unspecific spectrum (4); [2] FDR LogWorth= -log (FDR p-value); [3] rFC, relative fold change (=mean intensity (stored)/mean intensity(fresh)).

Variety-specific and group-specific effect

Table S4. Metabolites showing a variety differences. –The names of known compounds and relative fold changes of significant differences were set in bold. Differences with a fold change of >1.2 or < 0.8 were considered as relevant.

cID	**Identification**		**Single cultivars**										**Cultivar groups**						
	Name	**MSI**[1]	**FDR LogWorth**[2]	**Relative fold change vs. StR**									**FDR LogWorth**[2]	**Letter code**[3]			**Relative fold change**		
				Bif	**PdV**	**ErL**	**Stu**	**ZiG**	**WBr**	**Rij**	**Bol**	**JdC**		**I**	**II**	**III**	**II/I**	**III/II**	**III/I**
Amino acids																			
A0277	**Glutamine 4TMS**	2	10.0	1.81	0.69	3.00	1.45	2.93	9.08	**11.18**	7.12	2.96	10.6	C	B	A	**1.91**	**3.83**	**7.33**
A0299	**Glutamine 3TMS**	1	14.5	1.41	1.04	**1.76**	1.25	1.53	**2.73**	**2.93**	**2.31**	1.42	14.6	C	B	A	**1.30**	**1.77**	**2.31**
A0235	**Asparagine 3TMS**	1	10.1	1.33	1.55	1.30	1.27	1.50	**1.90**	**2.69**	**2.13**	1.00	8.1	B	B	A	1.05	**1.66**	**1.75**
A0202	Similar to Asparagine 4TMS	3	4.7	1.11	1.19	1.21	1.18	1.11	1.35	**1.82**	**1.49**	1.07	5.1	B	B	A	1.06	**1.34**	**1.42**
A0211	**Glutamic acid 3TMS**	1	11.2	1.16	1.04	0.99	1.14	1.06	1.21	**1.71**	**1.41**	1.12	7.8	B	B	A	1.00	**1.37**	**1.36**
A0161	**Aspartic acid 3TMS**	1	9.5	0.89	**0.75**	0.97	1.22	0.95	1.32	**1.46**	1.24	1.00	8.4	C	B	A	**1.20**	**1.27**	**1.52**
A0080	**Proline 2TMS**	1	3.5	1.13	0.94	1.26	1.49	1.07	1.51	**2.37**	1.18	0.74	2.0	B	AB	A	1.25	1.33	**1.66**
A0033	**Proline TMS**	2	n.s.	0.97	0.61	1.14	0.98	1.13	1.17	1.62	1.38	0.84	2.3	B	AB	A	1.25	1.30	**1.63**
A0153	**5-Oxoproline 2TMS**	1	8.9	**1.33**	0.96	1.19	1.03	1.14	**1.45**	**1.66**	**1.35**	0.92	6.5	B	B	A	1.02	**1.34**	**1.36**
A0208	**Ornithine 3TMS**	2	4.8	1.32	**1.60**	**1.43**	1.25	1.35	1.29	**1.54**	**1.43**	0.84	n.s.	A	A	A	1.02	1.06	1.09
A0324	**Ornithine 4TMS**	1	6.1	1.08	1.33	**1.39**	1.01	1.33	1.05	1.36	**1.40**	**0.60**	n.s.	A	A	A	1.09	1.03	1.13
A0344	**Lysine 3TMS**	1	2.8	1.12	0.94	1.26	1.33	1.18	1.31	**1.58**	**1.45**	1.38	4.3	B	A	A	**1.23**	1.16	**1.42**
A0015	**Alanine**	1	11.0	1.43	1.03	1.90	1.93	1.47	**3.14**	**4.07**	**1.96**	0.89	7.5	C	B	A	**1.52**	**1.74**	**2.64**
A0123	**beta-Alanine 3TMS**	1	4.1	1.51	0.68	2.38	**1.78**	1.45	**2.41**	**2.94**	1.50	1.20	3.2	B	A	A	**1.73**	1.23	**2.14**
A0162	**gamma-Aminobutyric acid 3TMS**	1	8.1	1.22	0.63	1.31	1.19	0.93	1.22	**2.39**	1.66	0.61	4.4	B	B	A	1.20	**1.56**	**1.87**
A0032	**2-Aminobutyric acid 2TMS**	2	3.9	1.05	1.08	1.83	**1.80**	1.60	1.49	**2.37**	1.28	**0.61**	2.6	B	A	A	**1.67**	0.99	**1.66**
A0042	**Dehydroalanine 2TMS**	1	11.2	**1.44**	**1.38**	1.22	1.12	**1.38**	**1.45**	**1.53**	**1.57**	0.80	3.8	B	B	A	0.98	**1.22**	**1.19**
A0081	**Glycine 3TMS**	1	8.6	1.08	0.83	1.09	1.14	1.11	1.29	**1.70**	**1.32**	0.91	7.1	C	B	A	**1.15**	**1.30**	**1.49**
A0110	**Threonine 3TMS**	1	5.1	0.92	0.84	1.25	1.43	1.12	1.34	**1.87**	1.27	0.88	4.2	B	A	A	**1.38**	1.18	**1.63**
A0072	**Leucine 2TMS**	1	7.7	1.03	0.90	1.84	1.47	0.93	1.76	**2.59**	**1.61**	1.58	5.6	C	B	A	**1.43**	**1.43**	**2.04**
A0029	**Leucine TMS**	2	6.8	0.95	0.82	1.48	1.03	1.05	1.50	**1.86**	**1.89**	1.67	8.1	B	B	A	1.28	**1.50**	**1.91**
A0045	**Valine 2TMS**	1	5.7	0.97	0.88	1.30	**1.45**	1.18	1.39	**1.80**	1.19	0.96	4.7	B	A	A	**1.38**	1.12	**1.54**
A0078	**Isoleucine 2TMS**	1	4.0	0.80	0.88	1.43	1.44	0.99	**1.53**	1.65	1.23	1.22	4.3	B	A	A	**1.43**	1.15	**1.64**
A0034	**Isoleucine TMS**	2	n.s.	0.72	0.75	1.10	0.97	1.10	1.30	1.15	1.35	1.28	3.5	B	A	A	**1.28**	1.20	**1.53**
A0014	**Valine TMS**	2	n.s.	0.80	0.67	0.98	0.96	1.24	1.06	1.19	1.31	1.00	2.2	B	AB	A	1.29	1.12	**1.44**
A0209	**Phenylalanine 2TMS**	1	14.5	0.87	1.05	1.17	1.18	**0.76**	**1.39**	**1.71**	**1.35**	**1.81**	8.6	B	B	A	1.06	**1.45**	**1.53**
A0389	**Tyrosine 3TMS**	2	2.1	0.92	0.93	1.09	1.06	1.09	1.08	1.20	1.14	0.85	2.6	B	A	A	**1.14**	1.06	**1.20**
A0155	**Methionine 2TMS**	1	7.8	1.27	1.34	1.36	1.08	1.09	**2.07**	**2.55**	**1.76**	1.69	8.5	B	B	A	0.97	**1.82**	**1.77**
A0136	**Methiin derivative**	1	3.9	1.54	1.19	1.52	1.16	1.49	2.22	**2.60**	1.41	1.11	3.0	B	B	A	1.11	**1.50**	**1.67**
A0167	**Methiin derivative**	1	8.1	1.60	1.27	1.30	1.05	1.58	**2.80**	**2.48**	**2.62**	**2.56**	9.5	B	B	A	1.01	**2.05**	**2.06**
A0283	**Methiin derivative**	1	6.6	1.34	1.33	1.29	1.06	1.25	**2.26**	**2.39**	**1.99**	**1.94**	8.4	B	B	A	0.98	**1.85**	**1.81**

Table S4. Continued.

cID	Identification		Single cultivars										Cultivar groups						
	Name	**MSI**[1]	**FDR LogWorth**[2]	**Relative fold change vs. StR**									**FDR LogWorth**[2]	**Letter code**[3]			**Relative fold change**		
				Bif	**PdV**	**ErL**	**Stu**	**ZiG**	**WBr**	**Rij**	**Bol**	**JdC**		**I**	**II**	**III**	**II/I**	**III/II**	**III/I**
									Amino acids										
A0237	**Methiin derivative**	1	3.1	1.42	1.16	1.39	1.01	1.15	**2.16**	**2.07**	**1.66**	**1.69**	4.2	B	B	A	0.98	**1.67**	**1.64**
A0121	***S*-Methylcysteine 2TMS**	2	2.1	1.00	1.02	1.11	1.20	0.94	**1.49**	**1.50**	1.05	1.32	2.0	B	B	A	1.08	**1.24**	**1.33**
A0198	Amino acid derivative	3	3.5	1.22	1.43	1.06	1.24	1.38	1.36	**1.73**	1.58	**0.46**	n.s.	A	A	A	1.01	1.26	1.28
A0204	Amino acid derivative	3	13.4	**1.39**	1.25	1.22	1.18	**1.36**	**1.44**	**1.64**	**1.64**	0.74	6.2	B	B	A	1.03	**1.26**	**1.30**
A0212	Amino acid derivative	3	2.5	0.87	0.67	1.06	1.11	0.65	1.31	1.67	1.33	1.41	4.2	B	B	A	1.14	**1.52**	**1.74**
A0223	Amino acid derivative	3	14.8	**1.35**	1.27	1.17	1.14	**1.39**	**1.50**	**1.62**	**1.64**	**0.73**	7.4	B	B	A	1.03	**1.28**	**1.32**
A0232	Amino acid derivative	3	11.6	**1.32**	1.22	1.15	1.10	1.24	**1.41**	**1.52**	**1.55**	**0.70**	6.2	B	B	A	0.99	**1.29**	**1.27**
A0243	Amino acid derivative	3	6.4	**1.68**	**1.45**	1.23	1.18	1.43	**1.45**	**1.55**	**1.63**	0.72	n.s.	A	A	A	0.93	1.21	1.12
A0251	Amino acid derivative	3	15.4	**1.36**	**1.24**	1.15	1.10	**1.34**	**1.34**	**1.56**	**1.58**	**0.75**	6.0	B	B	A	1.00	**1.25**	**1.25**
A0253	Amino acid derivative	3	14.9	**1.37**	**1.29**	1.17	1.16	**1.34**	**1.44**	**1.58**	**1.57**	**0.73**	6.3	B	B	A	1.00	**1.25**	**1.26**
A0254	Amino acid derivative	3	12.6	1.42	1.29	1.19	1.17	**1.40**	**1.48**	**1.60**	**1.63**	0.76	5.6	B	B	A	1.01	**1.25**	**1.27**
A0261	Amino acid derivative	3	13.7	**1.42**	**1.29**	1.22	1.17	**1.36**	**1.45**	**1.60**	**1.60**	0.76	5.8	B	B	A	1.01	**1.25**	**1.26**
A0280	Amino acid derivative	3	7.9	1.24	1.13	1.17	1.08	1.26	**1.71**	**2.07**	**1.86**	0.79	8.1	B	B	A	1.03	**1.61**	**1.67**
A0423	Amino acid derivative	3	8.4	1.35	1.43	1.47	1.35	**2.00**	**2.17**	**2.69**	**2.81**	1.37	9.2	B	B	A	1.25	**1.61**	**2.01**
A0428	Amino acid derivative	3	3.2	1.26	1.26	1.26	1.17	1.83	1.59	1.87	2.37	0.75	2.6	B	AB	A	1.23	1.37	**1.68**
									Acids										
A0179	**Erythronic acid 4TMS**	1	7.9	1.26	0.99	1.59	1.70	2.04	2.24	3.41	**4.61**	**4.28**	6.8	C	B	A	**1.68**	**1.91**	**3.21**
A0360	**Dehydroascorbic acid deriv.2**	2	11.3	0.95	0.75	0.95	1.53	1.64	**2.13**	**2.27**	**1.93**	**2.71**	10.1	C	B	A	**1.57**	**1.49**	**2.34**
A0341	**Dehydroascorbic acid deriv.1**	2	10.0	0.90	**0.78**	0.92	**1.24**	1.17	**1.31**	1.21	**1.22**	**1.36**	7.0	C	B	A	**1.25**	**1.12**	**1.39**
A0390	**Dehydroascorbic acid deriv.4**	2	3.1	0.84	0.68	1.01	1.22	1.26	1.25	1.23	1.36	1.11	4.3	B	A	A	**1.39**	1.09	**1.52**
A0068	**Phosphoric acid 3TMS**	1	4.2	1.12	1.20	0.91	1.12	0.96	**1.48**	1.18	**1.31**	1.10	4.1	B	B	A	0.90	**1.31**	**1.19**
A0003	**Pyruvic acid MEOX-TMS**	1	2.5	0.85	**0.63**	1.81	1.18	0.70	1.03	0.95	0.81	**0.71**	n.s.	A	A	A	1.46	0.77	1.12
A0142	**Malic acid 3TMS**	2	17.1	0.92	**0.84**	1.08	**1.12**	**0.87**	1.09	1.08	**1.22**	**1.14**	7.0	B	A	A	**1.11**	1.11	**1.23**
A0327	**Citric acid 4TMS**	1	7.8	1.00	1.08	**0.65**	1.08	1.21	0.75	0.90	**0.59**	0.80	3.3	A	A	B	0.97	**0.75**	**0.73**
A0097	**Fumaric acid 2TMS**	1	6.6	0.67	**0.45**	0.97	1.12	0.62	0.99	**0.51**	0.61	**0.35**	n.s.	A	A	A	1.30	0.76	0.99
									Sterols										
A0573	Sterol	3	7.1	1.44	1.35	**1.95**	0.81	1.08	**1.80**	1.21	0.87	1.29	n.s.	A	A	A	1.01	0.98	1.00
A0569	Sterol	3	2.8	1.38	1.03	**1.54**	0.97	0.93	1.35	1.21	1.02	0.95	n.s.	A	A	A	1.02	1.04	1.06
A0576	**beta-Sitosterol TMS**	1	n.s.	1.04	0.80	1.04	1.04	1.03	1.15	1.17	1.11	0.96	2.6	B	AB	A	1.09	1.10	**1.21**
									Sugar compounds										
A0135	Dialdose derivative	3	5.0	1.24	1.08	1.23	1.11	1.01	**1.85**	2.07	**2.08**	1.74	7.3	B	B	A	1.00	**1.81**	**1.81**
A0197	C5 sugar	3	9.9	0.97	0.95	0.78	1.01	**1.47**	0.96	**0.44**	**0.56**	0.92	5.0	A	A	B	1.13	**0.58**	**0.66**
A0217	Sugar-like	3	2.9	0.28	0.86	**0.22**	**1.68**	0.80	0.91	1.25	0.74	**0.45**	n.s.	A	A	A	1.26	0.98	1.24

Table S4. Continued.

cID	Identification		Single cultivars										Cultivar groups					
	Name	MSI[1]	FDR LogWorth[2]	Relative fold change vs. StR									FDR LogWorth[2]	Letter code[3]			Relative fold change		
				Bif	PdV	ErL	Stu	ZiG	WBr	Rij	Bol	JdC		I	II	III	II/I	III/II	III/I
				Sugar compounds															
A0227	**Xylose MEOX-4TMS deriv.1**	1	14.2	**0.52**	0.79	1.21	**1.70**	**1.72**	**2.19**	**2.43**	**2.52**	**2.76**	13.7	C	B	A	**1.96**	**1.53**	**3.01**
A0233	**Xylose MEOX-4TMS deriv.2**	1	20.9	0.99	1.21	1.30	**1.37**	**1.49**	**1.59**	**2.00**	**2.21**	**2.42**	15.4	C	B	A	**1.31**	**1.40**	**1.83**
A0248	**Ribose 5TMS**	1	21.6	**0.64**	0.88	1.26	1.42	1.02	**2.33**	**1.97**	**1.87**	**3.88**	15.3	C	B	A	**1.47**	**1.66**	**2.43**
A0271	**Arabitol 5TMS**	1	3.8	0.96	0.98	1.12	1.29	1.12	1.16	1.32	1.34	**1.40**	4.1	B	A	A	**1.20**	1.09	**1.30**
A0281	Sugar or sugar-like	3	8.8	1.09	0.91	**0.57**	0.97	1.27	0.94	**0.38**	**0.47**	0.94	4.2	A	A	B	0.95	**0.61**	**0.58**
A0288	Sugar acid	3	9.5	**1.47**	1.08	1.08	1.18	1.22	**1.50**	**1.31**	1.23	**1.77**	n.s.	A	A	A	0.98	1.15	1.13
A0295	Sugar	3	7.2	1.04	0.97	0.88	0.95	0.99	0.85	0.79	0.81	**0.30**	n.s.	A	A	A	0.94	0.86	0.81
A0297	Sugar acid	3	6.8	1.11	0.87	1.91	1.15	2.38	1.84	3.82	3.75	2.21	5.6	C	B	A	**1.93**	**1.66**	**3.20**
A0311	Sugar or sugar-like	3	16.0	0.81	1.26	**1.58**	1.16	1.45	**1.71**	**2.41**	**2.56**	**4.16**	10.2	C	B	A	**1.34**	**1.62**	**2.18**
A0326	Sugar or sugar-like	3	4.6	0.27	0.75	0.98	1.19	1.64	1.89	2.34	2.22	2.98	5.1	B	B	A	1.71	**1.68**	**2.87**
A0343	Sugar or sugar-like	3	20.3	**0.58**	0.86	**1.83**	**1.95**	**2.44**	**3.37**	**3.01**	**3.33**	**3.95**	20.6	C	B	A	**2.56**	**1.55**	**3.97**
A0355	Sugar / glycoside	3	29.1	**0.49**	1.07	**1.75**	1.31	**1.63**	**2.57**	**3.82**	**3.55**	**7.75**	20.1	C	B	A	**1.78**	**2.16**	**3.85**
A0356	Sugar or sugar-like	3	23.0	0.65	1.26	1.39	1.56	1.57	2.07	**2.28**	**3.05**	**10.28**	7.7	C	B	A	**1.54**	**1.64**	**2.53**
A0362	Sugar / glycoside	3	40.4	**0.55**	1.03	**1.87**	1.47	**1.81**	**2.56**	**3.33**	**3.77**	**8.94**	20.5	C	B	A	**1.99**	**1.90**	**3.78**
A0365	**Fructose MEOX-5TMS**	1	45.9	0.79	1.09	**1.32**	1.15	**1.26**	**1.61**	**1.95**	**2.00**	**4.08**	21.9	C	B	A	**1.30**	**1.50**	**1.95**
A0376	**Glucose MEOX-5TMS**	1	30.3	0.69	0.85	**1.57**	**1.69**	**1.88**	**2.37**	**2.30**	**2.45**	**2.77**	27.5	C	B	A	**2.04**	**1.38**	**2.81**
A0388	Sugar or sugar-like	3	15.3	0.85	0.99	1.28	1.31	1.63	**1.85**	**1.76**	**1.97**	**3.78**	7.3	C	B	A	**1.49**	**1.32**	**1.97**
A0397	Sugar or sugar-like	3	16.6	0.94	1.21	0.72	0.45	0.70	0.78	1.25	1.21	**5.28**	2.1	A	B	A	**0.60**	**1.73**	1.04
A0400	Sugar / glycoside	3	32.5	0.57	0.83	**2.09**	**2.34**	**2.76**	**3.96**	**3.76**	**4.07**	**5.50**	28.8	C	B	A	**3.00**	**1.63**	**4.90**
A0408	Sugar or sugar-like	3	30.4	0.52	1.14	**2.68**	**2.88**	**3.39**	**5.04**	**5.05**	**5.27**	**8.08**	25.2	C	B	A	**3.26**	**1.71**	**5.58**
A0412	Sugar / glycoside	3	20.2	0.62	0.90	**2.17**	**2.62**	**3.36**	**5.67**	**5.06**	**5.45**	**5.90**	24.6	C	B	A	**3.26**	**1.97**	**6.41**
A0415	Sugar or sugar-like	3	19.4	0.81	1.11	**2.82**	**3.08**	**3.71**	**5.01**	**4.76**	**5.67**	**7.67**	18.9	C	B	A	**3.27**	**1.60**	**5.23**
A0431	Sugar / glycoside	3	29.2	0.56	0.80	**1.91**	**2.02**	**2.18**	**2.80**	**2.86**	**2.95**	**4.03**	25.2	C	B	A	**2.60**	**1.41**	**3.66**
A0441	***myo*-Inositol 6TMS**	2	4.5	1.11	1.00	1.15	**1.16**	1.14	**1.17**	1.15	1.06	**1.29**	2.4	B	A	A	**1.11**	0.98	**1.08**
A0447	Sugar or sugar-like	3	3.3	1.46	1.13	1.90	3.10	3.85	4.40	3.99	4.58	4.79	4.4	C	B	A	**2.67**	**1.44**	**3.86**
A0448	Sugar acid	3	3.5	2.04	1.31	1.43	2.05	1.70	2.25	1.93	2.29	2.47	2.5	B	AB	A	1.18	1.24	**1.46**
A0484	Sugar or sugar-like	3	7.7	1.06	0.81	1.05	1.19	1.33	1.26	**1.61**	**1.53**	1.25	7.7	C	B	A	**1.26**	**1.23**	**1.54**
A0491	**Glucose-6-phosphate MEOX-6TMS**	1	9.2	1.02	0.95	0.78	0.84	**1.61**	1.51	**2.31**	1.49	**1.97**	5.2	B	B	A	1.12	**1.60**	**1.79**
A0505	Disaccharide	3	12.4	1.04	1.05	0.98	0.97	0.98	**0.77**	0.85	0.81	**0.33**	5.0	A	A	B	0.95	**0.83**	**0.79**
A0509	Disaccharide	3	4.0	1.22	0.89	**0.42**	1.15	0.94	0.52	0.87	0.60	0.77	n.s.	A	A	A	0.84	0.78	0.65
A0510	Disaccharide	3	3.0	1.17	0.71	0.60	0.92	0.79	0.74	1.12	0.98	**0.34**	n.s.	A	A	A	0.81	1.23	0.99
A0517	Disaccharide	3	7.6	1.04	0.89	0.80	0.94	0.83	**0.77**	0.81	0.76	**0.40**	2.7	A	B	B	**0.88**	0.91	**0.80**
A0523	Disaccharide	3	9.2	1.05	**0.57**	**0.61**	0.95	**0.73**	0.76	0.81	0.85	**0.31**	n.s.	A	A	A	0.88	1.05	0.93

Table S4. Continued.

	Identification		Single cultivars										Cultivar groups						
cID	**Name**	**MSI**[1]	**FDR LogWorth**[2]	**Relative fold change vs. StR**									**FDR LogWorth**[2]	**Letter code**[3]			**Relative fold change**		
				Bif	**PdV**	**ErL**	**Stu**	**ZiG**	**WBr**	**Rij**	**Bol**	**JdC**		**I**	**II**	**III**	**II/I**	**III/II**	**III/I**
								Sugar compounds											
A0524	**Sucrose 8TMS**	2	11.6	**0.89**	**0.78**	**0.85**	1.00	**0.89**	0.96	0.96	0.96	**0.72**	n.s.	A	A	A	1.03	1.05	1.08
A0530	Disaccharide	3	13.4	**0.29**	1.27	0.75	**1.49**	1.24	1.81	**1.77**	**1.59**	**2.90**	5.2	C	B	A	**1.38**	**1.46**	**2.01**
A0536	Disaccharide	3	27.5	**0.29**	2.01	0.88	**1.90**	2.04	**4.24**	**3.35**	**3.09**	**14.83**	5.7	C	B	A	**1.49**	**2.16**	**3.22**
A0539	**Trehalose 8TMS**	1	5.7	1.05	0.97	1.00	1.02	**1.29**	0.94	**1.21**	1.06	1.12	n.s.	A	A	A	1.10	0.97	1.07
A0560	**Galactinol 9TMS**	1	20.3	0.91	1.78	1.68	1.62	1.36	**2.21**	**4.54**	**3.17**	0.52	11.2	B	B	A	1.26	**2.17**	**2.73**
A0561	Galactinol-like	3	3.9	0.76	0.65	0.81	1.10	0.93	1.28	0.92	1.02	**0.57**	n.s.	A	A	A	1.16	1.10	1.28
A0568	Trisaccharide	3	8.0	0.97	1.03	**0.66**	**0.62**	0.70	**0.34**	**0.44**	**0.33**	**0.22**	11.3	A	B	C	**0.66**	**0.57**	**0.37**
A0570	Trisaccharide	3	5.7	1.38	1.09	0.87	0.88	0.96	0.78	0.95	1.03	**0.19**	n.s.	A	A	A	0.78	1.02	0.80
A0574	Trisaccharide	3	13.9	1.14	0.90	0.85	0.99	0.97	0.87	0.88	0.88	**0.12**	n.s.	A	A	A	0.93	0.93	0.86
A0579	**1-Kestose 11TMS**[4]	1	20.8	**0.81**	**0.58**	**0.75**	0.97	0.85	0.87	**0.65**	**0.62**	**0.05**	n.s.	A	A	A	1.07	0.83	0.89
A0581	Trisaccharide	3	3.2	0.57	1.42	0.95	**2.40**	1.79	**2.38**	1.75	1.43	1.42	2.5	B	A	A	**1.76**	1.04	**1.84**
A0582	Trisaccharide	3	2.1	0.45	1.14	**0.48**	1.70	1.19	1.63	1.07	0.76	2.03	n.s.	A	A	A	1.28	0.97	1.24
A0586	Tetrasaccharide	3	n.s.	1.08	0.84	0.65	0.80	0.90	0.59	0.76	0.48	n.d.	2.2	A	A	B	0.82	**0.75**	**0.61**
A0587	Tetrasaccharide	3	3.0	1.05	0.90	0.83	0.87	0.75	0.72	**0.51**	0.72	n.d.	4.2	A	AB	B	0.83	0.79	**0.66**
A0590	**Nystose 14TMS**	1	10.8	0.93	**0.73**	**0.74**	0.85	0.83	**0.65**	**0.38**	**0.31**	**0.04**	9.2	A	A	B	0.91	**0.54**	**0.49**
A0591	Major tetrasaccharide 2	3	9.5	0.88	0.73	**0.72**	0.81	0.83	**0.60**	**0.36**	**0.28**	0.02	8.9	A	A	B	0.90	**0.51**	**0.46**
								Unknowns											
A0284	Unknown	4	6.5	0.68	0.81	1.73	1.96	2.54	**2.67**	2.79	**3.76**	2.76	7.5	C	B	A	**2.51**	**1.49**	**3.73**
A0291	Unknown	4	12.8	0.71	0.89	1.76	**1.88**	**2.30**	**2.66**	**2.89**	**3.08**	**2.58**	15.5	C	B	A	**2.30**	**1.45**	**3.33**
A0127	Unknown	4	3.8	1.42	1.11	1.17	1.00	1.12	2.31	2.36	2.64	3.08	6.6	B	B	A	0.93	**2.22**	**2.07**
A0186	Unknown	4	8.6	0.93	0.74	1.24	1.29	0.73	2.14	2.94	2.06	1.69	9.3	B	B	A	1.21	**2.21**	**2.69**
A0257	Unknown	4	5.7	1.37	0.98	1.49	1.08	1.38	**2.05**	**2.40**	**1.89**	0.71	5.6	B	B	A	1.17	**1.62**	**1.89**
A0236	Unknown	4	5.3	1.42	0.94	**1.56**	1.06	1.24	1.93	**2.36**	**1.85**	**0.59**	4.9	B	B	A	1.14	**1.61**	**1.83**
A0485	Unknown	4	2.5	1.50	1.15	1.52	1.22	1.26	1.45	1.24	1.26	**2.17**	n.s.	A	A	A	1.10	0.97	1.07
A0216	Unknown	4	2.2	1.23	1.42	1.32	1.22	1.69	1.48	**2.15**	**2.06**	0.83	2.5	B	B	A	1.13	**1.35**	**1.52**
A0195	Unknown	4	2.9	1.26	1.12	1.30	1.82	1.11	**2.04**	**2.05**	**1.76**	**2.08**	3.6	B	B	A	1.26	**1.37**	**1.73**
A0255	Unknown	4	8.4	1.23	0.90	**1.51**	1.10	1.23	**1.75**	**2.01**	**1.75**	1.18	9.6	B	B	A	1.22	**1.45**	**1.76**
A0466	Unknown	4	2.4	1.36	1.39	1.35	1.24	1.62	1.31	**1.64**	**1.99**	1.12	n.s.	A	A	A	1.11	1.19	1.33
A0282	Unknown	4	14.0	**1.60**	**1.46**	**1.41**	**1.35**	**1.61**	**1.68**	**1.96**	**1.92**	0.86	6.7	B	B	A	1.08	**1.27**	**1.38**
A0444	Unknown	4	11.5	1.38	1.23	1.20	1.19	1.47	**1.59**	**1.77**	**1.94**	0.95	8.8	B	B	A	1.07	**1.38**	**1.48**
A0457	Unknown	4	8.8	1.32	1.20	1.27	1.16	**1.43**	1.46	**1.73**	**1.88**	1.00	7.8	B	B	A	1.10	**1.32**	**1.45**
A0114	Unknown	4	5.3	1.05	0.96	0.84	0.93	**1.67**	0.99	**0.35**	**0.49**	1.01	3.1	A	A	B	1.15	**0.53**	**0.61**
A0487	Unknown	4	5.8	**1.58**	1.28	1.57	0.87	1.27	**1.61**	1.19	0.87	**1.54**	n.s.	A	A	A	0.95	0.99	0.94

Table S4. Continued.

cID	Identification		Single cultivars										Cultivar groups						
	Name	MSI[1]	FDR LogWorth[2]	Relative fold changes vs. StR									FDR LogWorth[2]	Letter code[3]			Relative fold changes		
				Bif	**PdV**	**ErL**	**Stu**	**ZiG**	**WBr**	**Rij**	**Bol**	**JdC**		**I**	**II**	**III**	**II/I**	**III/II**	**III/I**
	Unknowns																		
A0174	Unknown	4	2.4	1.44	1.46	0.80	0.86	1.06	**1.60**	1.10	1.21	1.00	n.s.	A	A	A	0.70	1.40	0.98
A0220	Unknown	4	15.3	**1.37**	**1.24**	1.16	1.10	**1.37**	**1.34**	**1.54**	**1.59**	**0.73**	5.6	B	B	A	1.01	**1.24**	**1.25**
A0222	Unknown	4	13.5	1.35	1.23	1.14	1.10	**1.33**	**1.42**	**1.48**	**1.57**	**0.70**	5.6	B	B	A	1.00	**1.25**	**1.25**
A0270	Unknown	4	4.7	0.50	0.40	0.68	0.58	0.31	1.06	1.48	1.02	0.95	5.6	B	B	A	0.97	**2.17**	**2.11**
A0229	Unknown	4	2.1	1.21	1.03	1.04	0.91	1.19	1.00	1.17	**1.43**	0.80	n.s.	A	A	A	0.97	1.15	1.12
A0313	Unknown	4	3.5	1.04	0.92	1.07	1.01	1.15	0.89	0.85	0.97	**0.29**	n.s.	A	A	A	1.09	0.84	0.92
A0111	Unknown	4	2.0	1.00	0.97	1.04	1.01	1.05	1.02	**1.09**	1.07	1.04	2.9	B	A	A	**1.05**	1.03	**1.08**

[1] Metabolomics Standards Initiative identification levels: Identity confirmed by spiking with reference compound (1), compound tentatively identified by matching against internal or external spectral libraries, including retention indices (2), compound class tentatively identified by matching against spectral libraries or due to the presence of marker fragments (3), no assignment to compound class possible due to unspecific spectrum (4); [2] FDR LogWorth = -log(FDR *p*-value), [3] Letter code indicating significance according to Tukey HSD post-hoc test;[4] Coleuting with another trisaccharide with identical EI spectrum; n.s., not significant; n.d., not detected. Group I= Bif, PdV; Group II= ErL, Stu, ZiG; Group III= WBr, Rij, JdC. [3] rFC, relative fold change (=mean intensity (stored)/mean intensity(fresh)).

Chapter 4 – Sodium accumulation has minimal effect on the metabolome profile of onion bulbs

M.L. Romo-Pérez, C.H. Weinert, B. Egert, B.L. Franzisky, S.E. Kulling, C. Zörb

Under Review

Sodium accumulation has minimal effect on metabolite profile of onion bulbs

M. L. Romo-Pérez[1], C. H. Weinert[2], B. Egert[1], B. L. Franzisky[1], S. E. Kulling[2], C. Zörb[1]

[1] University of Hohenheim, Institute of Crop Science, Quality of Plant Products 340e, Schloss Westflügel, 70599 Stuttgart, Germany

[2] Max Rubner-Institut, Department of Safety and Quality of Fruit and Vegetables, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany

Abstract

Onions (*Allium cepa* L.) are considered as a salt-sensitive crop. However, to date, little evidence supports this claim, and information about the physiological and metabolomic effects of Na^+ accumulation in onion plants is lacking. The purpose of our research has been to assess changes in the leaves and bulbs of three different onion cultivars after soil and foliar applications with moderate doses of chloride-free Na_2SO_4. The antioxidative defense mechanism in onion and the transport of Na^+ within the plant has also been analyzed. Our findings demonstrate that Na^+ is mainly transported via the xylem, and that therefore foliar application does not lead to Na^+ accumulation in the bulbs. Soil application with Na_2SO_4 results in an accumulation of Na^+ in the leaves and bulbs, but with the exception of one onion variety, this does not alter the metabolite profile of onions significantly. Even the K^+ concentration and organic solute levels are unchanged after application of Na^+. Nevertheless, after Na_2SO_4 treatment, the antioxidative defense system moderately increases in onion bulbs. This study demonstrates that onion plants have the ability to exclude Na^+ at moderate Na_2SO_4 treatment, and that the potential for quality onion production in saline soils with increased sodium concentration is much higher than previously assumed.

Keywords: *Allium cepa* L., sodium, metabolomics, antioxidant defense systems, foliar application

1. Introduction

Onion, after tomato, is the second most cultivated vegetable in the world, reaching almost 100 billion tons in production during 2018 alone [1]. As a crop, it is considered moderately difficult to grow, mostly because of soil properties such as salinity [2]. Under saline conditions, Na^+ accumulation is one of the primary risk factors for toxic ion-specific damage affecting many crop plant species including onions [3-6]. Because of the similarity of the hydrated ionic radii of Na^+ and K^+, transporters cannot discriminate between them, leading to Na^+ accumulation in plant cells and to the inactivation of essential cellular functions [7, 8]. Furthermore, a high Na^+ content in soil has been shown to have an antagonistic effect on the absorption of K^+, Ca^{2+}, and Mg^{2+} [9, 10]. Sodium in combination with chloride (NaCl) has been found to be more toxic than sodium with sulfate in forage species and maize when administered as Na_2SO_4 [11]. Moreover, compared with the well-known Na^+ toxicity, the toxicity of sulfate remains unknown, because it is not accumulated in large amounts in plant tissues [12, 13]. In order to understand the response of Na^+ accumulation in onions, results obtained at both physiological and metabolic levels have to be closely examined, particularly with respect to possible effects not only in vegetative tissue, such as leaves, but also in the final product, namely the bulbs.

To mitigate oxidative stress resulting from *inter alia* Na^+ accumulation, plants have developed a defense system that consists of enzymatic (*e.g.,* glutathione reductase) and non-enzymatic components, such as antioxidants, including glutathione [14]. In response to adverse conditions, the antioxidative defense system becomes active, and levels of glutathione increase [15]. In onions, glutathione is a fundamental part of the metabolic pathway linked to aroma and flavor precursors. For this reason, a stress-related increase in onion glutathione levels is also expected to alter its flavor. Chang and Randle [16] have reported some physiological effects and changes in the levels of flavor precursors of onions in response to Na^+ accumulation. However, changes in the levels of other relevant metabolites and related enzymes such as glutathione and glutathione reductase still need to be explored, in particular, because sulfur-containing tri-peptide (Glu-Cys-Gly) is one of the central but least researched metabolic components in onion metabolism.

Onions produce two types of leaves: green foliage leaves, in which photosynthesis occurs, and non-green leaves (scales), in which the lateral cell expansion of the lower sheaths occurs to form the storage tissue, also known as the onion bulb [17]. Leaves are readily permeable to plant nutrient solutions, and for this reason, foliar application/fertilization is used as an alternative to provide plants with fertilization in an effective, environmentally friendly, and target-oriented way compared with soil application. The pathway of nutrient solution uptake into the plant depends on multiple factors, *e.g.*, the characteristics of the leaf surface and the chemical properties of the foliar spray formulation applied. The timing of application during growing stages also plays a crucial role [18, 19]. Foliar fertilization might serve as an effective alternative for providing onions with nutrients during bulb formation, when plants are preparing to transfer photosynthate from green foliage leaves to their sinks, and might minimize soil contamination with fertilizers

In this study, three onion varieties with different quality properties [20, 21] were subjected to Na_2SO_4 treatment by soil and leaf application. A moderate level of Na^+ application was chosen to avoid any symptoms of, for example, early senescence, or secondary side effects, such as visible leaf symptoms. This allowed us to analyze primary physiological and metabolic responses in the best possible way without sodium toxicity, i.e., we determined the effects of mild sodium treatment, rather than sodium stress or toxicity. The distribution and transport routes of Na^+ within plant tissues and the resulting physiological responses were also analyzed.

A comprehensive study of onion metabolism, including flavor and aroma precursors, has thus been conducted, for the first time, after the uptake of Na_2SO_4 by roots and leaves. We have investigated whether Na^+ application has a significant influence on the metabolism of onions, their quality, aroma, and antioxidative defense system. We have analyzed the metabolite profiles of onion bulbs by using untargeted two-dimensional gas chromatography-mass spectrometry (GC×GC-MS) [22] and with additional targeted methods. The results compiled by this study answer the following questions: (*i*) is Na^+ effectively transported into onion bulbs; (*ii*) does Na^+ accumulation affect the metabolic composition of onion bulbs; (*iii*) does Na^+ accumulation alter sulfur-containing aroma precursors and onion pungency over the whole growth stage?

2. Material and Methods

2.1. *Plant cultivation, sodium sulfate application, and sample preparation*

In spring 2018, seeds of three onion genotypes, Red Baron (RB, Bejo seeds), Sturon (StR, Bingenheimer Saatgut AG), and Birnförmige (Bif, Kultursaat e.V.) were sown under a control environment in a greenhouse at day/night set point temperatures of 18 °C/25 °C and natural photoperiods. After six weeks, seedlings were transplanted into 5 L Mitscherlich-pots containing soil mixture (4 seedlings/pot), placed in an open environment, and exposed to the natural light and temperature conditions of the area (48° 42' 29.149" N 9° 12' 42.25" E). The soil was a homogeneous mixture of subfloor loam soil and sand. In order to adjust the pH of the mixture, 5 % (w/w) of sour turf was added to the total subfloor-sand mixture adjusting the final pH to 7.1. The initial properties of the soil mixture are described in Table1. For fertilization, a total of 1 g $Mg(NO_3)_2$, 1 g NH_4NO_3, 0.5 g $Ca(NO_3)_2$, 3 g KH_2PO_4, and 0.3 g Fetrilon-combi micronutrient solution (Ag-Nova Technologies Pty Ltd) was added per pot. Basic fertilization was divided into two parts: 50 % at transplantation time and the other 50 % one month later. The setup was completely randomized by using six replications per treatment and variety (Figure 3). The treatments were: (LA) Na_2SO_4 leaf application; (SA) Na_2SO_4 soil application; (C) control well-supplied plants without Na^+ but with 0.5 g $MgSO_4$ added to the general fertilization as a substitute for the sulfate. Of note, in the control (C) group, doses of sulfur were applied in order to highlight the effect of Na^+ and not sulfur in this study. In the soil application (SA) und leaf application (LA) groups, Na_2SO_4 was gradually added in three parts during the bulbing stages of onion plants: 1) early bulbing, 2) active bulbing, 3) maturation. The final dose of LA was 400 mM Na_2SO_4 added to the leaves by brushing, and for SA, 4 g/pot Na_2SO_4 was added to the soil. To prevent drift and to facilitate Na_2SO_4 penetration / retention in the leaf, 0.04% KANTOR® adjuvant (agroplanta GmbH & Co. KG) was added to the liquid solution of the LA plants. To ensure equal treatments, plants of the SA and C groups also received 0.04% of the KANTOR® adjuvant with double-distilled H_2O (ddH_2O) instead of Na_2SO_4 solution. Leaf applications took place in the morning in order to avoid leaf burning by high irradiation and to encourage the opening of stomata. To minimize losses, the leaching of elements from leaves, and contamination with Na_2SO_4 from the leaves to the soil in the LA plants, the pots were transported to the interior of a greenhouse during heavy rain. To combat onion fly infestation during plant growth and bulb development, a monthly treatment with E605 Forte was applied to all plants. Because of an accidental contamination with nitrogen residues in some pots, the respective replicates were excluded from analysis, thus, leaving at least 4 replicates per variety and treatment ($n \geq 4$).

At harvest, the foliage of all plants was carefully washed with ddH_2O in order to remove residues of Na_2SO_4 and adjuvant. Subsequently, selected green parts of leaves were removed from the bulbs and shock-frozen in liquid nitrogen for metabolite profiling and mineral element analysis. Soil samples were also collected and air-dried for 48 h at 100°C in an oven before an analysis of minerals, ions, and electrical conductivity. The bulbs were cured at ambient greenhouse temperatures for three weeks and then weighed before analysis. The base plate was removed from each of the four plants per pot. Wedges of each bulb were cut longitudinally and then pooled. One half was immediately shock-frozen with liquid nitrogen, then freeze-dried, and finally milled with a ball mill (model MM 301, Retsch, Haan, Germany) for 60 s at 30 Hz. These samples were used for the analysis of onion metabolome, minerals, ions, antioxidants, and antioxidant enzymes. The other half was homogenized in a blender (Büchi Mixer B-400, Büchi Labortechnik AG, Flawil, Switzerland) and used for the analysis of pyruvic acid, non-structural carbohydrates, and dry matter.

Table 1 Basic properties of soil mixture at the transplanting time before general fertilization. EC, electrical conductivity

pH-value	*NH4-N mg/kg:*	*NO3-N mg/kg:*	S_{min} *mg/kg:*	*K mg/kg:*	*P mg/kg*	*EC* $mS\ cm^{-1}$
7.06	1.4	1.4	5.27	75	5.4	0.10

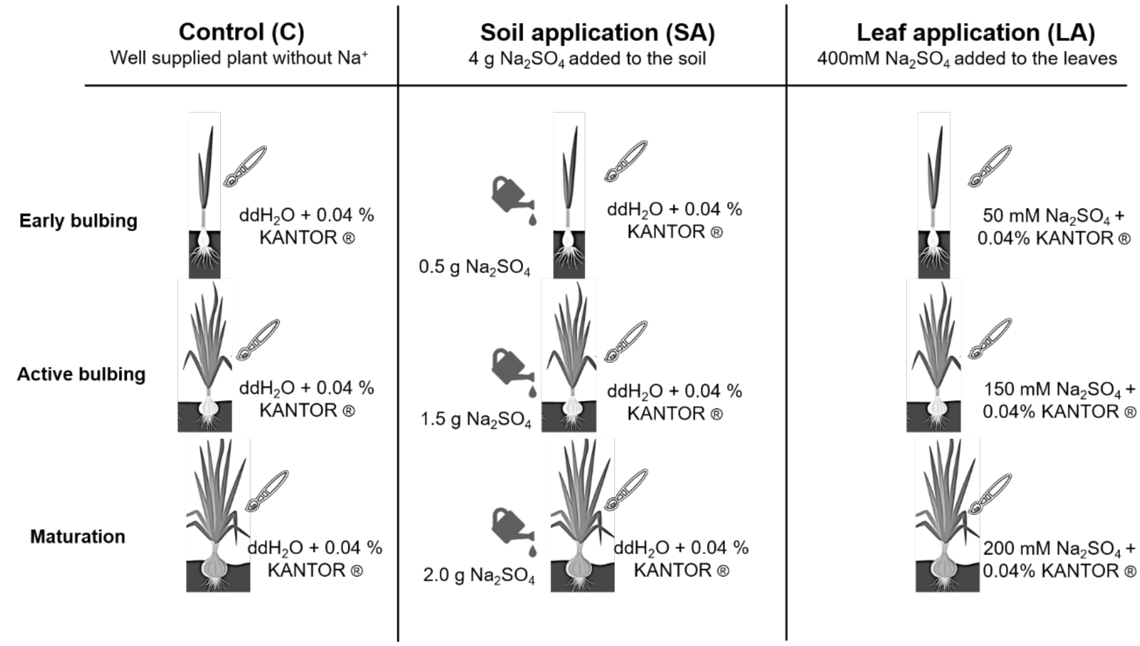


Figure 3 Schematic view of the experimental setup. Pot experiment with three different treatments. Control (C) without Na^+ and 0.5 g of $MgSO_4$ added to the general fertilization. Soil application (SA), final dose of 4 g Na_2SO_4 / pot added to the soil. Leaf application (LA), final dose of 400 mM Na_2SO_4 added to the onion leaves by brushing. SA and LA application were applied in three increments: during early bulbing, active bulbing, and maturation. Adjuvant KANTOR® was added to all plants in all treatments, respectively. For further information, see details in Material and Methods ($n \geq 4$).

2.2. *Total sulfur, carbon, nitrogen, potassium, magnesium, calcium, and sodium analysis*

For cation analysis (Na^+, K^+, Mg^{2+}, Ca^{2+}), approximately 50 mg freeze-dried leaf material and 100 mg bulb and soil material were solubilized in 8 ml 69 % HNO_3 (v/v) and 4 ml H_2O_2 by microwave digestion for 25 min at 190 °C (CEM, Mars 5, Matthews, USA). To verify the extraction procedure, standards and blanks were also digested. Cation concentrations were analyzed by atomic absorption spectrometry (3300 series; Thermo Fisher Scientific, Dreieich, Germany).

For analysis of total sulfur, carbon, and nitrogen, 30 mg freeze-dried leaf and bulb and soil material were examined in a CNS elemental analyzer (Vario max CNS, Elementar Analysensysteme GmbH, Hanau, Germany). The values presented refer to dry mass.

2.3. Targeted metabolic analysis

The determination of dry matter content, non-structural carbohydrates, onion pungency (pyruvic acid), and antioxidant activity was conducted as described by Romo Pérez et al. 2018 [21]. For the dry matter content, 20 g homogenized samples were air-dried in an oven for 48 h at 65 °C followed by 3 h at 105 °C. Non-structural carbohydrates were analyzed based on the Official Analytical Chemist (AOAC) method and the Megazyme fructan-assay kit using p-hydroxybenzoic acid hydrazine (PAHBAH) [23]. The determination of onion pungency was conducted using the improved method of Anthon and Barrett [24] and background pyruvic acid according to the method of Yoo and Pike [25]. The antioxidant activity of onion bulb was carried out according to Brand-Williams, Cuvelier, and Berset [26] using the ´2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method. Total glutathione was analyzed using an enzymatic method according to Saqib, Zörb, and Schubert [27] with some minor modifications. In brief, 50 mg freeze-dried material was homogenized in 2 ml buffer, containing 6 % metaphosphoric acid and 1 mM EDTA. The sample was than centrifuged at 11.500 *x g* for 15 min at 4 °C, and the supernatant was collected. For the stock solution, 1 mM glutathione oxidized (GSSG) was prepared and diluted in 6 % metaphosphoric acid + 1 mM EDTA buffer. The following solutions were prepared for the calibration curve: 0, 4, 8,10, 15 µM GSSG. For total glutathione, 540 µl extracts/standards/blank were mixed on ice with 40 µl 6 % metaphosphoric acid, 1 mM EDTA buffer, and 60 µl triethanolamine. For the measurements, 150 µl aliquots, 700 µl NADPH solution, and 100 µl DNTB solution were pipetted into a cuvette. The addition of 50 µl glutathione reductase (10 U/mL) started the reaction. The rate of absorption change was monitored in a dual beam spectrophotometer SPECORD 50 (Analytic Jena AG, Jena, Germany), at 412 nm every 15 s for 3 min. The concentrations are expressed as µM g^{-1} DM. The analysis of the glutathione reductase activity was measured according to the improved method of Elavarthi and Martin, 2010 [28].

2.4. Untargeted metabolomic analysis

The metabolite analysis was conducted as described by Romo-Perez, Weinert, Haussler, Egert, Frechen, Trierweiler, Kulling, and Zorb [20], with some minor modifications and adaptations to the leaf matrix. Pooled QC samples were prepared for each sample matrix separately by combining material from selected samples from all varieties and treatments. 20 ± 0.16 mg (freeze-dried bulb samples) or 10 ± 0.11 mg (freeze-dried leaf samples) were weighed into 2 ml reaction tubes. Samples were extracted twice with methanol, evaporated to dryness in a vacuum centrifuge and subsequently derivatized (methoximated and trimethylsilylated). Samples of both matrices were analyzed by untargeted GC×GC-MS by using a combination of a 30 m x 0.25 mm unpolar Rxi-5SilMS column in the first dimension and a 2.2 m x 0.15 mm medium-polar BPX50 column in the second dimension. Programmed temperature vaporization injection was performed using an OPTIC4 injector and an optimized temperature program to avoid carry-over of higher fructan oligomers (DP>4) in the bulb samples. A ZOEX ZX2 was employed for cryogenic modulation at a modulation period of 2.7 s. Data were acquired using a fast-scanning single-quadrupol MS instrument at a data acquisition rate of 33 s^{-1}. For a detailed description of the analytical procedures see section 1 of the Supplemental Information. GC×GC-MS data were processed with the SquareDance algorithm implemented in R [29, 30]

2.5. *Statistical analysis*

For the statistics of the targeted analysis, two-way ANOVA followed by the post-hoc Tukey HSD were used to display the variety and the treatment effect. Data were analyzed using linear models, and differences were considered as significant when $p > 0.05$. Principal component analysis (PCA) and a correlation matrix were employed to provide a graphical description of variety characteristics and treatment effect. All values were centralized and standardized. Data were plotted with "ggplot2" [31] and PCA "factoextra" [32]. All analyses were performed with R [30].

The untargeted GC×GC-MS metabolomics dataset was analyzed in two steps by using JMP 15.1.0 (SAS Institute Inc., Cary, NC, 1989-2019). At first, data matrices were prepared for all varieties together and for the single varieties. Variables with ≥ 25% non-detects were excluded and replaced by random numbers between 5,000-10,000. The resulting data matrices comprised between 224-242 and 208-218 metabolites (leaf and bulb samples, respectively). Next, PCA was performed to assess the impact of the fertilization treatment on the overall metabolite profiles of leaves and bulbs. To assess further the treatment effect at the univariate level, an ANOVA screening analysis including an FDR correction [33] was performed for each variety and each sample matrix separately by using the Response Screening platform of JMP. Potentially relevant compounds in the samples of the variety StR were selected visually using so-called "FDR LogWorth vs. Effect Size" plots, and significance was further verified by the Tukey-HSD post-hoc test.

3. Results

3.1. *Physiological reactions of Allium cepa L. caused by Na^+*

A mild Na^+ dose of 4 g Na_2SO_4 per pot for SA and 400 mM Na_2SO_4 for LA were chosen to avoid any visible toxicity or senescence symptoms, such as leaf necrosis or chlorosis. Therefore, the metabolic reaction of plants was not based on the secondary effects of toxicity and degeneration but relied on primary Na^+ accumulation. Regardless of the variety and the treatment, all onions developed well, and no stress symptoms were observed (Figure 4). Moreover, no significant difference between the treatments were determined with respect to the fresh weight of onion bulbs; values were between 160 - 231 g for four plants per pot. Similarly, the height and diameter of the onions showed no significant differences with respect to the treatment (Table 2). Therefore, we concluded that we had induced a non-stressful condition for the plants, as had been planned. However, significant differences were found in relation to the variety. The Bif variety, for example, has a typical elongated shape that resembles a pear (the German name "Birnenförmige" means "pear-shaped") and demonstrates a significantly higher dry matter content compared with the other two varieties. Nevertheless, the Na^+ treatment did not affect the dry matter levels in any of the onion bulb varieties.

Figure 5 shows that Na^+ accumulated significantly in the leaves after application of Na_2SO_4 by SA and LA. The highest level of Na^+ was recorded in leaves exposed to SA, with concentrations above 9 mg Na^+ g^{-1} DW. On the other hand, in onion bulbs, significantly elevated levels of Na^+ were only detected in plants exposed to SA, whereas LA did not lead to Na^+ accumulation.

Potassium concentrations in all varieties were not significantly affected in bulbs or leaves after SA and LA treatment with Na_2SO_4 (Figure 5).

In comparison with control plants, SA treatment led to slight increases of total sulfur levels in the leaves of all varieties. In contrast to the leaf pattern, onion bulbs exhibited no increase in sulfur levels, and no variety effect could be established.

Concentrations of magnesium, calcium, total nitrogen, and total carbon in leaves and bulbs showed only minor differences in all varieties (Table S2). Na^+ accumulation did not lead to significant changes of other ion concentrations, suggesting the lack of severe secondary effects following Na^+ treatment.

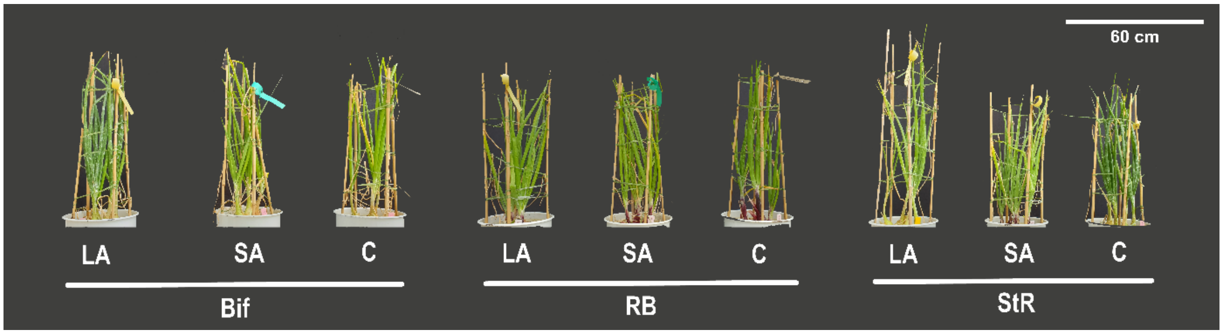


Figure 4 Onion plants (4 plants/pot) one week after the third and last application with Na_2SO_4, namely 4 g Na_2SO_4 by SA (soil application), 400 mM Na_2SO_4 by LA (leaf application), and without Na^+ in C (control). Birnförmige (Bif), Red Baron (RB), Sturon (StR).

Table 2 Total onion weight per pot (4 plants/pot). Height, diameter, and dry matter of onion bulbs**.**

Variety	*Treatment*	*Total onion bulb weight / pot [g]*	*Height [mm]*	*Diameter [mm]*	*Dry matter [%]*
Bif	C	171.48 ± 14.56 a	74.71 ± 4.65 a	34.98 ± 1.88 c	19.10 ± 0.41 a
	LA	169.93 ± 12.91 a	71.37 ± 3.98 a	35.16 ± 1.69 c	18.54 ± 0.36 a
	SA	160.15 ± 12.17 a	72.39 ± 4.03 a	35.64 ± 1.71 c	18.90 ± 0.36 a
RB	C	230.61 ± 19.59 a	58.36 ± 3.25 abc	47.25 ± 2.27 ab	12.85 ± 0.28 d
	LA	170.28 ± 11.81 a	46.94 ± 2.39 c	52.44 ± 2.30 a	13.66 ± 0.24 bcd
	SA	208.66 ± 17.72 a	50.48 ± 3.15 bc	52.60 ± 2.83 ab	13.24 ± 0.28 cd
StR	C	230.18 ± 19.55 a	62.41 ± 3.89 ab	41.23 ± 2.22 bc	14.87 ± 0.32 b
	LA	211.23 ± 17.94 a	59.95 ± 3.74 abc	42.80 ± 2.30 abc	13.49 ± 0.29 bcd
	SA	231.77 ± 19.69 a	61.54 ± 3.83 ab	42.64 ± 2.29 abc	14.52 ± 0.31 bc

Data are mean ± SE. Significance test by Tukey's HSD ($p < 0.05$, after two-way ANOVA, indicated by different letters). Birnförmige (Bif), Red Baron (RB), Sturon (StR). Control (C), Leaf application (LA), Soil application (SA). ($n \geq 4$).

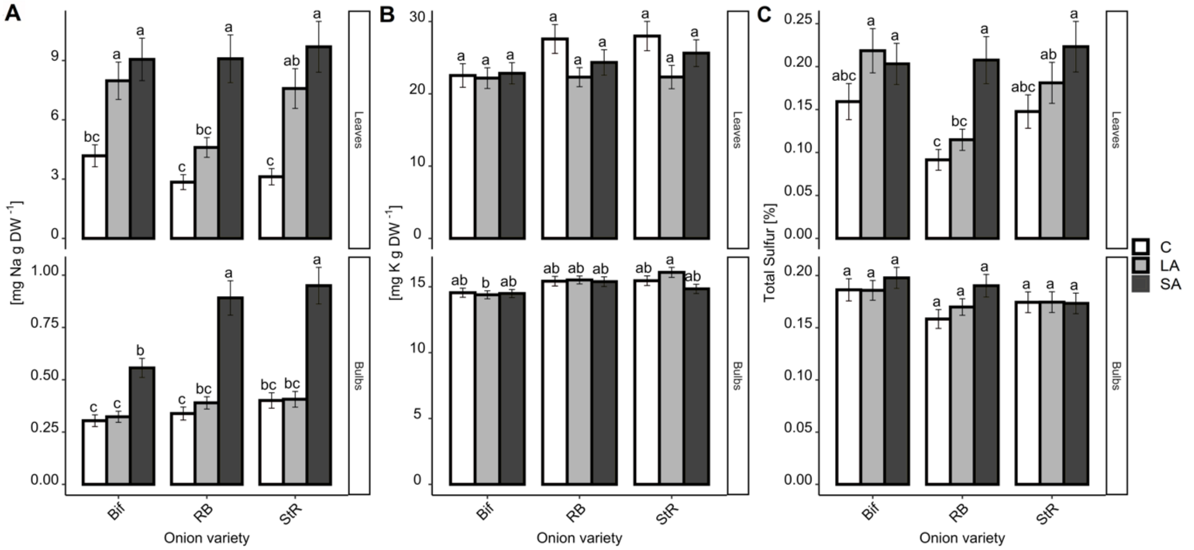


Figure 5 Ion concentrations in leaf and bulb, of three varieties, Birnförmige (Bif), Red Baron (RB) and Sturon (StR) of Allium cepa L. “Treatment” as indicated right; Control (C), Leaf application (LA), Soil application (SA). A: Sodium concentration in leaf and bulb of onions. B: Potassium concentration in leaves and bulbs of onions. C: Total sulfur, DW of leaf and bulb. Data are mean ± SE. Significant test by Tukey’s HSD ($p< 0.05$), after two-way ANOVA, indicated by different letters. ($n \geq 4$).

3.2. *Na^+- related metabolic responses*

Figure 6 shows a principal component analysis (PCA) plot of a targeted analysis of onion bulbs. The first two components represent 51.2 % of the total variance of the data set of onion bulbs, of which 39.2 % belongs to the first principal component and 12 % to the second one. Regarding the first principal component, the individual PCA allows us to distinguish two groups based on their similarities (Figure 6A): in the left-hand quadrant, the variety Birnförmige (Bif), and in the right-hand quadrant, the varieties Sturon (StR) and Red Baron (RB) sharing a high degree of similarity. The PCA loading plot (Figure 6B) highlights the contribution to the first two principal components of all-measured parameters: pyruvate, dry matter, fructan, reducing sugar, sucrose, total sugar, total carbon, total nitrogen, total sulfur, K^+, Na^+, Mg^{2+}, Ca^{2+}, total bulb weight/pot (yield), antioxidative activity (AOX), glutathione, and glutathione reductase. Within the PCA, variables clustering together are considered highly positively correlated, for example, AOX and total glutathione. The x-axis (PC1) mainly describes dry matter content, fructan, and total sugar concentrations, which are responsible for the separation of the Bif variety in the left quadrant and of the StR and RB varieties in the right quadrant. The y-axis (PC2) relates to the accumulation of Na^+ in onion bulbs. Responses in onion leaves are presented in Figure S3.

Figure 7 A-C highlights the absolute concentrations of highly relevant quality compounds in onion bulbs, such as fructan, reducing sugars, and pyruvate. Pyruvate, a pungency marker, ranged between 5.0 (variety StR) and 6.3 µmol g^{-1} FW (variety Bif), showing no significant differences of pungency between varieties or treatments. The concentrations of non-structural carbohydrates, such as fructan, sucrose, and reducing sugars, showed nosignificant differences depending on the treatment. However, significant variety differences were found for fructan and reducing sugars. The variety Bif stood out for its high levels of fructan, reaching 169 mg g^{-1} FW. On the other hand, RB and StR only attained 70 and 96 mg g^{-1} FW, respectively.

A broad overview of the impact of Na^+ on the variety and the treatment effect was obtained by untargeted metabolomics. The two-dimensional chromatograms of leaves and bulbs (see supplemental figure S1 A-B) visualize the similarities and differences between the metabolite profiles of both plant organs. Whereas most of the common primary metabolites were detected in both organs, specific features of the bulb samples were, for example, the presence of tri- and tetrasaccharides and the larger amounts of several sulfur-containing amino acid derivatives in accordance with a recent study [20]. Results of the metabolites are represented in a PCA (Figure 8). Compared with the PCA in Figure 6, a clearer variety differentiation was detected using untargeted metabolomics, revealing three groups: in the left quadrant, the Bif variety; in the middle, the variety StR; in the right quadrant, the variety RB (Figure 8B). Interestingly, variety differences were only apparent in onion bulbs, but was not be detected in leaves (Figure 8A). Overall, no relevant effect of Na^+ accumulation was found for the metabolite profiles of leaves and bulbs. However, as PCA is not suitable for the detection of weaker but potentially relevant differences at the single metabolite level, we applied an ANOVA-based screening approach (Figure 8C). This approach confirmed that Na^+ accumulation had no effect on leaf and bulb metabolites of the varieties Bif and RB. Nevertheless, some metabolites of the variety StR exhibited a weak Na^+ effect, with some of them being detached from the "main cloud of metabolites" and some of them even reaching the threshold FDR p = 0.1 (shown in the right-hand panel of Figure 8C). These metabolites were further investigated using the Tukey HSD post-hoc test, and relative fold changes were calculated and compared with those of the control plants (SA/C). As shown in Table S4, compared with the control, soil application caused increases of a C6 sugar/sugar-like compound, xylose, and arabinose in leaves. On the other hand, despite the fact that the threshold FDR p = 0.1 was never reached in the bulbs, soil application led to reduced levels of citrulline and tryptophan compared with the control.

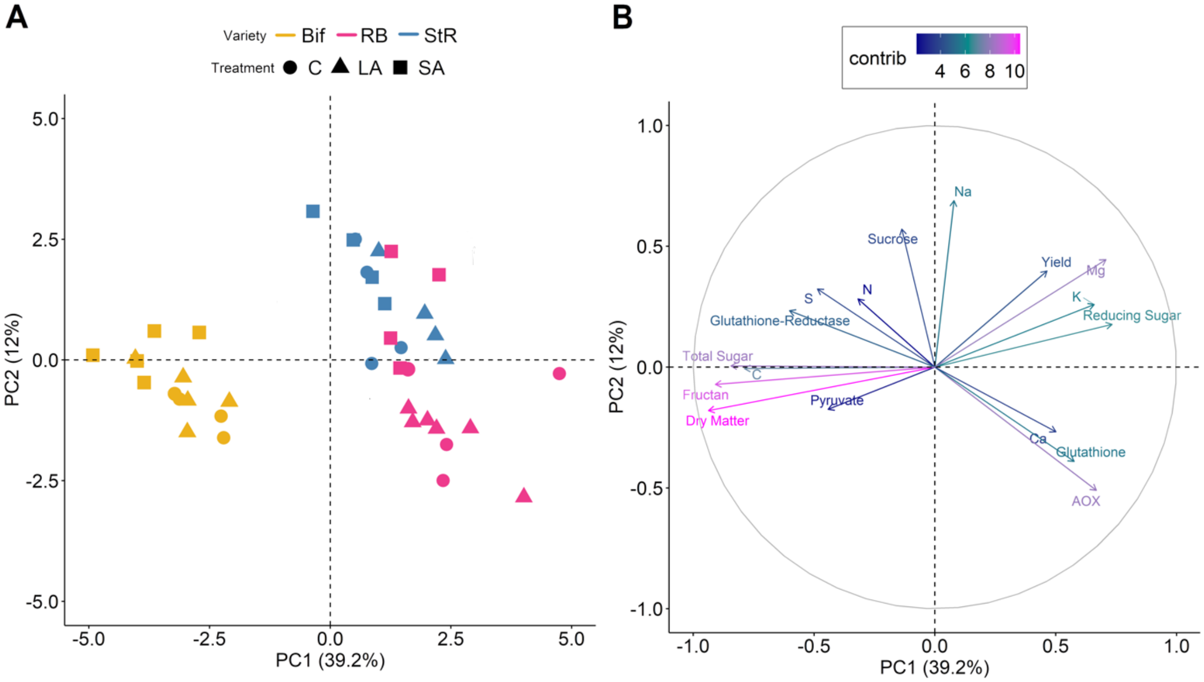


Figure 6 Targeted analysis. Response of onion bulbs to Na_2SO_4. Data were visualized by principal component analysis (PCA) of the first two components. The graph represents 51.2 % of the total variance of the data set. A: PCA scores plot of the targeted analysis. Treatments: control plants (●), leaf treated plants (▲) soil treated plants (▪). Varieties are represented in different colors: Birnförmige (Bif), Red Baron (RB), and Sturon (StR). B: PCA loading plot with the total contribution of variables to the first two principal components. Variables are colored based on their weight of the contribution to the two axes shown. (n ≥ 4).

3.3. Na^+- related responses of the antioxidative defense system of Allium cepa L.

The antioxidative defense system of onion bulbs reacted significantly to Na^+ accumulation. Figure 7D shows the results of antioxidant activity. Significant differences were found depending on the variety; the variety RB presents the highest levels (20.6 µmol TE g^{-1} DW), whereas the variety StR shows the lowest levels at 7.1 µmol TE g^{-1} DW. Compared with the bulbs of control plants, antioxidant activity responded to the Na^+ accumulation. Thus, a reduction in antioxidant activity in onion bulbs was found in all varieties subjected to SA treatment, this being particularly significant for the variety RB in which the levels dropped by half compared with those in the control (C). Similar to the antioxidative activity levels, glutathione levels (Figure 7E) also decreased with the accumulation of Na^+, resulting in lower levels in the bulbs treated with SA, except in the RB variety. On the other hand, glutathione reductase activity presented increased levels (Figure 7F) in the bulbs treated with SA compared with the C group.

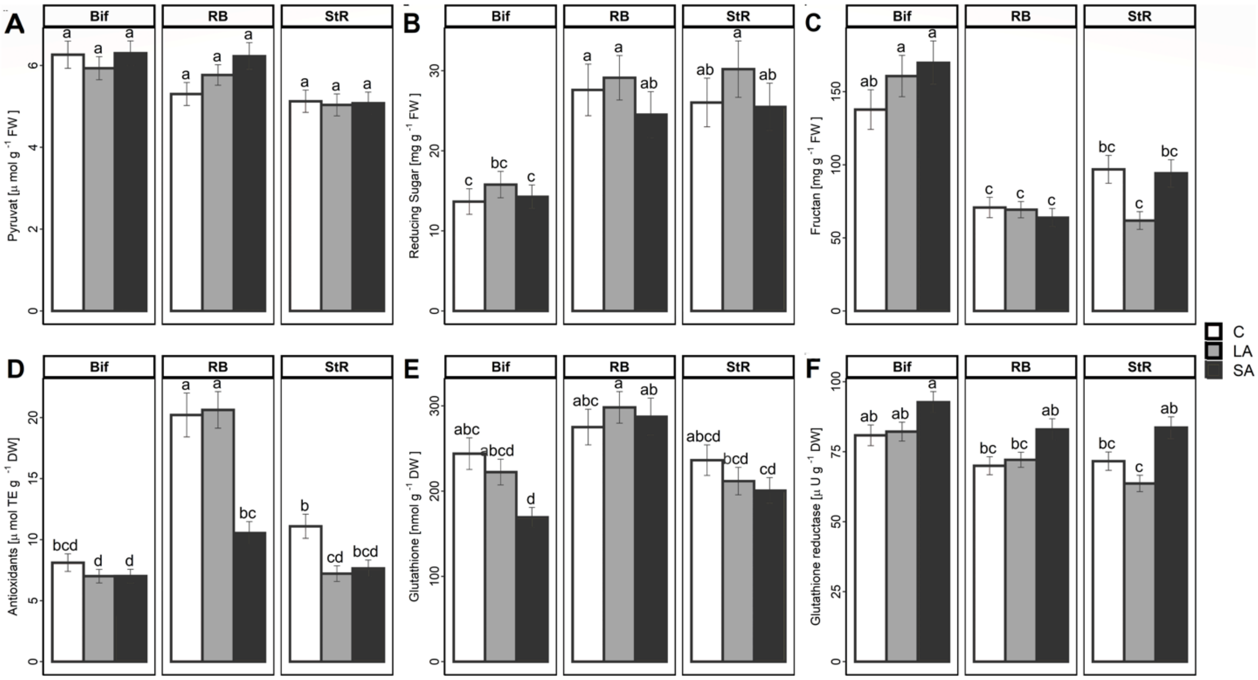


Figure 7 Absolute concentrations of relevant compounds in the bulbs of the varieties: Birnförmige (Bif), Red Baron (RB), and Sturon (StR). Treatment; control (C), leaf application (LA), soil application (SA). A: Pyruvate (onion pungency), B: Reducing sugar, C: Fructan, D: Antioxidants (antioxidant activity). E: Total glutathione, F: Glutathione reductase activity. Data are mean ± SE. Significance test by Tukey's HSD ($p< 0.05$), after two-way ANOVA, indicated by different letters. ($n \geq 4$).).

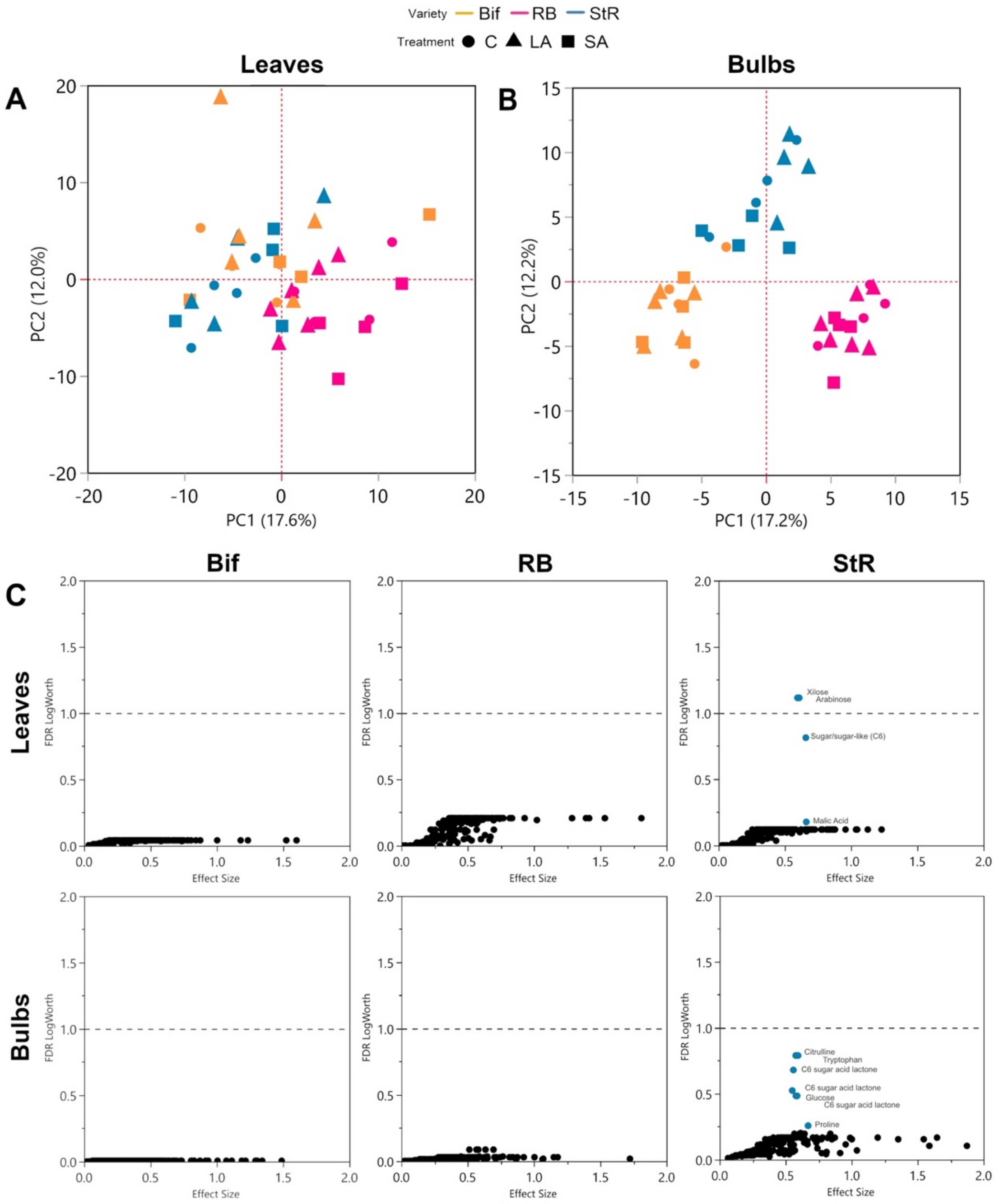


Figure 8 Untargeted metabolomics. Response of onion bulbs and leaves to Na_2SO_4. Data were visualized by principal component analysis (PCA) of the two first components. A: Onion leaves; the graph represents 29.6 % of the total variance of the data set. B: Onion bulbs; the graph represents 29.4 % of the total variance of the data set. Treatment: control plants (●), leaf treated plants (▲) soil treated plants (▪). Variety is represented in different colors: Birnförmige (Bif), Red Baron (RB), and Sturon (StR) ($n \geq 4$)).C: Univariate variety- and organ-specific ANOVA-based response. Screening analysis as shown by "FDR LogWorth vs Effect Size" plots. Each dot represents one analyte variable. Significance according to the ANOVA screening analysis is plotted on the y-axis, expressed as negatively log-transformed p-values (FDR LogWorth = $-\log_{10}$(FDR p-value). A high FDR LogWorth means high significance. The dashed line highlights FDR LogWorth of 1, which corresponds to a screening threshold of $p = 0.1$. Only in the case of the variety StR were a few metabolites detached from the "main cloud of analytes" in leaf and bulb samples (blue dots).

4. Discussion

4.1. Na^+ is mainly transported via xylem in onion plants.

Uptake responses to foliar fertilizers might differ immensely depending on the plant species. One prerequisite for species-specific differences in uptake responses might be a difference of the biochemical property of the leaf surface, such as cuticular waxes [18, 19]. In general, leaves are more sensitive to Na^+ than roots, because higher doses of Na^+ in leaves cause the inhibition of photosynthesis and other leaf-based processes [34]. Despite onion leaves accumulating Na^+ after foliar applications (Figure 5), the plants show no visible symptoms of Na^+ toxicity. Moreover, we demonstrate that Na^+ is not transported from the leaf to the bulb in the LA plants, arguing against a relevant translocation of Na^+ via the phloem. This finding is in accordance with the suggested unidirectional transport of Na^+ within plants [5] and explains the low Na^+ levels in phloem-loaded tissues, such as reproductive organs or storage tissues, *e.g.,* onion bulbs, even when the plant is exposed to saline conditions [35]. In contrast, Na^+ is transported to shoots by the transpiration stream via the xylem when Na^+ is applied to the soil and enters through the roots (Figure 5). This is the reason that Na^+ in the xylem sap can reach concentrations of up to 100 mM, whereas in the phloem, Na^+ concentrations remain lower at up to 20 mM [35] further suggesting the fast sequestration of Na^+ into leaf vacuoles [34, 36] because of its toxic nature at higher concentrations.

4.2. Susceptibility of onions to Na^+ accumulation

For this study, we selected the application of Na_2SO_4 rather than of NaCl, since the aim of the study was to analyze the effect of Na^+ without the possible negative influence of Cl^-. Sulfate was selected as a companion of Na^+, because its toxicity remains unknown, and it accumulates more slowly than Na^+ in plant tissue. Richter, Behr, Erban, Kopka, and Zorb [12] have demonstrated the poor accumulation of sulfate in the shoots and roots of *Vicia faba* L., and Tarchoune, Degl'Innocenti, Kaddour, Guidi, Lachaâl, Navari-Izzo and Ouerghi [13] its paucity in the leaves of *Ocimum basilicum* L. In both studies, although Na^+ accumulates in high concentrations, no accumulation of sulfur has been observed after Na_2SO_4 administration. Similarly, we have seen no increase of total sulfur in onion bulbs treated with Na_2SO_4 (Figure 5).

In most crop species, the accumulation of Na^+ into leaf tissue leads to Na^+-specific damage such as the necrosis of older leaves and reductions in growth and yield [5]. Based on previous studies [2, 37-42], onions have been classified as being sensitive to salt stress. An accumulation of 1 mg Na^+ g^{-1} DW is reported to lead to a reduction of more than 40% of the fresh weight of onion leaves and bulbs [38]. In our study, Na^+ accumulates up to 1 mg Na^+ g^{-1} DW without showing any yield reduction or leaf necrosis symptoms in onion plants. In contrast to other studies, no increases of soluble solids [38], dry matter [37], or sugar levels [43, 44] have been found after Na^+ accumulation (Figure 6). Moreover, concentrations of non-structural carbohydrates, including fructans, remains unchanged (Figure 7). One possible explanation for such low susceptibility at moderate Na^+ accumulation is that onions have large distinctive vacuoles that are able to store, for example, sulfate in high quantities [41]. We assume that Na^+ ions are also compartmentalized in these large vacuoles and are therefore unable to exert any damaging effects within the cytosol. The large vacuoles of onions might thus contribute to mitigating Na^+ accumulation and minimizing metabolite changes in onion bulbs (Figure 6, Figure 8). Onion plants are hence less sensitive to Na^+ ions than other crops, *e.g.*, *Vicia faba* L. [12, 45].

4.3. Na^+ accumulation has only a minimal effect on the metabolome but induces the antioxidative system of onion plants

To mitigate the osmotic and ionic effects of Na^+ accumulation, plants usually accumulate compatible organic solutes such as soluble carbohydrates or proline [5]. Our results detailing the targeted and untargeted metabolomics demonstrate that bulbs and leaves in two out of the three investigated varieties, show no significant increases regarding any of these components. However, amino acids and carbohydrates (*e.g.,* xylose and arabinose) are differentially affected by Na^+ accumulation in the variety StR (Figure 6C, Table S4) indicating that at least a mild metabolic effect is induced by Na^+ accumulation. Nevertheless, the observed effect on the variety StR is weak and might have been an initial reaction to the elevated Na^+ concentrations. Overall, our study reveals no or only a slight reaction of compatible organic solutes in onion plants in terms of Na^+ accumulation, suggesting that the extent of Na^+ accumulation is below (or at) the critical level needed to hamper ion homeostasis and metabolism.

Glutathion plays a significant role for the storage and transport of reduced sulfur and is also part of the antioxidative defense mechanism. The significantly positive correlation ($r = 0.7$) between glutathione levels and antioxidant activity has been confirmed in this study (Figure S2). In accordance with previous results [46], we have observed a reduction of antioxidants and glutathione levels in bulbs with Na^+ accumulation. In terms of total antioxidative activity, we have demonstrated a variety-specific pattern. The RB variety, which has red bulbs, shows particularly high antioxidative activity level because of its naturally high polyphenol concentrations, as previously reported in other studies [21]. However, in our study, bulbs of the RB variety respond to Na^+ accumulation by drastically reducing levels of antioxidative activity, although glutathione concentrations are not affected (Figure 7). This suggests that, unlike yellow onions, other antioxidants in red onions, such as anthocyanins, are first deployed in the antioxidative defense mechanism, before glutathione is involved.

In summary, the response to Na^+ accumulation in the three onion varieties occurs only at the antioxidative level, whereas the typically metabolic signatures of high Na^+ accumulation, such as increases in proline and of other organic solutes and decreases of K^+ concentrations by Na^+/K^+ antagonism, are not present. Therefore, K^+ homeostasis and, in turn, enzyme activation and cellular metabolism [33] are not affected by moderate Na^+ application.

4.4. Na^+ accumulation does not alter the pungency of onions

Little information is available about the effect of Na^+ accumulation on the flavor and aroma of onions. Few relevant studies have shown any effects of Na^+ on enzymatically produced pyruvate (pungency marker) or on precursors such as S-alkenyl cysteine sulfoxide (ACSOs) and other components closely related to onion flavor [16, 37, 41]. Chang and Randle [37] have reported that the application of NaCl at the early bulbing stages leads to significant decreases in aroma precursors, their intermediates, and pyruvate levels. Based on these results, we expected changes in the levels of aroma precursors, ACSOs, and onion pungency after the application of moderate Na^+ treatments at early bulbing. Despite increased concentrations (0.6 - 1 mg g^{-1} DM) of Na^+ in the SA onion bulbs, this accumulation did not cause a significant change in the pungency of the bulbs and their precursors (Figure 7A and Figure S6). The use of NaCl in the studies of Chang and Randle [16, 37] and the additive effects of Cl^- might have contributed to the change in pungency levels in onion bulbs. Future experiments and comparisons with doses of other salts such as KCl, NaCl, and Na_2SO_4 are required if we are to understand the influence of Na^+ and Cl^- separately. We also need to analyze the osmotic component present in onion plants under saline conditions.

5. Conclusion

Foliar application with Na_2SO_4 leads to an overall accumulation of Na^+ into onion leaves but is not transferred into the bulbs, suggesting the limited translocation of Na^+ via the phloem. On the contrary, soil applications lead to an accumulation of Na^+ in leaves and bulbs, demonstrating the transport of Na^+ via xylem transpiration stream. Compared with previous studies, no reduction in plant growth, or sulfur components have been demonstrated in the investigated varieties. Weak changes in the metabolite profile have been detected only in the variety StR after Na^+ accumulation. Pungency and the precursors involved in this characteristic of onions are not affected after mild Na^+ accumulation.

6. Acknowledgement

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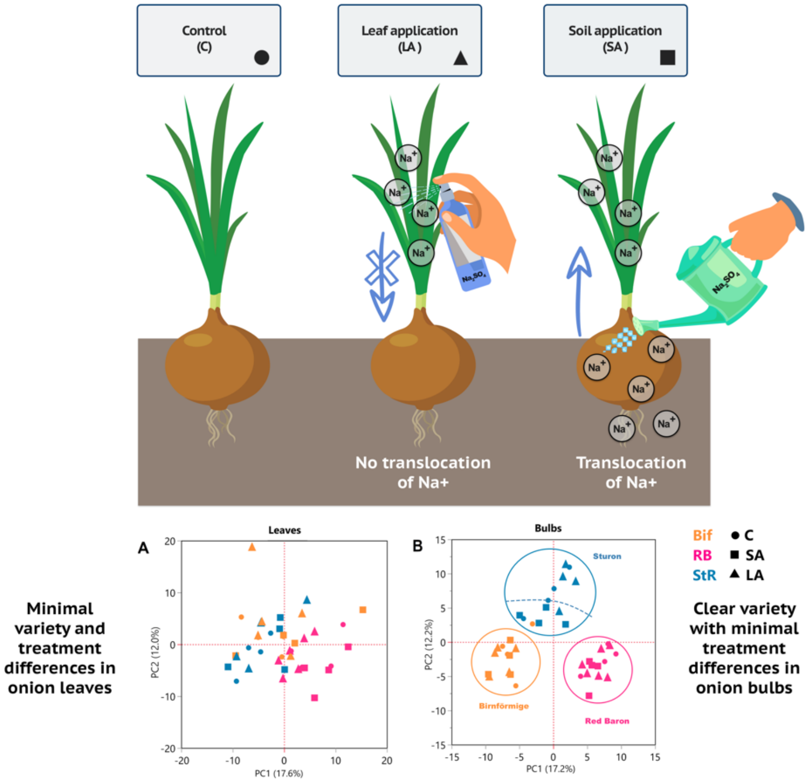
Supplementary material

Graphical Abstract



Untargeted GC×GC-MS metabolome analysis

Sample extraction, evaporation and derivatization

Work step	Action
	Extraction
1	Thaw study samples and QC sample aliquots on ice
2	Add 750 µL (bulbs) or 400 µL (leaves) of methanol
3	Add 20 µL (bulbs) or 10 µL (leaves) of a mixture of internal standards containing hexylamine (5 mM), 1-*O*-methyl-2-desoxy-D-ribose (5 mM), 2-chlorophenylacetic acid (3 mM), *p*-chloro-L-phenylalanine (2.5 mM), D –pinitol (5 mM), 3-*O*-methyl-D –glucopyranose (1 mM), 5-bromo-2,4-dihydroxybenzoic acid (1 mM), and phenyl-β-D-glucopyranoside (1 mM) in methanol/water
4	Mix samples vigorously
5	Incubate samples for 10 min at 35 °C and 1,400 rpm in a shaker
6	Remove insoluble matter by centrifugation for 1 min at 4 °C and 16100 × g
7	Transfer 700 µL (bulbs) or 350 µL (leaves) of the supernatants into new 2 mL reaction tubes
8	Re-extract the samples (repeat steps2, 4, and 5)
9	Combine and mix both supernatants
10	Sediment insoluble matter by centrifugation for 3 min at 4 °C and 16100 × g
11	Transfer 30 µL of the combined supernants to screw-capped glass vials with 300 µL micro inserts
	Evaporation
12	Dry samples in a rotary vacuum concentrator (Thermo Scientific/Savant SPD131DDA) for 40 min at 45 °C (for the first 10 minutes) and p< 1 mbar
13	Re-dissolve samples again in 20 µL of methanol
14	Dry samples again to remove traces of water (20 min, 45 °C (for the first 10 minutes), p< 1 mbar)
	Derivatization
15	Add 25 µL of methoxylamine-hydrochloride in pyridine (20 mg/mL)
16	Incubate for 1 h at 40 °C and 1,000 rpm in a shaker
17	Add 70 µL of MSTFA with 1 % TMCS
18	Incubate for 1 h at 65 °C without shaking
19	Add 8 µL of retention index marker mixture only to daily blanks

GC×GC-MS system

Instrument component	Name	Manufacturer
Gas chromatograph (single oven)	GC-2010	Shimadzu Corp, Kyoto, Japan
Single quadrupol mass spectrometer	QP2010 Ultra	Shimadzu Corp, Kyoto, Japan
Autosampler	AOC-20i	Shimadzu Corp, Kyoto, Japan
PTV Injector	OPTIC-4	GL Sciences, Eindhoven, The Netherlands
Loop-type cryogenic modulator	ZX2	ZOEX Corp., Houston, USA
Roughing pump	RV-3	Edwards, Crawley, UK

GC×GC method and consumables

Carrier gas	Helium 5.0
GC mode	Constant velocity
Purge flow	3 mL/min
Initial column head pressure	218.1 kPa
Liner type	Deactivated, fritted split liner with quartz wool
Primary column	Rxi-5SilMS, ${}^{1}L$ = 30 m plus 5 m of an integrated pre-column, ${}^{1}d_c$ = 0.25 mm, ${}^{1}d_f$ = 0.25 µm (Restek, Bellefont, USA)
Secondary column	BPX50, ${}^{2}L_{total}$ = 2.2 m, including a "separation segment" of ${}^{2}L_{sep}$ = 0.7 m, ${}^{2}d_c$ = 0.15 mm, ${}^{2}d_f$ = 0.15 µm (Trajan Scientific, Ringwood Victoria, Australia)
GC temperature ramp	90 °C → 2 °C/min → 150 °C → 2.5 °C/min → 190 °C → 4 °C/min → 220 °C → 5 °C/min → 250 °C → 3.5 °C/min → 270 °C→ 5 °C/min → 330 °C → 40 °C/min → 340 °C (hold 10 min (bulbs) or 7.54 min (leaves))
Run time	87.5 min. (bulbs) or 85 min (leaves)
Injection mode	Cold split
PTV temperature program	90 °C → 60 °C/s → 220 °C (hold 1 min) → 60 °C/s→ 280 °C (hold until end of run)
Split ratio program	1:5 (hold 1 min) → 1:100 (hold until end of run)
Injection volume	1.2 µL
Injector syringe	10 µL, with PTFE-tipped plunger (Trajan Scientific, Ringwood Victoria, Australia)
Syringe wash solvent 1	Acetone
Syringe wash solvent 2	Heptane
Interface temperature	320 °C

Modulator settings

Modulation period (P_M)	2.7 s
Cold jet temperature	-90 °C
Hot jet temperature program	200 °C (hold until 25 min) → 250 °C (hold until 48 min) → 300 °C (hold until 60 min) → 360 °C (hold until 85 min (bulbs) or 83 min (leaves)) → 200 °C (hold until end of run)
Hot jet pulse duration	250 ms

MS settings

Interface temperature	320 °C
Ion source temperature	200 °C
Ionization mode	EI (70 eV)
MS Mode	Scan
Scan speed	20.000 u/s
Scan range	m/z 60-550
Event time	30 ms
Data acquisition frequency	33 s^{-1}
Data acquisition period	4.5 -85 min (bulbs) or 84 min (leaves)
Detector voltage windows (bulbs)	4.50 – 22.50 min: 1.25 kV 22.50 – 22.95 min: 1.10 kV 22.95 – 41.55 min: 1.25 kV 41.55 – 43.75 min: 1.00 kV 43.75 – 61.57 min: 1.25 kV 61.57 – 64.35 min: 1.10 kV 64.35 – 73.40 min: 1.25 kV 73.40 – 85.00 min: 1.20 kV
Detector voltage windows (leaves)	4.50 – 22.50 min: 1.28 kV 22.35 – 22.78 min: 1.10 kV 22.78 – 41.18 min: 1.28 kV 41.18 – 43.50 min: 1.00 kV 43.50 – 61.40 min: 1.28 kV 61.40 – 62.33 min: 1.10 kV 62.33 – 84.00 min: 1.28 kV

Results

Table S1 Soil properties after the pot experiment. Soil electrical conductivity, soil pH, total carbon, total nitrogen, total sulfur, potassium and sodium concentration. ($n \geq 4$).

Parameter	*C*	*LA*	*SA*
Soil electrical conductivity (*mS cm*$^{-1}$)	0.19 ± 0.01 b	0.19 ± 0.01 b	0.55 ± 0.04 a
Soil pH	8.03 ± 0.12 a	8.06 ± 0.12 a	7.86 ± 0.12 a
Total Carbon (%)	0.55 ± 0.04 a	0.51 ± 0.04 a	0.44 ± 0.03 a
Total Nitrogen (%)	0.02 ± 0.002 a	0.02 ± 0.002 a	0.02 ± 0.001 a
Total Sulfur (%)	0.003 ± 0.00 b	0.000 ± 0.00 c	0.005 ± 0.00 a
Potassium (mg g^{-1})	0.11 ± 0.02 a	0.07 ± 0.02 a	0.05 ± 0.01 a
Sodium (mg g^{-1})	0.04 ± 0.004 b	0.04 ± 0.004 b	0.13 ± 0.005 a

Data are mean ± SE. Significant test by Tukey's HSD ($p < 0.05$) are indicated by different letters. Control (C), Leaf application (LA), Soil application (SA).

Table S2 Absolute ion concentrations in two different plant organs of three *Allium Cepa* L. varieties treated with Na_2SO_4 as soil application (SA) and leaf application (LA). Control (C) without Na^+.

Organ	*Variety*	*Treatment*	*Ion*			
			Ca^{2+} (mg g^{-1} DW)	*Mg^{2+} (mg g^{-1} DW)*	*Total N (%)*	*Total C (%)*
Leaves	*Bif*	*C*	28.10 ± 1.95 abc	2.92 ± 0.24 a	0.75 ± 0.07 a	41.80 ± 0.29 a
	Bif	*LA*	33.03 ± 2.05 a	2.71 ± 0.20 a	0.74 ± 0.06 a	41.93 ± 0.26 a
	Bif	*SA*	31.30 ± 1.94 ab	2.66 ± 0.20 a	0.83 ± 0.07 a	41.48 ± 0.25 a
	RB	*C*	21.89 ± 1.52 c	2.62 ± 0.22 a	0.68 ± 0.07 a	42.17 ± 0.29 a
	RB	*LA*	24.14 ± 1.37 bc	2.64 ± 0.18 a	0.60 ± 0.05 a	42.15 ± 0.24 a
	RB	*SA*	27.03 ± 1.88 abc	2.31 ± 0.19 a	0.79 ± 0.08 a	41.37 ± 0.28 a
	StR	*C*	34.64 ± 2.40 a	2.92 ± 0.25 a	0.67 ± 0.07 a	41.20 ± 0.28 a
	StR	*LA*	33.55 ± 2.33 a	2.22 ± 0.19 a	0.75 ± 0.07 a	41.19 ± 0.28 a
	StR	*SA*	30.93 ± 2.15 ab	2.26 ± 0.19 a	0.75 ± 0.07 a	41.27 ± 0.28 a
Bulbs	*Bif*	*C*	0.99 ± 0.88 a	0.55 ± 0.01 c	0.64 ± 0.03 ab	42.54 ± 0.10 ab
	Bif	*LA*	0.99 ± 0.07 a	0.56 ± 0.01 bc	0.62 ± 0.02 ab	42.55 ± 0.09 ab
	Bif	*SA*	1.13 ± 0.08 a	0.55 ± 0.01 c	0.61 ± 0.02 ab	42.61 ± 0.09 a
	RB	*C*	1.20 ± 0.10 a	0.60 ± 0.01 abc	0.55 ± 0.02 b	42.00 ± 0.10 c
	RB	*LA*	1.32 ± 0.09 a	0.61 ± 0.01 ab	0.57 ± 0.02 ab	42.08 ± 0.08 c
	RB	*SA*	1.38 ± 0.09 a	0.62 ± 0.01 a	0.56 ± 0.02 ab	42.19 ± 0.10 abc
	StR	*C*	1.16 ± 0.10 a	0.63 ± 0.01 a	0.66 ± 0.02 a	42.15 ± 0.10 bc
	StR	*LA*	1.02 ± 0.09 a	0.64 ± 0.01 a	0.66 ± 0.02 ab	42.22 ± 0.10 abc
	StR	*SA*	1.04 ± 0.09 a	0.64 ± 0.01 a	0.58 ± 0.02 ab	42.26 ± 0.10 abc

Data are mean ± SE. Significance test values from Tukey's HSD ($p < 0.05$), after two-way ANOVA, are indicated by different letters. Birnförmige (Bif), Red Baron (RB), Sturon (StR). Control (C), Leaf application (LA), Soil application (SA).

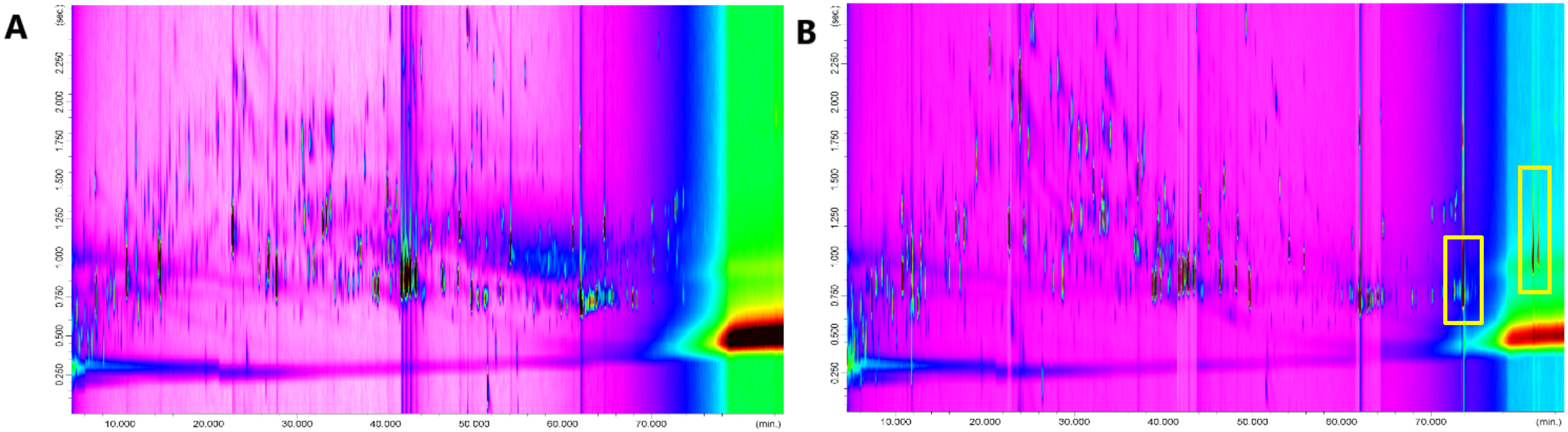


Figure S1 2D chromatograms of onion leaves (A) and bulbs (B) after optimization of GC-temperature program. The chromatograms visualize the specific metabolite profiles of both plant organs, with the presence of tri- and tetrasaccharides (i.e., trimeric and tetrameric fructans) only in the bulb samples (highlighted by yellow boxes in panel B) being one of the characteristic differences.

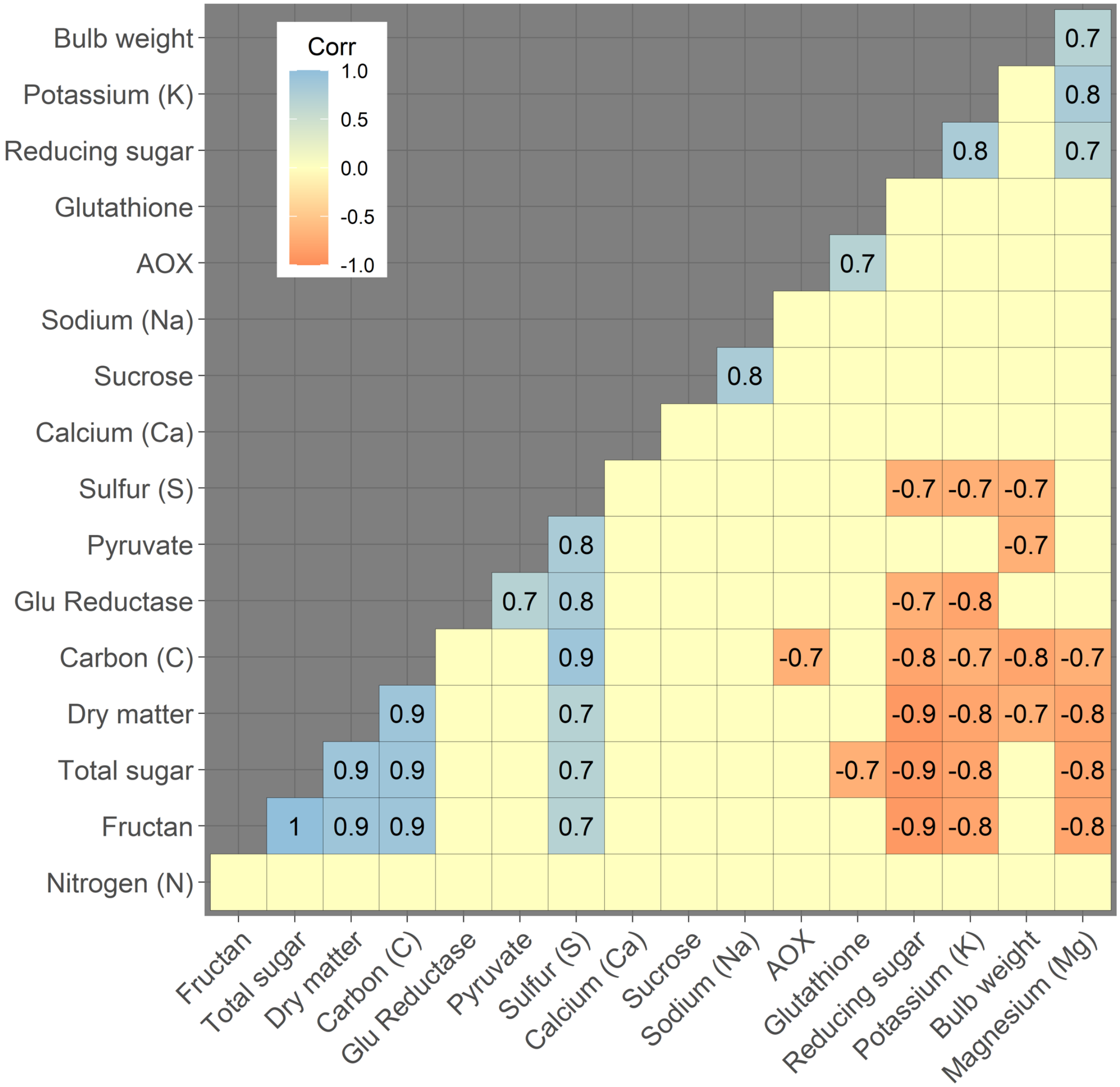


Figure S2 Correlation matrix of all analyzed parameters from targeted metabolomics in onion bulbs of the three varieties; Birnförmige (Bif), Red Baron (RB) and Sturon (StR) and after sodium-treatments; Na_2SO_4 soil-treated-plants (SA), Na_2SO_4 leaf-treated-plants (LA) and control (C) plants without Na^+. Correlations with p-value < 0.05 are considered as significant and are highlighted in this figure. Correlations with p > 0.05 do not appear in this figure.

Table S3 Absolute non-structural carbohydrate concentrations– Reducing sugar, sucrose, fructan, total sugar – in fresh-weight bulbs of three *Allium Cepa* L. varieties treated with Na_2SO_4 as soil application (SA), leaf application (LA) and control (C) without Na^+.

Variety	*Treatment*	*Non-structural carbohydrates*			
		Reducing Sugar (mg g^{-1} FW)	*Sucrose (mg g^{-1} FW)*	*Fructan (mg g^{-1} FW)*	*Total Sugar (mg g^{-1} FW)*
Bif	*C*	13.66 ± 1.60 c	7.68 ± 1.23 a	137.77 ± 13.56 ab	159.54 ± 13.40 ab
Bif	*LA*	15.77 ± 1.65 bc	9.03 ± 1.29 a	160.72 ± 14.15 a	185.77 ± 13.96 ab
Bif	*SA*	14.25 ± 1.49 c	10.60 ± 1.51 a	169.80 ± 14.95 a	195.42 ± 14.68 a
RB	*C*	27.60 ± 3.22 a	6.58 ± 1.05 a	70.79 ± 6.97 c	106.04 ± 8.91c
RB	*LA*	29.12 ± 2.78 a	7.75 ± 1.01 a	69.29 ± 5.57 c	106.54 ± 7.31 c
RB	*SA*	24.52 ± 2.86 ab	11.13 ± 1.77 a	63.82 ± 6.28 c	99.98 ± 8.40 c
StR	*C*	26.03 ± 3.04 ab	13.36 ± 2.13 a	96.91 ± 9.54 bc	136.98 ± 11.51 abc
StR	*LA*	30.19 ± 3.53 a	6.86 ± 1.09 a	61.83 ± 6.09 c	99.92 ± 8.39 c
StR	*SA*	25.47 ± 2.97 ab	9.58 ± 1.53 a	94.19 ± 9.27 bc	130.54 ± 10.97 bc

Data are mean ± SE. Significance test values with Tukey's HSD ($p < 0.05$), after two-way ANOVA, are indicated by different letters. Birnförmige (Bif), Red Baron (RB), Sturon (StR). Control (C), Leaf application (LA), Soil application (SA).

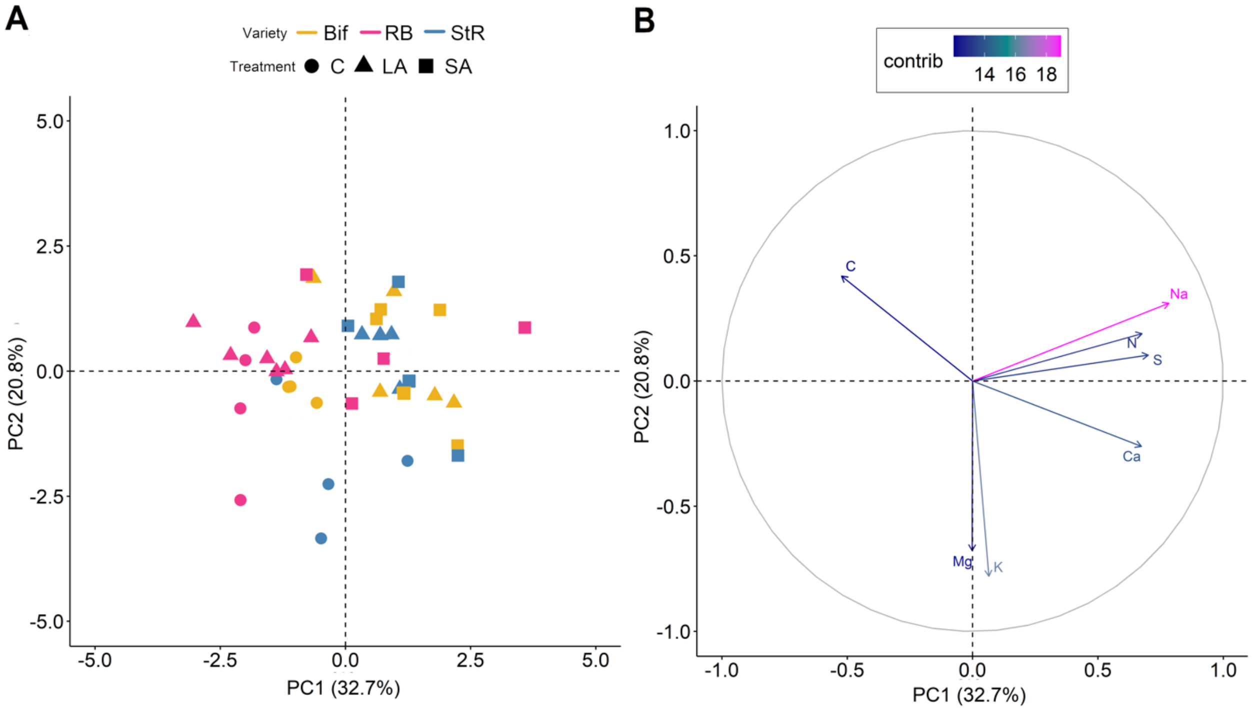


Figure S3 Targeted analysis. Ion responses in onion leaves to Na_2SO_4. Data was visualized by principal component analysis (PCA) of the first two components. The graphs represent 53.5 % total variance of the data set. **A:** PCA scores plot of the targeted analysis. Argument treatment: control plants are represented as (●), leaf treated plants as (▲) soil treated plants as (■). Argument variety is represented in different colors, Birnförmige (Bif), Red Baron (RB) and Sturon (StR). **B:** PCA loading plot with the total contribution of variables to the first two principal components. Variables are colored based on their weight of the contribution to the two axes showed.

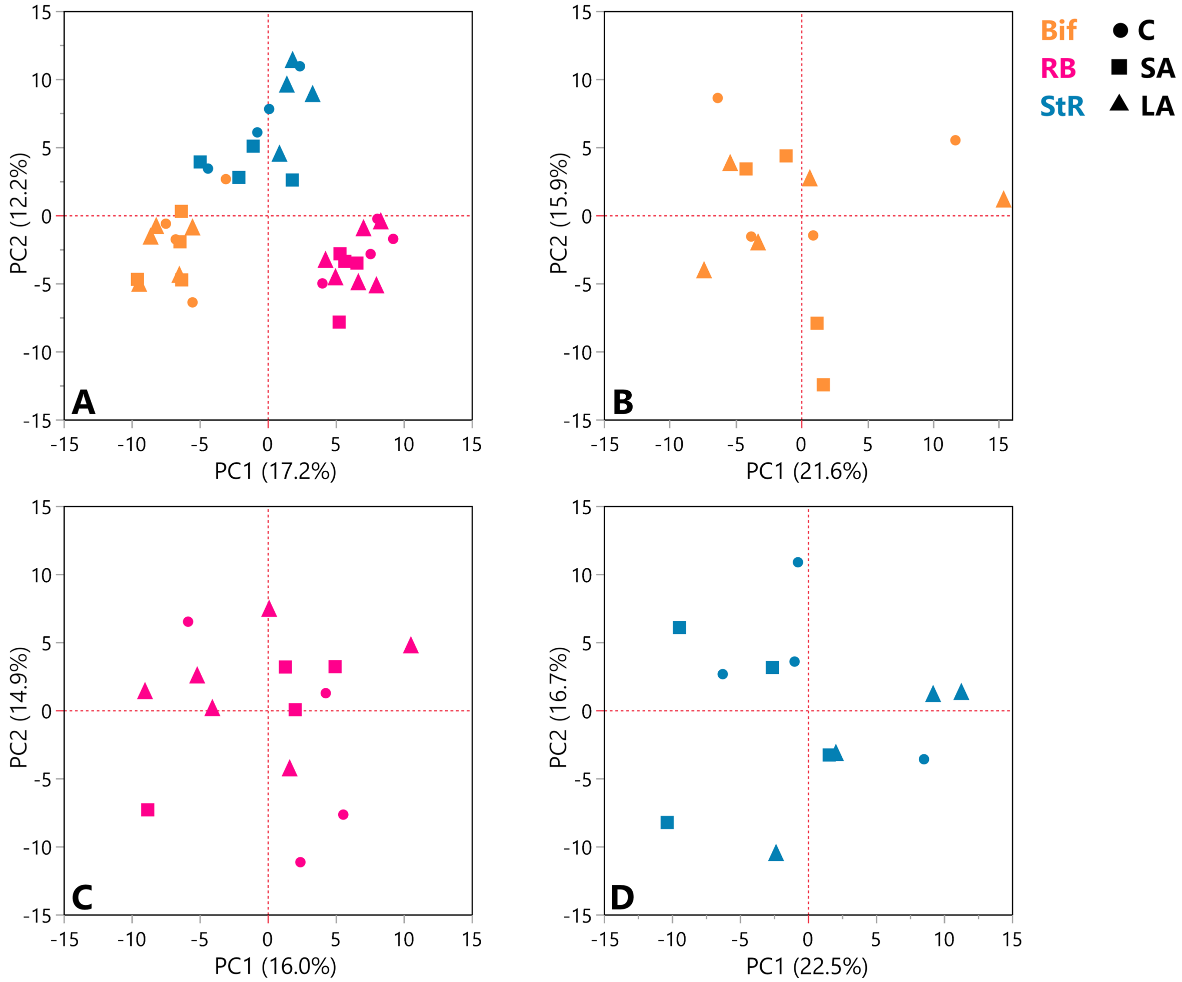


Figure S4 Untargeted metabolomics. Response of onion bulbs to Na_2SO_4. Data was visualized by principal component analysis (PCA) of the two fist components. **A:** all three varieties and treatments are represented here; the graph represents 29.4 % of the total variance of the dataset. **B:** Variety Bif, graph represents 37.5 % of the total variance of the dataset. **C:** Variety RB, graph represents 30.9 % of the total variance of the dataset. **D:** Variety StR, graph represents 39.2 % of the total variance of the dataset. Argument treatment: control plants are represented as (•), leaf treated plants as (▲) soil treated plants as (■). Argument variety is represented in different colors, Birnförmige (Bif), Red Baron (RB) and Sturon (StR).

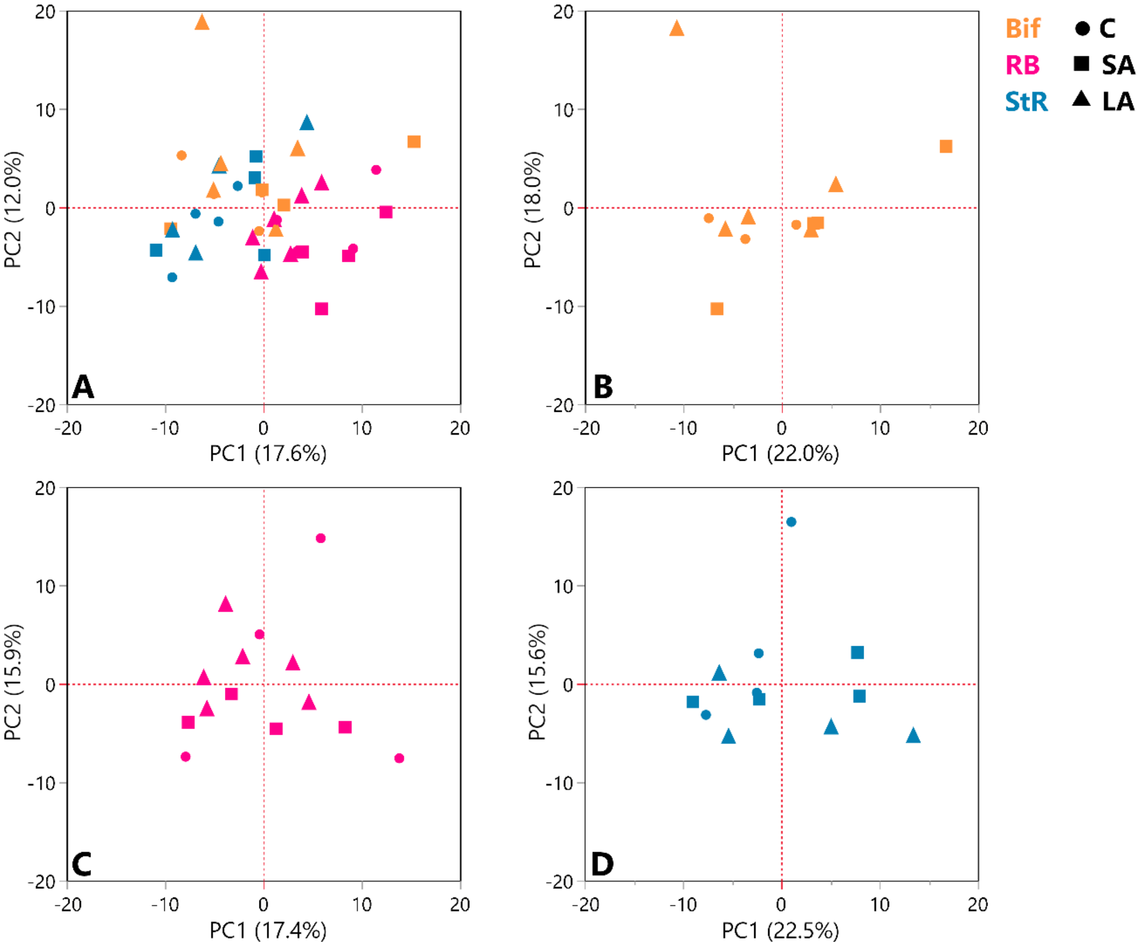


Figure S5 Untargeted metabolomics. Response of onion leaves to Na_2SO_4. Data was visualized by principal component analysis (PCA) of the two fist components. **A:** all three varieties and treatments are represented here; the graph represents 29.6 % of the total variance of the dataset. **B:** Variety Bif, graph represents 40 % of the total variance of the dataset. **C:** Variety RB, graph represents 33.3 % of the total variance of the dataset. **D:** Variety StR, graph represents 38.1 % of the total variance of the dataset. Argument treatment: control plants are represented as (•), leaf treated plants as (▲) soil treated plants as (■). Argument variety is represented in different colors, Birnförmige (Bif), Red Baron (RB) and Sturon (StR).

Table S4 Results of the Response Screening analysis and the post-hoc testing of the selected metabolites in leaf and bulbs samples of the variety StR. See also Figure 6 of the main document.

Analyte		Response Screening (ANOVA)			Letter code (Tukey)			Fold changes
		p[1]	FDR p[2]	FDR LogWorth[3]	C	LA	SA	SA/C
Leaves	Xylose MEOX-4TMS deriv. 1	0.0006	0.0767	1.12	B	B	A	1.23
	Arabinose MEOX-4TMS	0.0007	0.0767	1.12	B	B	A	1.24
	Sugar/sugar-like (C6)	0.0021	0.1535	0.81	B	B	A	3.35
	Malic acid 3TMS	0.0119	0.6637	0.18	A	B	A	0.91
Bulbs	Citrulline 2TMS*	0.0015	0.1623	0.79	A	A	B	0.40
	Tryptophan 3TMS	0.0013	0.1623	0.79	A	A	B	0.37
	C6 sugar acid lactone 3	0.0029	0.2088	0.68	B	A	B	0.68
	C6 sugar acid lactone 1	0.0055	0.2989	0.52	B	A	B	0.90
	Glucose MEOX-5TMS deriv. 1	0.0080	0.3278	0.48	B	A	B	0.97
	C6 sugar acid lactone 2	0.0090	0.3278	0.48	B	A	B	0.86
	Proline 2TMS	0.0177	0.5526	0.26	AB	A	B	0.86

[1] Uncorrected ANOVA p value; [2] FDR-corrected ANOVA p value; [2] Negatively log-transformed FDR p value; * Trace-level analyte.

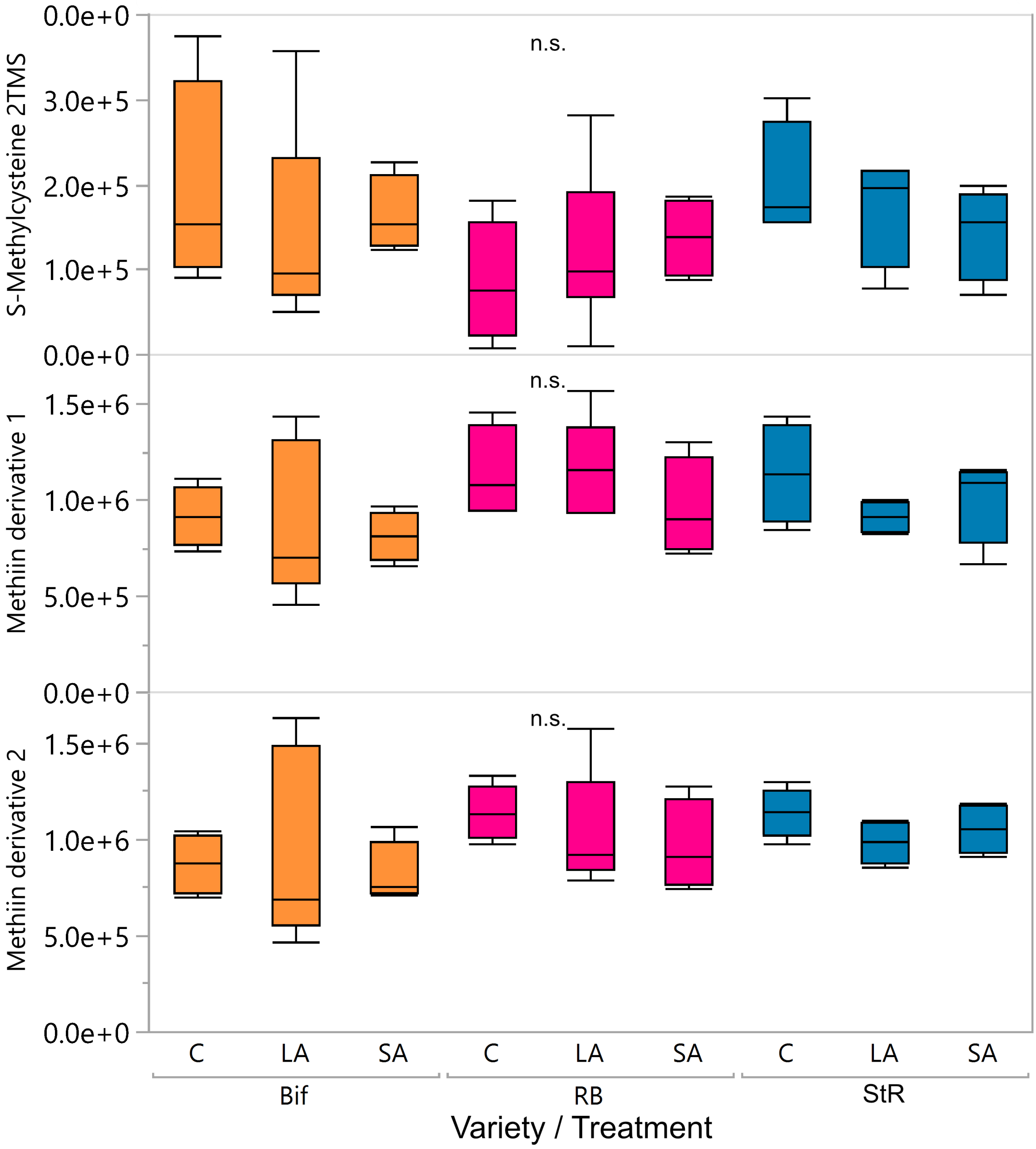


Figure S6 Boxplots showing non-significant treatment and variety differences in the levels of the sulfur-containing amino acids within onion bulbs, such as S-methylcysteine and methiin. While cysteine, methionine and alliin were only detected in trace amounts, other putatively identified but not fully characterized and more abundant derivatives of sulfur-containing amino acids showed the same behavior as S-methylcysteine and methiin. Onion varieties are represented in different colors. Birnförmige (Bif), Red Baron (RB) and Sturon (StR)

Chapter 5 – Acclimatisation of guard cell metabolism to long-term salinity

B.L. Franzisky, C.M. Geilfus, M.L. Romo-Pérez, I. Fehrle, A. Erban, J. Kopka, C. Zörb
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ORIGINAL ARTICLE

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Acclimatisation of guard cell metabolism to long-term salinity

Bastian Leander Franzisky[1] | **Christoph-Martin Geilfus**[2] | **Maria Luisa Romo-Pérez**[1] | **Ines Fehrle**[3] | **Alexander Erban**[3] | **Joachim Kopka**[3] | **Christian Zörb**[1]

[1]Institute of Crop Science, University of Hohenheim, Stuttgart, Germany

[2]Division of Controlled Environment Horticulture, Faculty of Life Sciences, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt-University of Berlin, Berlin, Germany

[3]Max-Planck-Institute of Molecular Plant Physiology, Potsdam-Golm, Germany

Correspondence
Christian Zörb, University of Hohenheim, Institute of Crop Science, 340e, Schloss Westflügel, 70593, Stuttgart, Germany.
Email: Christian.zoerb@uni-hohenheim.de

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Abstract

Stomatal movements are enabled by changes in guard cell turgor facilitated via transient accumulation of inorganic and organic ions imported from the apoplast or biosynthesized within guard cells. Under salinity, excess salt ions accumulate within plant tissues resulting in osmotic and ionic stress. To elucidate whether (a) Na^+ and Cl^- concentrations increase in guard cells in response to long-term NaCl exposure and how (b) guard cell metabolism acclimates to the anticipated stress, we profiled the ions and primary metabolites of leaves, the apoplast and isolated guard cells at darkness and during light, that is, closed and fully opened stomata. In contrast to leaves, the primary metabolism of guard cell preparations remained predominantly unaffected by increased salt ion concentrations. Orchestrated reductions of stomatal aperture and guard cell osmolyte synthesis were found, but unlike in leaves, no increases of stress responsive metabolites or compatible solutes occurred. Diverging regulation of guard cell metabolism might be a prerequisite to facilitate the constant adjustment of turgor that affects aperture. Moreover, the photoperiod-dependent sucrose accumulation in the apoplast and guard cells changed to a permanently replete condition under NaCl, indicating that stress-related photosynthate accumulation in leaves contributes to the permanent closing response of stomata under stress.

KEYWORDS
apoplast, assimilation, chloride, field bean, guard cells, metabolite, salt stress, sodium, stomata, transpiration

1 | INTRODUCTION

Legumes and particularly *Vicia faba* L. are sensitive to salt and, therefore, undergo fast physiological changes attributable to osmotic stress, as early as the first hour after exposure to high salinity. In addition to the inhibition of nitrogen assimilation and the accumulation of metabolites associated with the formation and scavenging of reactive oxygen species (Geilfus et al., 2015; Geilfus, Mithofer, Ludwig-Muller, Zörb, & Mühling, 2015), abscisic acid signalling results in the rapid closure of stomatal pores, which are formed by pairs of highly specialized guard cells (GCs) (Jezek & Blatt, 2017). With continuous exposure to high salinity, salt ions accumulate within roots and leaves and, in turn, cause symptoms of ion toxicity as a result of disturbed ion homeostasis (Munns, James, Gilliham, Flowers, & Colmer, 2016). Such abiotic stress condition perturbs plant metabolism, for example, by hindering enzyme function and lowering the availability of substrates (Obata &

Fernie, 2012). As part of an optimization of the biological system to the new environmental condition (Herrmann, Schwartz, & Johnson, 2019), metabolism acclimates by reconfiguring the metabolic network to adopt a new steady state (Obata & Fernie, 2012), which can help to avoid or mitigate harmful effects resulting from the prevailing stress condition (Schwachtje et al., 2019). The leaf metabolic acclimation of *V. faba* to salt stress is characterized by, for example, increased metabolite pools of *myo*-inositol, the presence of the general stress marker proline and decreased intermediates of the tricarboxylic acid (TCA) cycle and monosaccharides such as arabinose and xylose (Richter, Behr, Erban, Kopka, & Zörb, 2019), all of which reflect conserved patterns of metabolic acclimation to high salinity in legumes (Sanchez et al., 2011; Sanchez, Siahpoosh, Roessner, Udvardi, & Kopka, 2008). In contrast to the well-known metabolic acclimation of root and leaf tissues to high salinity, information about stress-related modifications of the specialized GC metabolome is limited.

Guard cells enable a controlled gas exchange between the atmosphere and the leaf internal space. This is important for balancing the trade-off between CO_2 intake and the concomitant water loss achieving CO_2 availability for Calvin cycle activity, the autotrophic production of organic compounds and the maintenance of the plant's hydration (Lawson & Blatt, 2014; McAusland et al., 2016), aspects essential for cell expansion and plant growth (Thompson, 2005). The adjustment of stomatal aperture in response to exogenous and endogenous cues is facilitated by the transient accumulation of osmotically active compounds within GCs thereby enabling rapid turgor changes (Jezek & Blatt, 2017). For this process, the import of K^+, Cl^- and NO_3^- from the apoplast is essential; however, organic solutes considerably contribute to the GC osmotic adjustment (Lawson & Matthews, 2020). Therefore, GC metabolism is not only important for feeding the high energy demand required for ion transport processes, but also for contributing to the built up of the osmotic gradient necessary for stomatal opening by the synthesis of organic solutes such as malate (Kollist, Nuhkat, & Roelfsema, 2014; Kopka, Provart, & Müller-Röber, 1997; Santelia & Lawson, 2016). In contrast to the increasing sucrose and starch content in mesophyll cells (Santelia & Lunn, 2017; Tcherkez, Boex-Fontvieille, Mahé, & Hodges, 2012), the degradation of starch (Daloso et al., 2017; Flütsch et al., 2020; Horrer et al., 2016) and the activation of glycolysis (Medeiros et al., 2018) occur in GCs in response to illumination indicating the demand for energy during stomatal opening. In agreement with this, the breakdown of lipid droplets and starch in GCs has been found to be essential for blue-light-stimulated stomatal opening (Horrer et al., 2016; McLachlan et al., 2016). The breakdown of fatty acids is proposed to favour adenosine triphosphate production via peroxisomal β-oxidation during the dark-to-day transition (McLachlan et al., 2016), whereas starch breakdown is suggested to replenish cytosolic sugar pools (Flütsch et al., 2020) and to stimulate the flux of carbon skeletons into mitochondria and the TCA cycle for energy production (Daloso et al., 2015; Daloso, Anjos, & Fernie, 2016; Lima et al., 2018; Medeiros et al., 2018). Guard cells in detached epidermal strips are able to respond to environmental cues such as light intensity and quality and changes in CO_2 solely (Mott, Sibbernsen, & Shope, 2008). Nevertheless, mesophyll derived sugars and organic acids have been shown to affect stomatal behaviour *in planta* (Antunes, de Menezes Daloso, Pinheiro, Williams, & Loureiro, 2017; Araújo et al., 2011; Kelly et al., 2013), providing evidence for metabolic feedback mechanisms correlating mesophyll photosynthetic demands with stomatal aperture. The accumulation of mesophyll-derived sucrose in the apoplast has been proposed as a mechanism for stimulating stomatal closure (Antunes et al., 2017; Granot & Kelly, 2019). This metabolic feedback of sucrose has been found to affect stomatal aperture via an abscisic acid pathway stimulating sugar-sensing hexokinase (Kelly et al., 2013; Lugassi et al., 2015), thereby coordinating mesophyll photosynthesis with transpirational water loss (Daloso et al., 2017; Lima et al., 2018).

The motivation behind the present study was to evaluate whether (a) long-term NaCl stress leads to Na^+ and Cl^- accumulation in GC preparations and whether (b) GCs reflect patterns of conserved metabolic response to high NaCl such as the accumulation of organic and amino acids. For this purpose, a non-targeted metabolomics workflow involving gas-chromatography mass-spectrometry was used to explore modulations of GC metabolism in comparison with that of leaves and the apoplast under long-term NaCl. To assess variation in the dark-to-light transition, we compared the physiological status of GCs under dark and light condition, that is, between closed and fully opened stomata.

2 | MATERIALS AND METHODS

2.1 | Cultivation of plant material

The *Vicia faba* L. varieties Fuego and Scoop (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth, Germany) were grown under hydroponic culture conditions in a climate cabinet (WEISS HGC1014, Heuchelheim, Germany) (14/10 hr day/night; 22/18°C; approx. 80/60% humidity, 300 μmol photons m^2/s at shoot level). Seeds were immersed in aerated $CaSO_4$ (0.5 mM) solution for 1 day at room temperature and were subsequently placed in moistened quartz sand. After 12 days of germination, seedlings were transferred into plastic pots containing 1/4-strength aerated nutrient solution. The concentration of the nutrient solution was incrementally increased to 1/2-strength after 2 days, 3/4-strength after 3 days and to full-strength after 4 days. The full-strength nutrient solution had the following composition: 0.1 mM KH_2PO_4, 1.0 mM K_2SO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.00464% (wt/vol) Sequestren (Ciba Geigy, Basel, Switzerland), 10 μM NaCl, 10 μM H_3BO_3, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.1 μM $CoCl_2$, 0.05 μM $(NH_4)_6Mo_7O_{24}$. After 4 days of growing under full-strength nutrient concentration, the NaCl treatment was introduced to the plants. During the following three consecutive days, the NaCl concentration was increased starting from 1/3-strength, over 2/3-strength to full-strength (100 mM NaCl), respectively. Concomitant to the replacement of the nutrient solution every third day, the plant roots were gently rinsed with deionized water. After 20 days of full-strength

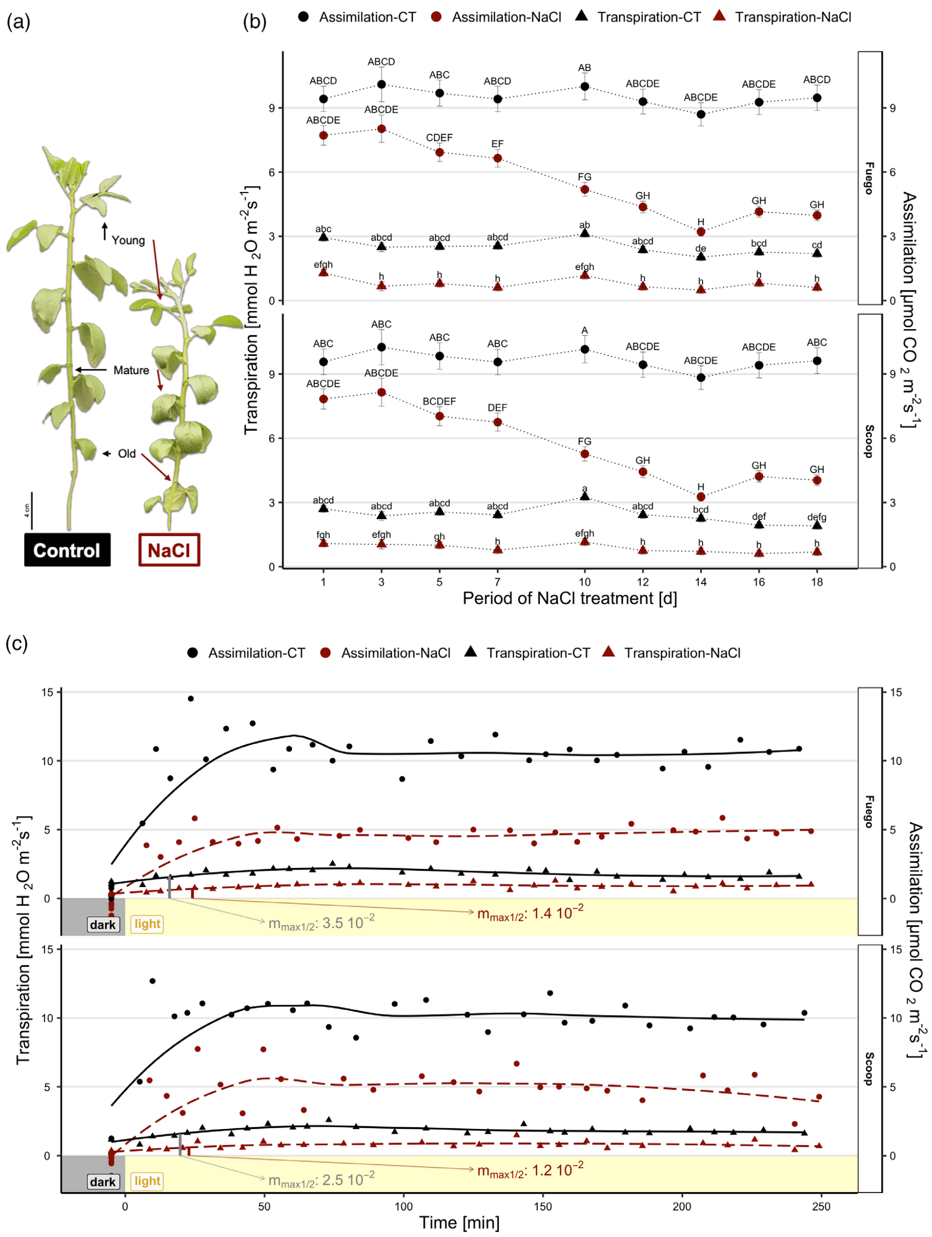

FIGURE 1 Gas exchange and the stomatal response to light in *Vicia faba* grown under NaCl. (a) Images of plant shoots from *V. faba* variety Fuego grown under control and 100 mM NaCl conditions (20 days). (b) Assimilation (A) and transpiration (E) rate of two *V. faba* varieties, Fuego and Scoop, grown under control and 100 mM NaCl. Measurements were conducted at fourth leaves from top after 100 mM NaCl treatment had been applied. Means ± SE; different letters indicate significant differences of means of comparison within variety (Tukey test; $p \leq .05$; $n = 5$). (c) The response of A and E rates of fourth leaves from top of Fuego and Scoop after 20 days NaCl stress and controls to light. Measurements of A and E rates with trend-line (local polynomial regression fit) during the transition from darkness to light indicated by labelled horizontal segments (grey: 0 µmol m^{-2} s^{-1}; yellow: 400 µmol m^{-2} s^{-1}). Vertical bars annotate the half of the maximum value (max$_{1/2}$) plus the slope (*m*) from the initial value to max$_{1/2}$ ($n = 3$) [Colour figure can be viewed at wileyonlinelibrary.com]

NaCl treatment, plant materials, all without salt-induced lesions, were harvested in a randomized order. Material referred to as light condition was collected starting from after 2.5 hr lights on. The material of the second, dark condition was collected identically, but the plants were kept in the darkness until harvest and the laboratory remained unlit ($\sim$1 μmol photons m^2/s) during the harvest (Figure S2).

2.2 | Isolation of guard cells

Guard cells for metabolomic analysis were isolated by the abaxial leaf epidermis being peeled off of at least 5 leaves as described earlier (Cornish & Zeevaart, 1986; Geilfus, Lan, & Carpentier, 2018). Peeled off strips were collected within 5 min in 10 ml 0.001% Tween20 on ice and then sonicated for 3 min by using 0.3 s pulses at approx. 35 W (SONOPULS HD 2070/UW 2070/M 73, Bandelin, Berlin, Germany). This procedure destroyed the epidermal cells, whereas the more robust GCs remained intact (Cornish & Zeevaart, 1986). After being rinsed with ice-cold deionized water in a sieve, isolated GCs (GC preparations) were shock-frozen in liquid nitrogen, lyophilized and stored at −80°C. To obtain sufficient material for ion analysis of GCs, a second GC isolation approach was used, namely, the ice blender method according to Bauer et al. (2013) with minor modifications. At least 6 leaves were blended (B-400, Büchi, Essen, Germany) in 200 ml deionized H_2O containing crushed ice for 30 s and, then, the tissue was collected and rinsed on a nylon mesh with a pore width of 210 μm. The collected tissue was subjected to a second blending step, as described above, and then, collected, shock-frozen in liquid nitrogen, lyophilized and stored at −80°C. The effectiveness of the two isolation methods was microscopically verified by means of viability stains (Geilfus et al., 2018; Geilfus, Mithofer, et al., 2015).

2.3 | Extraction of apoplastic washing fluids and sampling of leaf fractions

Cut leaves were infiltrated with deionized H_2O according to Lohaus, Pennewiss, Sattelmacher, Hussmann, and Mühling (2001). Apoplastic washing fluid (AWF) was collected within 3 min by carefully pulling the plunger of a syringe (without a needle) that was being gently pressed on the abaxial leaf side. Cytosolic AWF contamination (Floerl et al., 2008) was estimated by malate dehydrogenase (MDH) activity as described by Lohaus et al. (2001) (maximal relative activity of about 5%; Figure S1). The remaining leaf materials (symplastic leaf fractions) from which AWFs had been extracted, AWFs and non-treated leaf materials (non-sample controls) were shock-frozen in liquid nitrogen, lyophilized and stored at −80°C.

2.4 | Gas exchange and stomatal imprints

The transpiration and CO_2 assimilation rates of the fourth leaves (Figure 1a) were measured in the climate cabinet (see above) by using an LCi-*SD* ultra-compact photosynthesis system (ADC Bioscientific, U.K.) after 2.5 hr lights on. For the recording of the light-induced changes in gas exchange, sets of three plants of each condition were measured in rotation during dark-to-light (0–400 μmol photons m^2/s) transition until 4 hr after lights on. For recording of the stomatal closing response, leaf gas exchange was continuously measured of plants that had been transferred to dark. In addition to the transition to darkness, dry air (approx. 20% humidity) was supplied to the continuous flow leaf chamber. Epidermal imprints were taken under light (4 hr after lights on) and dark (4 hr after lights off at the end of the photoperiod) condition by applying a thin layer Formvar (2% in 1,2-dichlorethane). After being dried, the imprints were detached from the leaves by using adhesive strips and afterwards analysed microscopically.

2.5 | Ion extraction and measurement

Leaf ions were extracted and measured as described earlier (Franzisky, Geilfus, Kränzlein, Zhang, & Zörb, 2019). In brief, 50 mg ground plant material was solubilized by microwave digestion at 190°C for 25 min in 12 ml digestion solution (46% (vol/vol) HNO_3 and 10% (vol/vol) H_2O_2). Filtrated digestates were used for the analysis of cations by atomic absorbance spectroscopy whereas the Cl^- concentrations were measured by using the ferricyanide method (Munns, Wallace, Teakle, & Colmer, 2010). For the ion measurement of GC preparations and AWFs, approximately 15 mg DW or AWF aliquots of 100 μl were each added to 500 μl 0.5 M HNO_3, respectively, and then incubated at 85°C for 16 hr with repeated thorough mixing. Supernatants of centrifuged (20,800 g, 10 min 4°C) extracts were used for analysis. After the addition of internal Rh standard, concentrations were measured by using an inductively coupled plasma mass spectrometer (NexION 300 X, Perkin Elmer, Waltham, USA). AWFs were corrected by the subtraction of cytosolic ion contamination, which was calculated by the use of the respective fresh leaf ion concentrations and MDH activities (Figure S1). Although the ion concentrations of GC preparations do not refer to guard cell symplasts only (Raschke, 1979; Stevens & Martin, 1977), the approach allowed a qualitative analysis of the effect of the NaCl treatment on the ion contents.

2.6 | Metabolite extraction and metabolite profiling

Approximately 10 mg lyophilized leaf material or GC preparation was homogenized by using a Retsch MM300 TissueLyser (Haan, Germany). Soluble metabolites of leaves were extracted according to Richter et al. (2019) with modifications. In brief, metabolites were extracted in 360 μl methanol with added $U^{13}C_6$-sorbitol standard at 70°C for 1.5 hr. After the addition of 200 μl $CHCl_3$ the samples were agitated at 37°C for 0.5 hr. An aliquot of 400 μl bi-distilled H_2O was added to induce a liquid phase separation. After thorough mixing and centrifugation (20,800 g for 5 min), aliquots of 160 μl of the upper polar phase were dried in a vacuum concentrator overnight at room temperature. AWFs were derivatized without further extraction. Primary metabolites of GC preparations were extracted with 600 μl premix containing MeOH/H_2O/$CHCl_3$: 2.5/1/1 (vol/vol/vol) without liquid partitioning into chloroform (Erban et al., 2020). Aliquots of 500 μl were dried in a vacuum concentrator overnight at room temperature. Chemical derivatization, that is, methoxyamination and trimethylsilylation, and subsequent gas chromatography-electron impact/time-of-flight mass-spectrometry (GasC-EI/TOF-MS)-based metabolite profiling was carried out as described earlier (Dethloff et al., 2014) in splitless mode for GC preparations and in splitless and split 1:30 mode for leaves and AWFs.

2.7 | Metabolite data processing

GasC-EI/TOF-MS chromatograms were acquired, visually controlled, baseline-corrected and exported in the NetCDF file format by using ChromaTOF software (Version 4.22; LECO, St. Joseph, USA). The processing of GasC-EI/TOF-MS data into a standardized numerical data matrix and compound identification were performed by using TagFinder software (Luedemann, Strassburg, Erban, & Kopka, 2008). Compounds were identified according to standardized guidelines (Dethloff et al., 2014) by mass spectral and retention time index matching to a reference collection of authenticated standard substances and of frequently observed but not yet identified mass spectral tags from the Golm Metabolome Database, GMD, (http://gmd.mpimp-golm.mpg.de/search.aspx) (Hummel et al., 2010; Kopka et al., 2005). In this study, individual hexoses could not be resolved because of co-elution. Because the extraction of the GC preparation metabolites had been performed without phase separation, an analysis of fatty acids and lipids was performed for GC preparations only. Data processing and numerical analysis were carried out according to Richter et al. (2019). Metabolite abundancies were normalized to weights, internal standards and the sum of the intensities of the respective sample to enable the analysis of compositional changes of metabolites separately within each of the three sample types, namely leaves, AWFs and GCs. Values are presented as the percent of maximum. Fold-changes for the comparison of metabolic changes in response to experimental conditions were calculated by numerical subtraction after $\log_2$-transformation.

2.8 | Statistical analysis

All fractions for metabolome profiling, but non-sample controls, were sampled from two independent experiments with each of 5 biological replicates yielding 10 biological replicates for AWFs, symplastic fractions and GC preparations (exceptions with $n = 9$ are given in Table S1). Symplastic fractions (leaf material from which AWFs had been extracted) were used to restrict analysis of AWF profiles to metabolite pools that remained unaffected by the extraction procedure ($p \le .001$). Data processing, transformation, analyses of variance (ANOVA), models and the post-hoc test (Tukey's) were carried out by using R software for statistical computing (R Core Team, 2020). Data of repeated experiments were analysed by using the mixed model algorithm of "lmer" (Bates, Maechler, Bolker, & Walker, 2015) with the repeated experiments as a random factor by applying a significance threshold of $p < .05$. Other data were analysed by using linear models and applying the same threshold unless stated otherwise. Prior to computing the principal component analysis by using "stats", the data were restricted to metabolites with less than 10% missing values (NA); remaining NA were replaced by half of the minimum value. Data were plotted by using "ggplot2" (Wickham, 2016). Trend-lines were fitted with local polynomial regression by using "stats". Heatmaps and Euler diagrams were drawn by using "pheatmap" (Kolde, 2019) and "VennDiagram" (Chen & Boutros, 2011), respectively.

3 | RESULTS

3.1 | Leaf physiological measures

The two *Vicia faba* L. varieties Fuego and Scoop were selected to allow the evaluation of the broad stress response occurring in both varieties, which had shown plasticity in withstanding salt stress in a previous experiment (Franzisky et al., 2019). In addition, in this experiment, some plants of the variety Fuego showed salt-induced stress symptoms such as necrotic spots on leaves a few days earlier than Scoop. However, for the collection of plant material and physiological measurements only plants without symptoms were considered.

Under control conditions, mature leaves (Figure 1a) of both varieties exhibited similar assimilation and transpiration rates, with values of about 9 μmol CO_2 m^{-2}/s and 3 mmol H_2O m^{-2}/s, respectively (Figure 1b). In response to NaCl treatment, the transpiration rate significantly decreased by about 60% in comparison with controls. This reduction was constant over the measuring period for both varieties. Similar to the transpiration, the rate of CO_2 assimilation was slightly reduced at the beginning of the stress period but decreased continuously with prolonged exposure to NaCl stress to values of about 4 μmol CO_2 m^{-2}/s, although gas exchange in terms of transpiration remained at a similarly reduced level (Figure 1b). To assess the opening response of the stomata to light, we recorded the gas exchange characteristics during the dark-to-light transition after 20 days of NaCl stress. After lights on, the photosynthesis rate of both varieties increased within 25 mins to the respective maximum with no temporal differences between NaCl stressed and control plants (Figure 1c). However, the maximal assimilation rate of NaCl treated plants was half of that of controls (Figure 1b,c). In accordance with the course of assimilation rates, the transpiration rates increased with the duration of illumination, but in the salt stressed plants, the maximum rates were again half of that of the controls. The transpiration rates of NaCl stressed Fuego and Scoop reached maximal values after about 60 mins, which was about 30% later than controls (Figure 1c). The comparison of the slopes from initial values recorded at darkness to half of the maximum ($\max_{1/2}$) illustrated that the speed of the light-

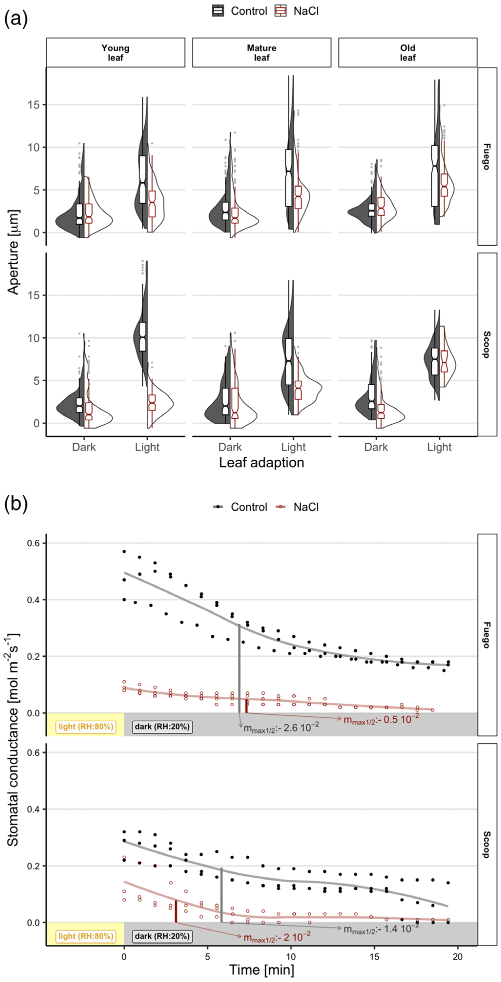

FIGURE 2 Analysis of stomatal apertures and the closing response in salt stressed *Vicia faba*. (a) Stomatal aperture of leaves of various developmental stages of two *V. faba* varieties, Fuego and Scoop, from control and 20 days 100 mM NaCl conditions. Epidermal imprints were taken from the abaxial side of young, mature and old leaves (Figure 1a) during dark and light. Data are presented as box plots featuring the maxima, 75 quartiles, medians, 25 quartiles and minima with density plots of individual apertures (*n* = 3). (b) The response of stomatal conductance of fourth leaves from top of Fuego and Scoop after 20 days NaCl stress and controls to combined darkness and dry air (approx. 20% humidity) as stomatal closing stimuli. Continuous measurement of stomatal conductance with trend-line (local polynomial regression fit) during the transition from light to dark and from 80 to 20% relative humidity (RH) indicated by labelled horizontal segments (yellow: 400 µmol m^{-2} s^{-1}, RH: ~80%; grey: 0 µmol m^{-2} s^{-1}, RH: ~20%). Vertical bars annotate the half of the maximum value (max$_{1/2}$) plus the slope (m) from the initial value to max$_{1/2}$ (*n* = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

induced increase of transpiration, that is, the opening of the stomatal pores, was about 50% slower in long-term NaCl stressed plants than in the controls, irrespective of the variety.

In agreement with the reduced transpiration rates, the stomatal apertures of leaves of various developmental stages were reduced under light after 20 days of NaCl stress in comparison with controls (Figure 2a). However, the aperture of the stomatal pores of leaves of various developmental stages was differently regulated because the salt stress-induced reduction was weaker in old leaves than in mature and young leaves in both varieties. Moreover, young leaves of Scoop had more opened stomata under control conditions in comparison with Fuego whereas the apertures of both varieties were similar under NaCl condition. In contrast to the differential aperture regulation in light, the stomata of NaCl-treated plants were closed 4 hr after the end of the photoperiod (dark), the same as in the controls (Figure 2a). To assess the closing response of stomata, we recorded gas-exchange of plants that had been transferred to dark and were treated with dry air (approx. 20% humidity). In response to the combined closing stimuli, the rates of stomatal conductance of all plants declined markedly within 20 min (Figure 2b). Starting from about 0.4 to 0.6 mol m^{-2} s^{-1}, the stomatal conductance of controls decreased by at least 50% within 20 mins reaching a constant, basal plateau. In agreement with the reduced gas-exchange under NaCl stress, the initial rates of stomatal conductance of NaCl stressed plants were lower than that of the controls with values of 0.1 mol m^{-2} s^{-1}, which decreased in response to the closing stimuli to zero within 20 and 10 mins in Fuego and Scoop, respectively (Figure 2b). The comparison of the slopes from initial values to max$_{1/2}$ illustrated that the stomatal conductance declined about 50% faster in Fuego than in Scoop under control conditions. Under long-term salt stress however, the response to the closing stimuli was faster in Scoop than in the respective control, and four-fold faster in comparison with salt stressed Fuego.

3.2 | Ion concentrations of leaves, apoplast and guard cell preparations

In response to NaCl treatment, Na$^+$ and Cl$^-$ concentrations increased in young and mature leaves of both varieties (Figure 3). Compared with younger leaves, the accumulation of Na$^+$ and Cl$^-$ and the reductions in K$^+$ were higher in mature leaves. Mature leaves also showed a significant reduction in Mg^{2+} concentration, whereas stress-responsive increases in Ca^{2+} were higher in young leaves of both cultivars. An enrichment of salt ions was also found in the apoplastic space, that is, cell walls, with Na$^+$ and Cl$^-$ concentrations being increased >100-fold and up to 26-fold in comparison with controls, respectively (Figure 3b). A similar trend was found for GC preparations because the Na$^+$ concentrations were increased three-fold and 7.6-fold in the NaCl-treated Fuego and Scoop varieties, respectively, whereas the Cl$^-$ concentrations remained similar to those of the control (Figure 3c). The concentrations of K$^+$, being the major inorganic osmolyte for GC osmotic adjustment, were significantly reduced (0.1 to 0.2-fold) in comparison with controls (Figure 3c). Divalent cations such as Ca^{2+} and Mg^{2+} remained unchanged, the slight reductions being non-significant.

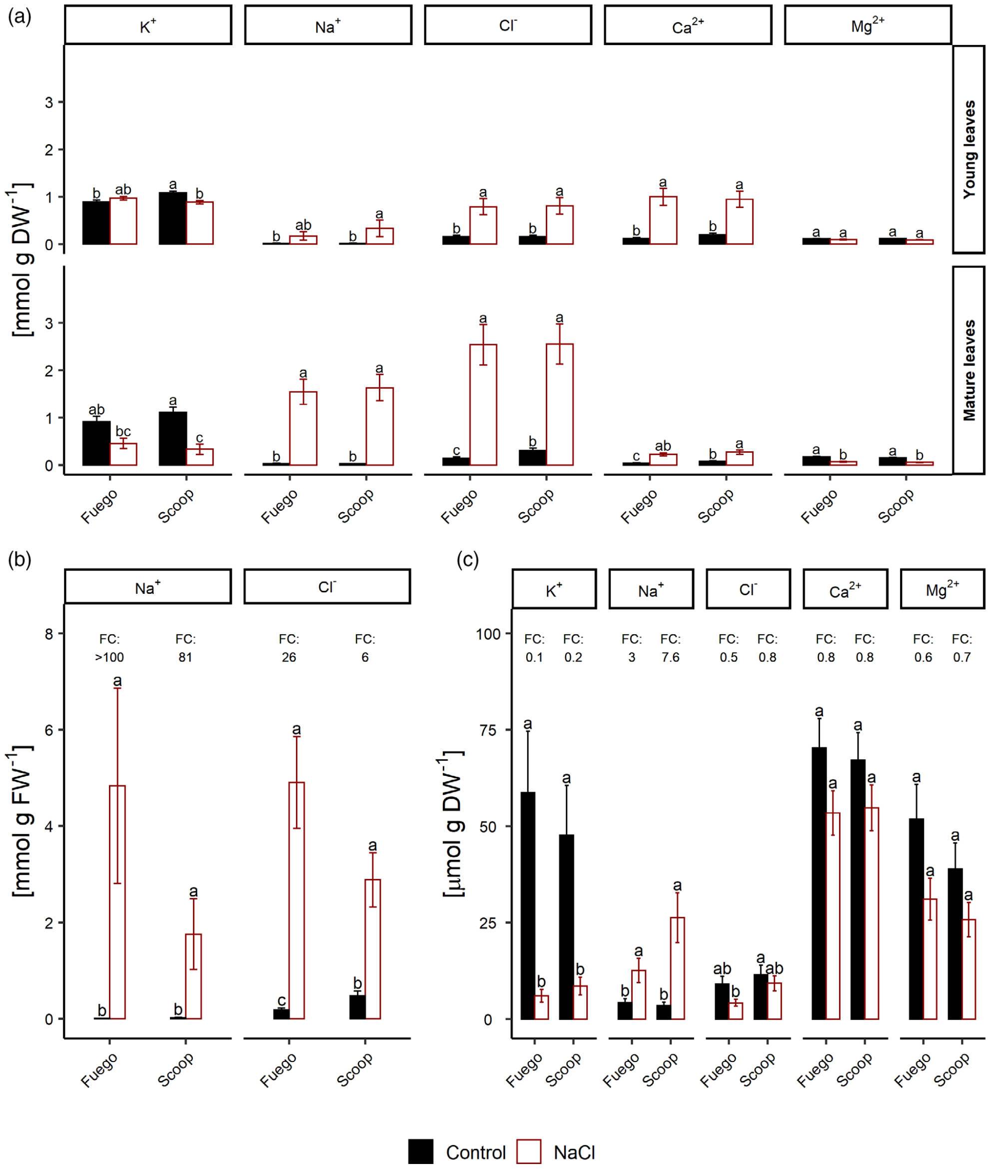

FIGURE 3 Ion compositions of various leaf fractions of *Vicia faba* grown under NaCl and control conditions. Ion concentrations of (a) young and mature leaves, (b) apoplastic washing fluids and (c) guard cell preparations of *V. faba* varieties, Fuego and Scoop, under control and 100 mM NaCl conditions. Concentrations of potassium (K^+), sodium (Na^+), chloride (Cl^-), calcium (Ca^{2+}) and magnesium (Mg^{2+}). Means ± SE; FC, fold change; different letters indicate significant differences of comparisons between varieties and treatments within ions; ($p \le .05$; $n = 5$) [Colour figure can be viewed at wileyonlinelibrary.com]

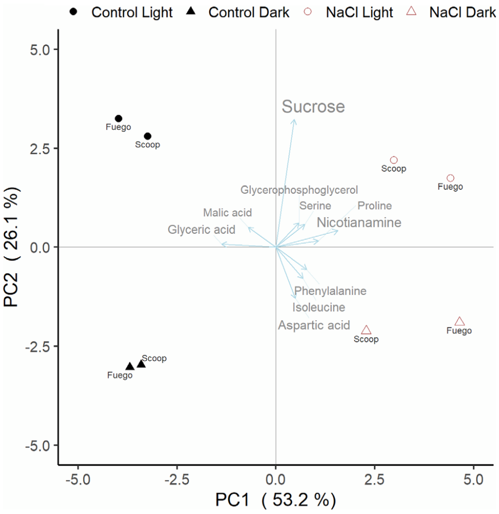

FIGURE 4 Unsupervised analysis of leaf primary metabolite content of *Vicia faba* in dark and light under NaCl and control conditions. Principal component analysis (PCA) of the relative leaf metabolite content of two *V. faba* varieties, Fuego and Scoop, under control and 100 mM NaCl, and dark and light conditions. Principal components (PCs) represent 79.3% of the total variance of the data with PC1 reflecting the differences between control and NaCl, and PC2 the differences between dark and light conditions. Top 10 influential metabolites of PC loadings are indicated by labels and blue arrows. The segment length and the size of the metabolite labels correspond to the influence on the separation (means of *n* = 5) [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Leaf metabolic acclimation to long-term NaCl

For a comparison of the metabolic signatures of GCs, the apoplast and the leaves in response to long-term NaCl during dark and light, we applied non-targeted gas chromatography mass-spectrometry. The evaluation of the metabolites in each fraction was restricted to manually identified mass spectral tags consisting of known metabolites and to non-identified compounds with known mass spectrum and retention index properties.

To overview the general effects of the experimental conditions on the metabolome of the mesophyll dominated whole leaf fractions, from which the GC preparations and the AWFs had been isolated, we analysed the principal components of the leaf metabolomic data (Figure 4). The latter analysis illustrated a separation according to the long-term NaCl treatment (horizontally) and the light conditions (vertically). Correspondingly, the first principal component gave high loadings to NaCl-responsive metabolites such as the compatible solute proline and other amino acids such as aspartic acid, phenylalanine, isoleucine and serine whereas the separation along the second principal component was largely influenced by the light-dependent changes in sucrose content. More specifically, the leaf metabolic response to long-term NaCl was mainly characterized by lower levels of organic acids related to the TCA cycle (aconitic-, maleic- and malic acid), minor carbon hydrate metabolism (threonic acid) and photorespiratory pathway associated glyceric acid whereas the compatible solute proline and other amino acids, the stress-responsive *myo*-inositol, TCA cycle intermediate succinic acid and anti-oxidative and membrane-related compounds (nicotiamide, glycerophosphoglycerol) were increased (Figure 5a). Although the changes in metabolite pools in response to NaCl exposure showed variety-specific differences in their extent, these general trends were the same for Fuego and Scoop (Figure 5).

3.4 | Guard cell metabolic response to long-term NaCl diverges from that of leaves

Qualitative analysis revealed decreasing leaf and GC preparation metabolite pools having partly overlapping patterns in response to NaCl treatment (Figure 5). Decreases of 6 metabolite pools were found in GC preparations, with 5 (butanoic acid, 4-amino- [GABA], citric-, malic-, glutamic- and glyceric acid) being common between the two *V. faba* varieties (Figure 5a,b). Although the reduction of citric acid was the same for both varieties, the reductions of GABA, malic and glutamic acid pools in Fuego were about two-fold in comparison with Scoop (Figure 5a). Common decreases between leaves and GC preparations were found for glyceric and malic acid, and for A170001, with the last-mentioned for Fuego only (Figure 5b). In comparison with leaves, the reductions of malic acid pools of GC preparations were higher for each variety, whereas glyceric acid was similarly reduced (Figure 5a). In GC preparations 7 metabolites increased in response to NaCl treatment of which the fatty acid tetradecanoic acid was similar for both varieties (Figure 5a,c). However, there was no common increase between metabolite pools of leaves and GC preparations (Figure 5c).

3.5 | Deviations in guard cell metabolic response to light under long-term NaCl

For a detailed analysis of the metabolic response to light (Stitt, Lunn, & Usadel, 2010; Stitt & Zeeman, 2012; Szecowka et al., 2013) throughout the three leaf fractions, we looked at the changes resulting from the transition from dark to light (Figure 6). Similar patterns of light-induced increases were found for sucrose and malic acid representing the major photosynthate and a TCA cycle intermediate with a central role in primary metabolism and in GC turgor adjustments, respectively. However, malic acid pools in the leaf and AWF fractions showed only minor light-related increases under control conditions, because the levels were similar in darkness and light (Figure 7e,f). In contrast, the light-induced increases in the malic acid

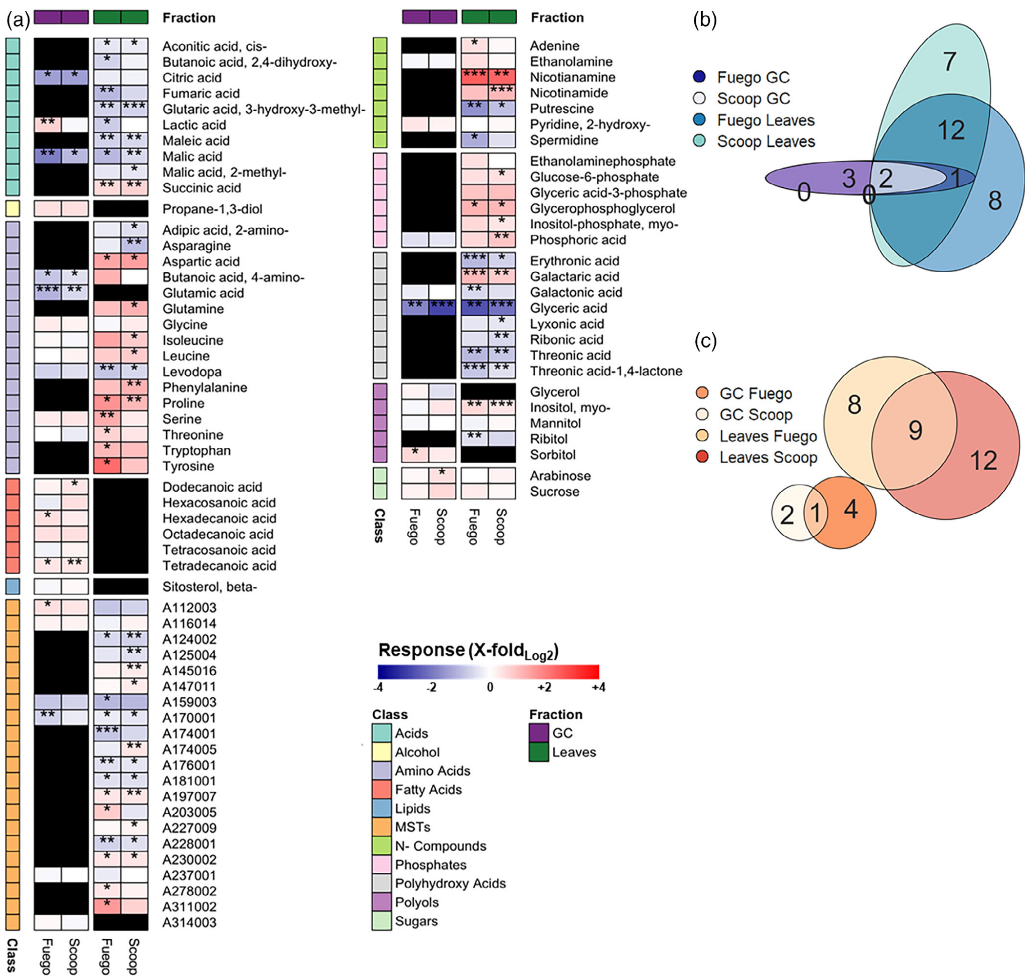

FIGURE 5 Analysis of common and specific metabolic changes in guard cell preparations and leaves of *Vicia faba* in response to NaCl. (a) Metabolic response to NaCl (100 mM, 20 days) in guard cell (GC) preparations and leaves of the *V. faba* varieties, Fuego and Scoop, under light condition. Means of the fold-change$_{log2}$ of the relative metabolite contents are indicated by a colour code. Asterisks indicate the level of significance (**p* ≤ .05; ***p* ≤ .01; ****p* ≤ .001; GC: *n* = 9–10; leaves: *n* = 5; MST, mass spectral tag; the shown metabolites are restricted to detections in GCs plus affected leave metabolites; full data set provided in supplementary Table S1). (b, c) Comparison of common, significantly affected metabolites in leaves and GC preparations under light condition. Results of the qualitative assessment of decreased (b) and increased (c) metabolite pools are plotted as Euler diagrams with numbers indicating common and unique metabolites [Colour figure can be viewed at wileyonlinelibrary.com]

pools of GC preparations were more prominent, because the levels were low at darkness and high after acclimation to light (Figure 7d). Under NaCl, malic acid pools of leaf and AWF fractions only slightly increased in response to light, whereas light-related increases in GC preparations were diminished (Figure 7d–f). The sucrose pools increased under all conditions and in all fractions in response to illumination; however, increases in AWFs and GC preparations were slightly lower under NaCl conditions (Figure 6; Figure 7a–c). In comparison with the control, the sucrose levels of GC preparations and AWFs of NaCl-treated plants were higher at dark (Figure 7a,b). Pools of amino acids tended to decrease in AWFs and leaves in response to light, except for the increasing pools of photorespiratory-pathway-

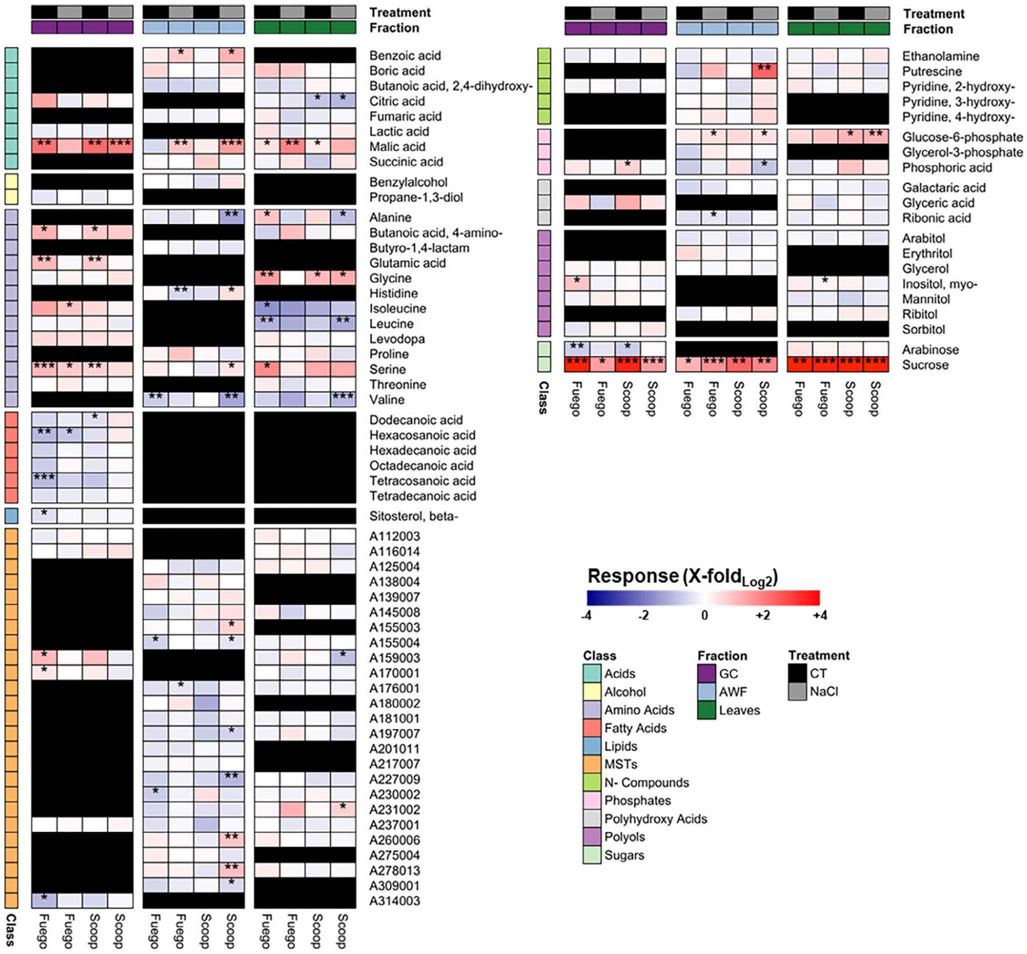

FIGURE 6 Metabolic response to light in guard cell preparations, apoplastic washing fluids and leaves of *Vicia faba*. Metabolic response to light in guard cell (GC) preparations, apoplastic washing fluids (AWF) and leaves of the *V. faba* varieties, Fuego and Scoop, under control and 100 mM NaCl conditions. Means of the fold-change$_{\log 2}$of relative metabolite contents are indicated by a colour code. Asterisks indicate the level of significance (**p* ≤ .05; ***p* ≤ .01; ****p* ≤ .001; GC: *n* = 9–10; AWF: *n* = 10; leaves: *n* = 5; MST, mass spectral tag; the shown metabolites are restricted to detections in GCs and AWFs; full data set provided in Table S1) [Colour figure can be viewed at wileyonlinelibrary.com]

associated glycine and serine in leaves (Figure 6). This pattern was partly seen in GC preparations, as serine increased together with polyhydroxic and glyceric acid in response to light. However, increases in glutamic acid, GABA and serine pools of GC preparations were seen under control rather than under NaCl conditions. Throughout the various leaf fractions, sucrose was the only sugar exhibiting a similar pattern. Arabinose pools remained mostly unchanged within leaf and AWF fractions. In control GC preparations, arabinose pools slightly decreased in response to light. In the respective NaCl fractions, this trend was weaker.

4 | DISCUSSION

4.1 | Similar physiological response to long-term NaCl in both *Vicia faba* varieties

Plant growth and nutrient uptake seems to be disturbed under high salinity such as when K^+ is diminished and Na^+ and Cl^- is increased (Flowers, Munns, & Colmer, 2015; Kotula, Garcia, Zörb, Colmer, & Flowers, 2020; Zörb, Geilfus, & Dietz, 2019). This well-known pattern has also been found here for both *V. faba* varieties (Figures 1a and

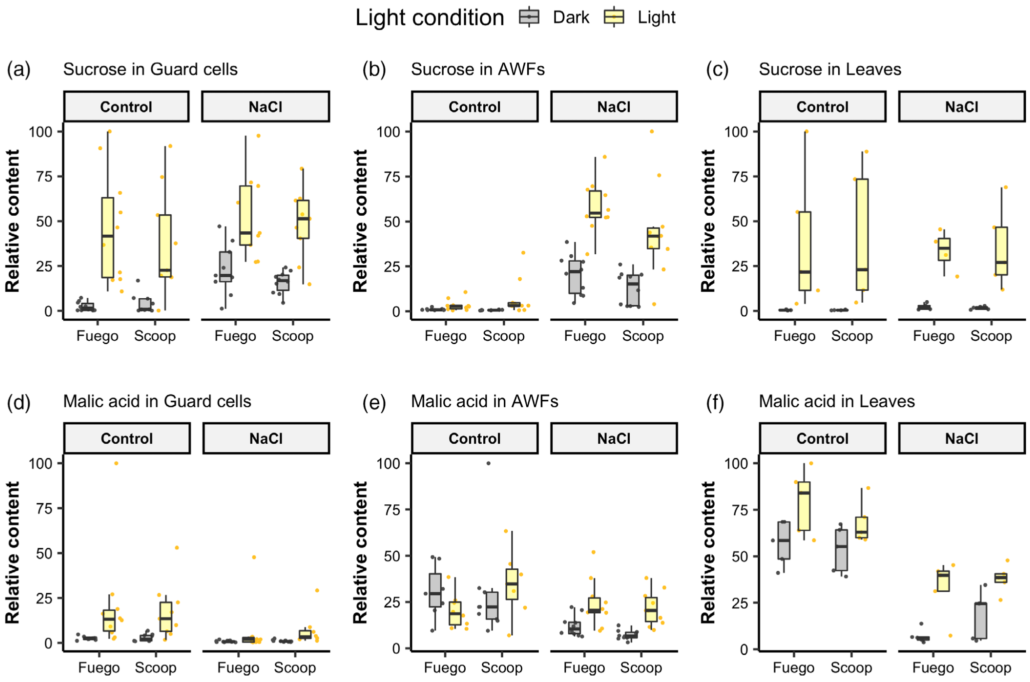

FIGURE 7 Sucrose and malate in various leaf fractions of *Vicia faba* in dark and light under salt and control conditions. Detail of sucrose (a–c) and malic acid (d–f) in guard cell preparations, apoplastic washing fluids (AWFs) and leaves, respectively, of the *V. faba* varieties, Fuego and Scoop, under control and 100 mM NaCl conditions at dark and light condition. Maximum scaled (%) relative metabolite contents presented as box plots featuring the maxima, 75 quartiles, medians, 25 quartiles and minima [Colour figure can be viewed at wileyonlinelibrary.com]

3a). In our experiment, K^+ was little changed in plant leaves of both varieties (Figure 3a) indicating that the NaCl stress was below the level of causing a K^+ deficiency (Zörb et al., 2019). In terms of the ion distribution in leaves of various ages, the younger leaves are clearly protected by the high salt ion load, which agrees with previous studies (Franzisky et al., 2019; Richter et al., 2019). The physiological stress response of the extent of gas exchange was similar in both varieties showing a stress-related delay in the stomatal opening (Figure 1c) and the reduction of transpiration more than half (Figure 1b) due to stomatal closure (Figure 2a). Both physiological reactions diminish plant productivity and water use efficiency in terms of unproductive water loss or the limitation of CO_2 diffusion into leaves (Lawson & Vialet-Chabrand, 2019). The assimilation of CO_2 decreased continuously over the stress period (Figure 1b) suggesting a salt stress-induced metabolic perturbation of photosynthesis beyond the limitation of CO_2 diffusion because the gas exchange in terms of transpiration was similarly reduced throughout the NaCl stress period. This illustrates that photosynthesis was negatively affected under long-term NaCl, but not at a severe level. Thus, the plants could produce sufficient assimilates and react with appropriate metabolic regulation to mitigate direct stress effects.

4.2 | Stomatal regulation resists increasing leaf Na^+ and Cl^- concentrations

The enrichment of acropetally transported Na^+ and Cl^- in leaves and apoplast was found in both *V. faba* varieties (Figure 3a, b) and is known to interfere with enzyme functioning and other metabolism at higher concentrations (Flowers et al., 2015; Geilfus, 2018; Munns et al., 2016). Increasing salt ion concentrations within the apoplast resulting inter alia from weak ion exclusion capabilities (Munns, Pasioura, Colmer, & Byrt, 2020), in particular that of Na^+, have previously been reported for salt-sensitive dicots such as *V. faba* (Shahzad, Zörb, Geilfus, & Mühling, 2013; Speer & Kaiser, 1991). Because inorganic ions are imported from the apoplastic reservoirs during stomatal opening (Felle, Hanstein, Steinmeyer, & Hedrich, 2000; Roelfsema & Hedrich, 2002), apoplastic salt ion accumulation might interfere with GC ion transport (Hedrich & Shabala, 2018). In addition to the apparent reductions in K^+ concentrations in GC preparations compared with controls, reductions that are primarily a consequence of the NaCl-induced suppression of stomatal opening (Kollist et al., 2014; Munemasa et al., 2015; Roelfsema, Hedrich, & Geiger, 2012; Zhu, 2002), Na^+ concentrations were increased in GC preparations

(Figure 3c). Unlike the values obtained from surrounding mesophyll and apoplast (Figure 3a,b), Cl$^-$ did not increase on NaCl treatment in the GC preparations of the two varieties (Figure 3c). The differential accumulation pattern of Na$^+$ and Cl$^-$ implies that GCs rely on their Cl$^-$ transport mechanisms (Jezek & Blatt, 2017) to avoid the accumulation of Cl$^-$, whereas the intake of Na$^+$ seems to be less well controlled. Although the stomatal pores of both varieties were closed 4 hrs after the end of the photoperiod same as the controls (Figure 2a), the stomatal response to darkness and low air humidity was markedly delayed in the variety Fuego (Figure 2b) suggesting a dysfunctional stomatal closing response as a result of Na$^+$ intake into GCs (Robinson, Véry, Sanders, & Mansfield, 1997; Thiel & Blatt, 1991) observed in Fuego but not in Scoop. Such a delayed closing response increases the probability of unproductive water loss (Lawson & Vialet-Chabrand, 2019; McAusland et al., 2016) and continued acropetal transport of deleterious salt ions to the shoot (Hedrich & Shabala, 2018). However, the general reduction of the stomatal pore widths (Figures 1b and Figure 2a), and thus, less intake of inorganic ions into GCs, might be advantageous to prevent accumulation of deleterious salt ions when apoplastic Na$^+$ and Cl$^-$ concentrations increase because of continuous exposure to salinity.

4.3 | Diverging signatures of leaf and guard cell metabolic acclimatisation to NaCl

Long-term exposure to NaCl results in the accumulation of salt ions in plant tissues (Figure 3), challenging ion homeostasis (Munns et al., 2016), nutrient availability (Zörb et al., 2019) and primary metabolism (Sanchez et al., 2008). The last-mentioned is markedly affected in *V. faba* (Richter et al., 2019) and other legumes such as Lotus (Sanchez et al., 2008; Sanchez et al., 2011). Accordingly, the leaf metabolic profiles reflected changes in response to the long-term exposure to NaCl and the light condition, that is, when the stomata were open or closed (Figure 4). Changes of the levels of compatible solutes such as proline and stress-responsive metabolites such as sugars, free amino acids and the reduction of TCA cycle intermediates (Figures 4 and 5a) were in agreement with known patterns of metabolic responses to osmotic and high NaCl stress (Fàbregas & Fernie, 2019; Richter et al., 2019; Sanchez et al., 2008).

As far as GC metabolism is concerned, we have shown that the amount of changed metabolites under salt conditions is lower than in whole leaves indicating that GCs, in general, are not as sensitive to saline conditions as leaves (Figure 5). This is in accordance to the findings above and therefore suggests that the stomata of *V. faba* can still function, that is, control their aperture and the transpiration rate, albeit at a slower rate (Figures 1b,c and 2), to reduce water loss and the amount of salt delivered to the shoot (Hedrich & Shabala, 2018; Robinson et al., 1997), even when the mesophyll is affected by high salinity. Conserved leaf metabolic responses to high NaCl exposure, for example, increases of stress-responsive metabolites such as *myo*-inositol and free amino acids (Sanchez et al., 2008; Sanchez et al., 2011), were not present in GC preparations (Figure 5a). In *V. faba*, the metabolic response of the mesophyll, with respect to TCA cycle intermediates such as fumaric and malic acid, and the stress-responsive amino acid proline, is more affected by salinity with Cl$^-$ (KCl, NaCl) than by exposition to Na$^+$ without Cl$^-$ (NaSO$_4$) (Richter et al., 2019). Therefore, the absence of a conserved NaCl-stress signature in guard cell metabolism might be associated with the prevention of Cl$^-$ accumulation (Figure 3c). In terms of increased photorespiratory pathway and reduced TCA cycle activity, GC and leaf metabolism were similarly affected, as associated metabolites were changed in response to NaCl exposure (Figures 4 and 5a) reflecting known signatures of leaf metabolic response to stress in many salt-sensitive glycophytes (Richter et al., 2019; Sanchez et al., 2008; Sanchez et al., 2011). In GC metabolism, however, the TCA cycle plays a pivotal role for osmolyte synthesis and energy production, which is required for energizing the proton pumps that drive the import of inorganic ions into GCs (Daloso et al., 2017; Robaina-Estévez, Daloso, Zhang, Fernie, & Nikoloski, 2017; Santelia & Lawson, 2016). In addition to Cl$^-$, organic anions such as malic acid counterbalance the positive charge resulting from K$^+$ that is accumulated during stomatal opening (Chen et al., 2012; Fernie & Martinoia, 2009; Hills, Chen, Amtmann, Blatt, & Lew, 2012; Horrer et al., 2016; Santelia & Lawson, 2016). In association with the decreased malic acid and increased GABA pools of the GC preparations (Mekonnen, Flügge, & Ludewig, 2016), the stomatal apertures of both the *V. faba* varieties were reduced in the light (Figure 2a) illustrating the tight correlation of GC metabolism and stomatal aperture (Daloso et al., 2017; Santelia & Lawson, 2016). In view of the impact of the stress hormone abscisic acid on GC sugar transport and the TCA cycle activity suggested by previous studies (Asai, Nakajima, Kondo, & Kamada, 1999; Jin et al., 2013; Yoshida et al., 2019; Zhu & Assmann, 2017) the observed changes of GC sucrose and malic acid pools might be part of the metabolic response to abscisic acid. Thus, GC metabolism under long-term NaCl seems to be primarily affected by stress-related abscisic acid signalling (Lee & Luan, 2012; Umezawa et al., 2010; Weiler, Schnabl, & Hornberg, 1982) rather than by direct NaCl stress effects as observed in the Na$^+$ and Cl$^-$ accumulating mesophyll-dominated whole leaf fractions (Figures 3a and 5a). Hence, the differential metabolic response of GC metabolism to long-term salinity indicates that the ability for transient turgor adjustments of GCs remains preserved under conditions of increasing salt ion loads in the leaves and the apoplast.

4.4 | Sucrose replete pattern in guard cells indicates metabolic feedforward function during long-term salinity

We observed increased sucrose pools in AWFs and GC preparations both at darkness and in the light under conditions of NaCl stress (Figure 7a–c), when stomata remained mostly closed (Figures 1b,c and 2a). Sucrose pools of controls were lower at darkness and then increased over the photoperiod in all fractions (Figure 7a–c). In *V. faba*, sucrose represents the primary photosynthate and transport form for sugars, which are translocated to the phloem with an apoplastic step. This leads to the transpiration-stream-driven enrichment of sucrose in the apoplast during the photoperiod (Ewert, Outlaw Jr, Zhang, Aghoram, & Riddle, 2000; Outlaw & De Vlieghere-He, 2001). Concomitant with sucrose accumulation in the apoplast during the late diel period, the stomatal aperture declines, although environmental conditions remain unchanged. In addition to a potential osmotic effect of apoplastic sucrose, Kelly et al. (2013) proposed a metabolic feedforward functioning of sucrose coordinating stomatal conductivity and photosynthesis by the stimulation of stomatal closure via a sugar-sensing hexokinase, which in turn stimulates the abscisic acid-signalling pathway in GCs resulting in stomatal closing. Such metabolic regulation has been hypothesized to be relevant under conditions of limited sink capacity and therefore saturated phloem loading in the late diel period (Lawson, Simkin, Kelly, & Granot, 2014; Lima et al., 2018) but might also apply to the physiological condition of *V. faba* under long-term NaCl exposure favouring leaf sugar accumulation of, for example, sucrose (Kempa, Krasensky, Dal Santo, Kopka, & Jonak, 2008; Krasensky & Jonak, 2012; Sanchez et al., 2008). We therefore hypothesize that the sucrose feedforward mechanism acts not only as a positive regulator of GC abscisic-signalling in the late diel period leading to stomatal closure (Kelly et al., 2013; Lugassi et al., 2015), but also under conditions of long-term NaCl, when sucrose accumulates in the apoplast and GCs as a consequence of stress-related carbon partitioning.

In conclusion, this study shows that NaCl exposure increased Na^+ in GCs and triggered responses that indicated a reduced TCA cycle and increased photorespiration activity, both of which are attributable to stress-related hormone signalling. Metabolic stress markers and compatible solutes accumulated in leaf tissue but not in GC preparations. Diverging metabolic regulation of GCs might be a prerequisite for maintaining GC functionality under long-term NaCl, that is, facilitation of a constant adjustment of GC turgor that affects stomatal aperture and water loss. In contrast to controls, the sucrose levels of the apoplast and GC preparations from NaCl-treated plants were high irrespective of the photoperiod indicating that a metabolic sucrose-mediated feedforward mechanism is involved in coordinating stomatal closure under conditions of long-term NaCl.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Bastian Leander Franzisky https://orcid.org/0000-0003-2590-2118
Christoph-Martin Geilfus https://orcid.org/0000-0002-9820-8111
Maria Luisa Romo-Pérez https://orcid.org/0000-0001-7554-0501
Christian Zörb https://orcid.org/0000-0003-0000-5138

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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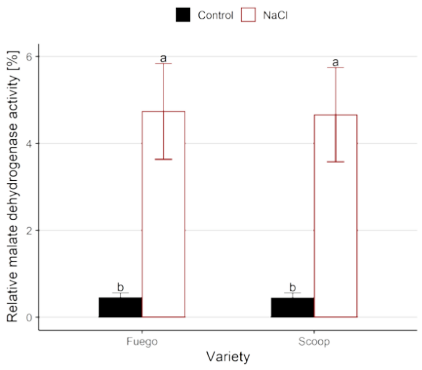
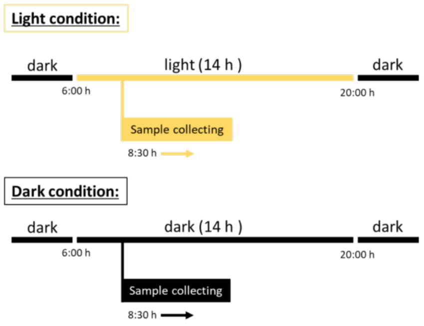
Supplementary material

Main text

Supplementary figure 1:



Supplementary figure 2:



Chapter 6 – General discussion

6. General discussion

6.1. Local onion landraces for the organic farming

In contrast to modern onion varieties with high production capacity and tolerance or resistance to certain pathogens, landraces have traditionally been valued for their yield stability and resistances against local adverse conditions (Zeven, 1998). Landraces are genetically diverse, open-pollinated varieties that generally carry a local name, and are characterized by their properties and qualities that are preferred in the local region (Raggi et al., 2012; Zeven, 1998). In recent decades, farmers have increasingly focused on breeding onion varieties that perform well in conventional farming systems and rely on chemical inputs, largely ignoring the potential of those suitable for organic agriculture (Osman et al., 2008). For this reason, today there is a substantial lack of varieties adapted to organic farming systems where chemical pesticides and fertilizers are prohibited. Screening onion landraces is an effective way of collecting material for future breeding programs, thus preserving and maintaining the local genetic variability and acting against genetic erosion. With this purpose, in year 2015 a first field trial was initiated under organic farming conditions with nine onion landraces that were evaluated and compared against two commercial varieties of the region (Chapter 2). The landraces were Jaune des Cévennes, Stunova, Bolstar, Wiener Bronzekugel, Rijnsburger 4, Birnförmige, Erfurter Lager, Zittauer Gelbe, Paille des Vertus, most of these of French, German, Austrian and Dutch origin. The other two commercial varieties Sturon and Red Baron were selected based on their easy acquisition as organic seeds and popularity in the local organic sector. Yield results and quality aspects in onion landraces demonstrated that, with the exception of the variety Jaune des Cevennes, all studied varieties prospered well under organic farming conditions of South West Germany. The most outstanding results were generated by the following varieties: Birnförmige, which produced good yield with bulbs free from diseases and good quality characteristics, *e.g.* high firmness and dry matter level (Chapter 2, Fig.

2, Tab. 2), the variety Rijnsburger 4, which showed lower bolting and diseases incidences, as well as the variety Stunova, which also produced higher yield than the control variety Sturon. Field, quality and storage studies demonstrated that the above-mentioned landraces performed similar or even better in yield and quality characteristics than commercial varieties, therefore signaling their potential for further studies and breeding programs for the regional organic farming.

6.2. Exploring the metabolite profile and storage potential of onion landraces

Apart from field and yield parameters (chapter 6.1.), internal bulb composition of onion landraces was evaluated in Chapter 3 of this thesis. In order to determine internal quality characteristics, the most important and well-known parameters in onion research, such as sugar, dry matter, pyruvic acid, and polyphenols, were analyzed through targeted/standard methods. Moreover, to get a detailed overview of those characteristics, untargeted metabolomics was performed. With the complementary combination of targeted and untargeted methods it was possible to explore differences among landraces and the other commercial variety Sturon, as well as impact of cold storage on the metabolite profile of onion bulbs.

According to Brewster (2008), onion varieties can be categorized according to their internal quality characteristics into two major production groups: 1) sweet types for fresh market, and 2) dehydrated, pungent onions for food processing. Sweet varieties have a mild-flavor, with high reducing sugar levels, while dehydrated varieties are distinguished by their high dry-matter content and a high fructan levels. Moreover, as described in figure 3 of this discussion, a third category of onion varieties are attracting the interest of consumers and the food industry alike, due to their chemical composition and high levels of key health compounds and aroma properties (Griffiths et al., 2002; Sharma et al., 2015). Results of targeted analysis demonstrate significant differences between the studied onion varieties by analyzing dry matter content, total soluble solids (TSS), non-structural carbohydrates, pyruvic acid and onion pungency, as well as total phenolics. As Fig. 5 in Chapter 2 demonstrates, from nine studied landraces, two in particular, showed the largest deviation from the others, Birnförmige and Jaune des Cévennes. In the variety “Birnförmige”, high levels of fructan, accompanied by high dry matter content and strong pungency were observed, meeting the “dehydrated onion” criteria. On the other hand, the variety “Jaune des Cevennes” demonstrated lower degree of pungency, lower content of dry matter and high levels of reducing sugar, which are distinctive of a “sweet variety” (Chapter 2, Fig. 3). Complementary results produced by untargeted metabolomics allowed us to confirm the above-mentioned variety differences. Furthermore, a clearer separation of the studied varieties into 3 groups (I, II, III) based on their similarities was distinguished; Group I (Birnförmige, Paille des Vertu, Sturon), group II (Erfurter Lager, Stunova, Zittauer Gelbe), and group III (Wiener Bronzekugel, Rijnsburger 4, Bolstar). Unaccompanied and far from the other groups of varieties was Jaune des Cévennes (Chapter 3, Fig. 3). Group I, including the variety Birnförmige, demonstrated high concentrations of tetrasaccharides, while the varieties of group III (in particular the variety Rijnsburger 4) showed, compared to the control variety Sturon consistently and substantially higher levels of amino acids and sugars, some of them with high potential of aromatic properties in onions (Chapter 3, Fig. 5). As mentioned by Brewster (2008) and Petropoulos et al. (2017) in their works, pungent and sweet onions have a different storage potential. Sweet onions, like Jaune des Cevennes, are well-known for their lower storability due to high water content and for this reason, this variety demonstrated rapid spoilage within the first two months of cold storage. On the other end of the spectrum, varieties with high dry matter content and high fructans (*e.g.* nystose) concentration, like variety “Birnförmige”, demonstrated good storability, making it and other varieties from group I, II and III, including the commercial Sturon, suitable for mid- to long-term storage.

In summary, with the results collected in Chapters 2 and 3 of this thesis, it could be concluded that the maximum yield and quality potential in commercial varieties has not yet been reached. Quality optimization in new varieties could be augmented through breeding programs that include local varieties, recovering the aromatic properties that have been lost in modern genotypes. Future studies at different locations are recommended with a limited selection of landraces (*e.g.* Stunova, Birnförmige, Rijnsburger 4) and other commercial varieties, including hybrids. The objective of these studies should be to verify the potential and adaptation of these landraces throughout temperate zones in order to expand the palette of varieties available for the market, thus avoiding genetic erosion.

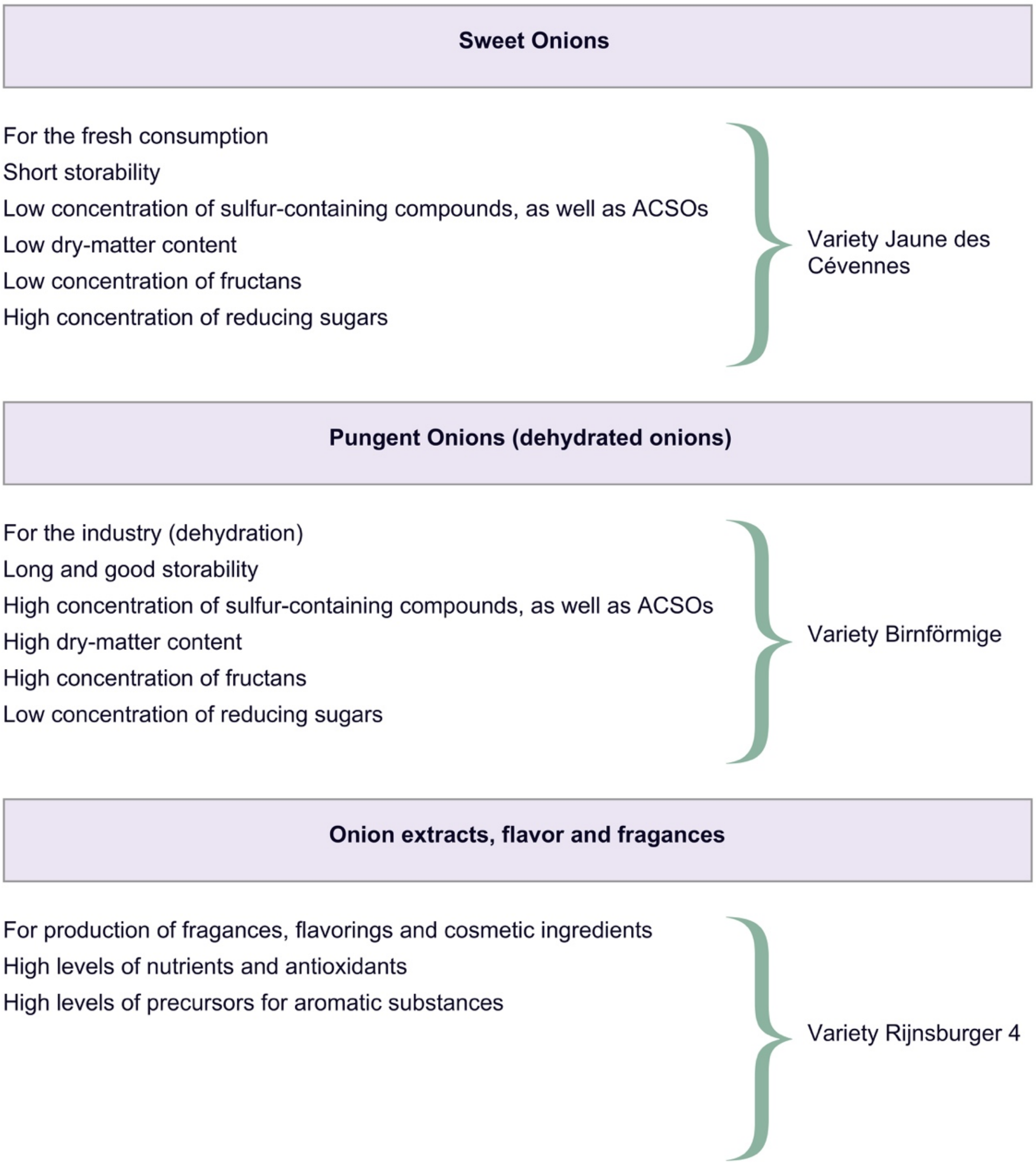


Figure 9 Overview of different production categories and market suitability according the internal quality characteristics of the most outstanding onion landraces.

6.3. Cold storage increases flavor and aroma properties in onion bulbs.

There is a wide range of studies about onion storage with a special focus on changes in S-containing amino acids - *e.g.* S-alkenyl cysteine sulfoxides (ACSOs) - (Lancaster and Shaw, 1991; Yoo et al., 2012) and carbohydrates (Ohanenye et al., 2019; Salamal et al., 1990), but insights into changes in other amino acids linked to aroma and flavor properties of onions are scarce. Results of this thesis showed that after five-month cold storage, non-visible, *i.e.*, internal sprouting was initiated in all varieties, indicating an elongation of the sprout leaf. This physiological change was reflected in the metabolite profile of all onion varieties showing that many non-structural carbohydrates and amino acids - especially those with acidic or basic side chain ones - were affected (Chapter 3, Fig. 4). Moreover, there was a substantial increase in other amino acids involved in the aroma and flavor pathways of onions such as Ser, Cys, Glu and Met, which led to a final increase in the concentrations of aroma precursors ACSOs and in the pungency of the onion. Besides the increased level of those amino acids linked to onion pungency, a significant increase of glutamic acid and aspartic acid, also called “umami amino acids” (Gong et al., 2016) was demonstrated in chapter 3, suggesting a development of onion sensory properties during cold storage. The overall increase of those amino acids could in part be linked to the strong decrease of fructan concentrations, since carbohydrate biosynthesis and N-assimilation of amino acids compete for input of energy and reduced carbon (Druege et al., 2000), and in part linked to the hydrolysis of γ-glutamyl peptides during cold storage (Petropoulos et al., 2017).

6.4. Sodium accumulation and its effect on onion plants

Onions have always been considered a very salt sensitive crop (Aghajanzadeh et al., 2018; Allen et al., 1998; Chang, 2003; Chang and Randle, 2004; Chang and Randle, 2005; Hanci et al., 2016; Turhan et al., 2020). However, to date only a few studies have attempted validating that claim and there is a distinct lack of information about the effect of salinity on onions. In order to understand the salt response of onions, it is necessary to understand physiological and also metabolic response of the whole plant to salinity. For this study, a pot experiment with three of the above-mentioned, contrasting and stable varieties (Chapter 2, figure 5) were selected; the commercial varieties Red Baron and Sturon, as well as the landrace Birnförmige. A moderate level of sodium salinity (4 g Na_2SO_4 per pot for soil application and 400 mM Na_2SO_4 for leaf application) was chosen to allow the analysis of primary physiological and metabolic responses, thus avoiding secondary effects of sodium toxicity. In addition, the transport route of sodium within the plant was analyzed after soil and leaf applications. Similar to the cold-storage study in chapter 3, this study also validated by complementary use of targeted and untargeted metabolomic methods.

Regarding the transport route of Na^+ within the plant, onion leaves accumulated high levels of Na^+ after leaf applications. However, this was not transported from the leaf to the bulbs, arguing against a relevant translocation of Na^+ via phloem. On the other hand, soil application led to an accumulation of Na^+ in bulbs and leaves, indicating the transport of Na^+ via xylem transpiration stream. These findings explain why phloem-loaded reproductive organs and storage tissues (*e.g.* tubers and bulbs) typically show very low Na^+ concentration after exposure to saline conditions (Maathuis et al., 2014). Physiological results of this study demonstrated that the selected treatments and Na^+ accumulation did not have a significant visual effect on onion plants. Neither stress symptoms nor reduction of the fresh weight were observed in the three studied varieties (Chapter 3, figure 2 and table 3). With regard to metabolic signatures, results of the targeted and untargeted metabolomics demonstrated that, in two out of three studied varieties, there were no significant changes on the metabolite profile (Chapter 3, figure 6). For instance, the landrace Birnförmige generally did not react to Na^+ accumulation (Chapter 3, figure 6) and further compared to Red Baron and Sturon, a significant lower level of Na^+ was accumulated in the bulbs of Birnförmige (chapter 3, figure 3). On the other hand, variety Sturon was the only one that showed a mild metabolic effect of Na^+ accumulation, where increases of xylose and arabinose were observed.

According to Allen et al. (1998) and Brewster (2008), onions are next to *Vicia faba* L. one of the most salt sensitive crops. To identify metabolic responses of the salt-sensitive *Vicia faba* L. to Na^+ accumulation, a study was conducted with two contrasting varieties (Chapter 4). Variety Fuego and Scoop were selected based on their plasticity to resist salinity, which has already been demonstrated in previous experiments (Franzisky et al., 2019). After 20 days exposure of *Vicia faba* L. to 100 mM NaCL, results of the untargeted metabolomics showed a significantly effect of Na^+ accumulation in both varieties (Chapter 4, figure 3a, 4, 5). The leaf metabolic response to Na^+ accumulation was mainly characterized by increases of compatible solute proline, as well as stress-responsive sugars and free amino acids, whereas organic acids related to TCA cycles (*e.g.* maleic-, and malic acid) decreased. Overall, in comparison to the results shown in varieties of *Vicia faba* L., the aforementioned metabolic responses induced by the exposure to saline conditions were missing in onions, arguing that the extent of Na^+ accumulation in the studied onion varieties was below a critical level to hamper ion homeostasis, metabolism as well as onion quality. Our findings suggest that there are onion varieties, as well as onion landraces, with greater salt tolerance than previously reported in available literature. However, future experiments and comparisons are needed to understand the influence of other salts such as KCL, NaCL on the onion metabolism.

Although Na^+ accumulation had only a minimal effect on onion metabolome, it induced the enzymatic and non-enzymatic antioxidative system in onion bulbs. In accordance with Tausz et al. (2004), who mentioned that glutathione levels could be reduced upon exposure to stress conditions, the results of this thesis demonstrated a slightly decrease of antioxidants and glutathione accompanied by an increase of glutathione reductase levels. This indicates that the antioxidant parameters of onions are the first indicators to appear long before other metabolic signatures are revealed as a result of exposure to saline conditions. Apart from its important role in the antioxidative defense mechanism of plants, glutathione is also a fundamental part of the metabolic pathway linked to formation of onion aroma and flavor precursors ACSOs (Chang and Randle, 2004). For this reason, it is expected that changes in onion glutathione levels lead to an alteration in the concentrations of ACSOs and pungency (figure 1 of general introduction). Despite the fact that the levels of glutathione decreased, no reduction of flavor and aroma precursors ACSOs, as well as the pungency, were demonstrated in this study. Nevertheless, it should be noted that glutathione levels were only slightly reduced and it is likely that intermediaries in the synthesis of flavor precursors have compensated for the drop of glutathione levels.

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Chapter 7 – Summary

Summary

Onion cultivation has been practiced for over 4000 years and does not exist as a wild species. Over time onions have adapted to different climates, creating a wide range of varieties and landraces. Today, in modern agriculture, commercial onion breeders focus almost exclusively on conventional farming which increases the demand for certain well-known varieties and hybrids, but lowers the diversity available in the mainstream market. Additionally, a clear need for new varieties of onions is heralded by organic farming systems, where the application of pesticides and chemical fertilizers is banned. A way to preserve biodiversity and enrich the range of varieties available in organic farming systems is to re-evaluate traditional landraces and introduce their benefits to the broader public.

Onions are known for their good storability, particular aroma, as well as for their health-promoting benefits due to the rich content of non-structural carbohydrates, flavonoids and S-containing compounds. However, quality status and sensorial characteristics of onions can be influenced through preharvest and postharvest factors. Some of those factors are genotype, soil and storage conditions. Preharvest abiotic factors such as soil salinity can lead to several reduction of yield and quality aspects. Much like many other vegetable species, onions have always been classified as a salt sensitive crop. However, to date there was very little evidence to that claim and information about the impact of salinity on onion quality and physiology is lacking.

The aim of this thesis is to characterize onion landraces and compare them using targeted and untargeted metabolomics with commercial cultivars when grown under organic farming conditions. A part of this is evaluating the differences of landrace metabolite profile and the storage impact after five months of cold storage. Furthermore, this thesis discusses the effect of increased soil salinity on the metabolism and physiology of onion plants.

In chapter 2, yield and quality aspects of studied onion varieties demonstrated that landraces can achieve similar or even better results than modern varieties Sturon and Red Baron when

grown under organic farming conditions of South-West Germany. Furthermore, differences between Sturon and landraces demonstrated that parts of the aromatic and flavor properties found in landraces have been lost in modern genotypes (Chapter 3). These results indicate that the maximum potential of the modern onion varieties has not yet been reached and a further optimization of their yield and quality parameter could be attained through future breeding programs that include local landraces. Among the studied landraces, Birnförmige, Stunova and Rijnsburger 4 are the most interesting and promising candidates. For instance, Birnformige demonstrated not only good storability, but also high levels of S-containing compounds and fructans. Stunova presented a good yield stability and capacity, while Rijnsburger 4 exhibited the highest levels of amino acids suitable as precursors of aromatic substances as well as a good storability.

Despite a number of reports claiming that onions are sensitive to salinity, chapter 4 of this thesis demonstrated there is no reduction in plant growth, quality or aroma in onion plants after moderate Na^+ treatments. Nevertheless, in comparison with the landrace Birnformige, modern variety Sturon showed a slight increase of compatible solutes by Na^+ accumulation, demonstrating that the potential of certain varieties for onion production under increased soil salinity is actually much higher than previously assumed.

Chapter 8 – Zusammenfassung

Zusammenfassung

Die Speisezwiebel wird seit mehr als 4000 Jahren kultiviert. Ihr genauer Ursprungsort ist unbekannt, eine Wildform existiert nicht. Über Jahrhunderte haben sich Zwiebeln jedoch an unterschiedliche Klimazonen angepasst, es ist eine Vielzahl an Landsorten entstanden. In der modernen Landwirtschaft liegt der Fokus oft im konventionellen Anbau, dies führt zu einer Limitierung des nutzbaren Sortenspektrums und zur Extensivierung der Erhaltungszüchtung von Populationssorten. Darüber hinaus werden neue Zwiebelsorten benötigt, die an Bedingungen im ökologischen Anbausystem (ohne Anwendungen von Pestiziden und chemischen Düngemitteln) angepasst sind. Ein Weg, um die biologische Vielfalt zu erhalten und das Sortenspektrum des ökologischen Landbaus zu bereichern, besteht darin, traditionelle Landsorten neu zu bewerten und ihre besonderen Charakteristiken herauszuarbeiten.

Zwiebeln zeichnen sich durch eine gute Lagerfähigkeit, ihr besonderes Aroma und ihre gesundheitsfördernde Wirkung, bedingt durch das Vorhandensein nicht-struktureller Kohlenhydraten, Flavonoide und S-haltige Verbindungen, aus. Diese Qualitätsparameter und sensorischen Eigenschaften können jedoch durch Vor- sowie Nacherntefaktoren beeinflusst werden. Zu diesen Faktoren zählen: Genotyp, Bodenbedingungen, und Lagerbedingungen. Vorerntefaktoren, wie beispielsweise ein erhöhter Salzgehalt des Bodens, können auch zu Ertragsminderungen und Qualitätsänderungen von Zwiebeln führen. Ähnlich wie bei anderen Gemüsearten wurden Zwiebeln bisher als salzempfindliche Pflanzen eingestuft. Bisher gibt es jedoch nur sehr wenige Hinweise für diese Behauptung, des Weiteren fehlen Informationen zu Auswirkungen des Salzgehalts auf die Zwiebelqualität sowie deren Physiologie. Das Ziel dieser Arbeit ist es, durch gezielte und nicht zielgerichtete Analyse von Metaboliten (Metabolomics) Zwiebellandsorten zu charakterisieren und diese mit kommerziellen Sorten, die unter ökologischen Landbau-Bedingungen erzeugt wurden, zu vergleichen. Zudem werden in dieser Studie auch die Auswirkungen von fünf-monatiger Kühllagerung und die Veränderung des Metabolitenprofils der Landsorten bewertet. Abschließend wird in der vorliegenden Arbeit der Effekt

eines erhöhten Salzgehalts im Boden auf den Stoffwechsel und die Physiologie von Zwiebelpflanzen erörtert.

In Kapitel 2, Ergebnisse von Ertrags- und Qualitätsaspekten der untersuchten Zwiebelsorten zeigen, dass Landsorten teilweise höhere Ertragswerte erreichten, als die modernen Sorten Sturon und Red Baron wenn diese unter Bedingungen des ökologischen Landbaus in Südwestdeutschland angebaut wurden. Außerdem zeigen Unterschiede des Metabolitprofils zwischen Sturon und Landsorten, dass ein Teil der in Landsorten gefundenen Aromaeigenschaften in modernen Genotypen verloren gegangen ist (Kapitel 3). Die Ergebnisse dieser Arbeit zeigen, dass das maximale Potenzial der modernen Zwiebelsorten noch nicht erreicht wurde und eine Optimierung ihrer Ertrags- und Qualitätsparameter durch Züchtungsprogramme unter Einbeziehung der Landsorten noch verbessert werden könnte. Interessante Landsorten sind z. B. Birnförmige, Stunova und Rijnsburger 4. Die Birnförmige hatte z. B. hohe Konzentrationen an S-haltigen Verbindungen und Fructanen. Die Sorte Stunova hatte einen guten Ertrag und Rijnsburger 4 hatte die höchsten Konzentrationen solcher Aminosäuren, die als Vorstufen aromatischer Substanzen dienen, sowie auch eine gute Lagerfähigkeit.

Trotz einiger Studien, die von einer hohen Empfindlichkeit der Zwiebeln gegenüber erhöhtem Salzgehalt in Böden berichten, zeigen die hier vorgelegten Ergebnisse in Kapitel 4, dass nach einer moderaten Na^+-Behandlung keine Verringerung des Pflanzenwachstums erfolgte und dass auch die Qualität der Zwiebeln erhalten blieb. Lediglich zeigte die moderne Sorte Sturon, im Vergleich zur Landessorte Birnförmige eine leichte Erhöhung von kompatiblen Soluten durch Na^+-Akkumulation. Die Ergebnisse zeigen das das Potenzial für die Zwiebelproduktion unter erhöhter Bodensalinität tatsächlich viel höher als bisher angenommen ist.

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List of publications

Publications of the present cumulative doctoral thesis have already been published, are accepted for publication or have the status “submitted”:

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Romo-Pérez M.L., Weinert C.H., Häußler M., Egert B., Frechen M.A., Trierweiler B., Kulling S.E., Zörb C. (2019) Metabolite profiling of onion landraces and the cold storage effect. Plant Physiology and Biochemistry 146, 428 – 437. https://doi.org/10.1016/j.plaphy.2019.11.007

Romo-Pérez M.L., Weinert C.H., Egert E., Franzisky B.L., Kulling S.E., Zörb C. (2021) Sodium accumulation has minimal effect on the metabolome profile of onion bulbs. Plant Science, status “Under Review”.

Franzisky B.L., Geilfus C.M., **Romo-Pérez M.L.**, Fehrle I., Erban A., Kopka J., Zörb C. (2020) Acclimatisation of guard cell metabolism to long-term salinity. Plant, Cell & Environment, status: “accepted”. https://doi.org/10.1111/pce.13964.

List of conferences

Romo-Pérez M.L., Merkt N., Zörb C., (2017). Poster: Evaluation of West-European onion landraces with highest quality potential for organic farming. Deutsche Gesellschaft für Pflanzenernährung (DGP), 60th Conference. University of Giessen, Giessen, Germany.

Romo-Pérez M.L., Merkt N, Zikeli S., Zörb C. (2018). Speech: Quality aspects in open-pollinated onion varieties from Western Europe (Qualitätsaspekte in samenfesten Zwiebelsorten von Ersteuropa). Deutsche Gesellschaft für Qualitätsforschung (DGQ), 52th Conference. University of Giessen, Giessen, Germany.

Romo-Pérez M.L., Weinert C.H., Häußler M., Egert B., Frechen M.A., Trierweiler B., Kulling S.E., Zörb C. (2018). Poster: Variety comparison and storage-related changes in the metabolite profile of open-pollinated onions. Deutsche Gesellschaft für Pflanzenernährung (DGP), 61th Conference. Hochschule Osnabrück, Osnabrück, Germany.

Romo-Pérez M.L., Weinert C.H., Häußler M., Egert B., Frechen M.A., Trierweiler B., Kulling S.E., Zörb C. (2019). Poster: Cold storage impact on the metabolome of open-polinated onion varieties. Deutsche Gesellschaft für Qualitätsforschung (DGQ), 53th Conference and 2nd Plant Spectroscopy Conference (IPSC). Free University of Berlin, Berlin, Germany.

Romo-Pérez M.L., Geilfus C.M., Zörb C. (2018). Poster: Effect of sodium sulfate foliar spray on antioxidants and glutathione levels in onions. Deutsche Gesellschaft für Pflanzenernährung (DGP), 62th Conference. Humboldt University, Berlin, Germany.

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Maria Luisa ROMO PÉREZ
Birth date:	18.07.1986
Birth place:	Guayaquil, Ecuador
E-mail:	m.romoperez@uni-hohenheim.de

EDUCATION

SCHOOL:

05.92 – 01.98	Primary school, “Escuela particular Santa Ana”. Guayaquil-Ecuador
05.98 – 01.04	Highschool, “Colegio bilingüe Santa Catalina”. Guayaquil-Ecuador

UNIVERSITY

05.06 – 10.07	German language school “Ökumenisches Zentrum Deutschkolleg” TestDaf and DSH-II certificates
10.08 – 06.13	University of Hohenheim, Bachelor of Science (B.Sc.) Agricultural Sciences. Major: Plant Sciences Thesis: Visualization and quantification of environmental variations in a greenhouse (Grade: 1.0)
04.13 – 11.15	University of Hohenheim, Master of Science (M.Sc.) Agricultural biology. Major: Agricultural biotechnology Thesis: Quality and yield of onion landraces under organic farming conditions (Grade: 1.3)
Since 07.16	University of Hohenheim, Doctorate agricultural sciences (Dr.agr.sc.) Department Quality of Plant Products Doctoral-Thesis: Preharvest and Postharvest factors affecting the quality profile of onion landraces

WORK EXPERIENCE

10.10 – 04.11	Student assistant Department of Plant Breeding, University of Hohenheim Stuttgart-Germany
04.12 – 06.12	Intern Department of Plant Breeding, University of Hohenheim Stuttgart-Germany
Since 07.16	Research assistant, Department of Quality of Plant Products, University of Hohenheim Stuttgart-Germany
05.18 – 04.20	Research assistant, Center for Organic Farming (ZÖLUH), University of Hohenheim Stuttgart-Germany

SCHOLARSHIPS AND GRANTS

SCHOLARSHIP:	Doctoral scholarship. University of Hohenheim, Faculty of Agricultural Sciences (07.2016 – 06.2018)

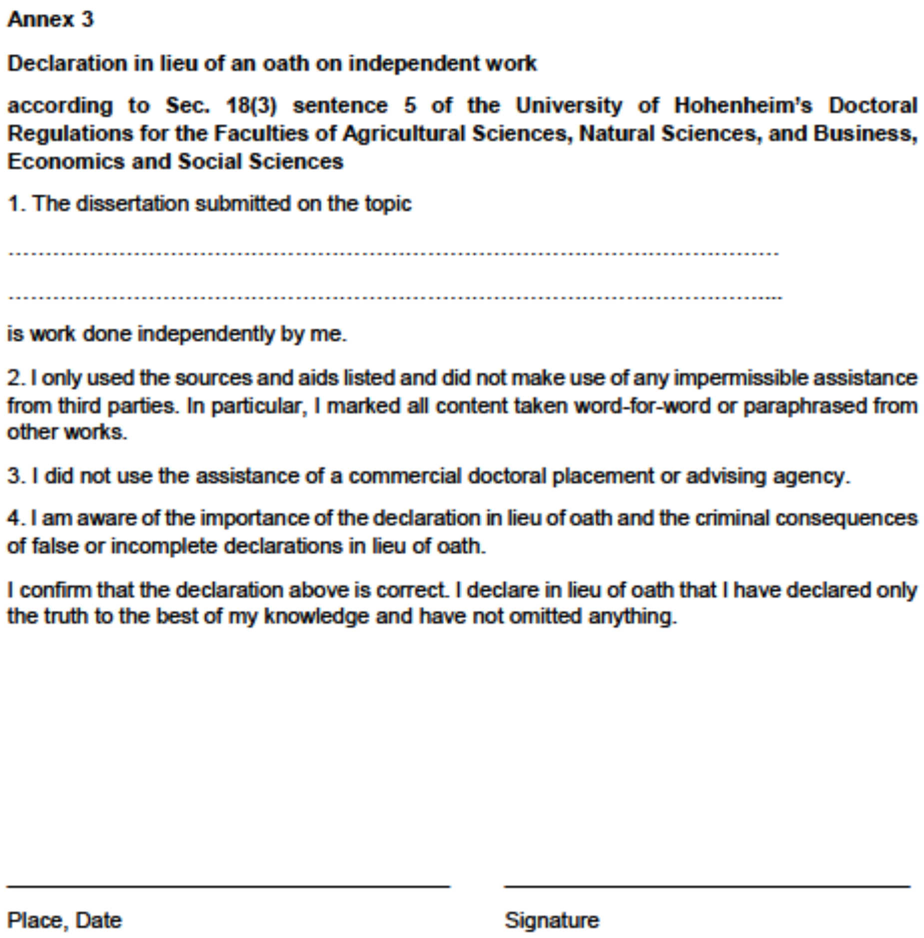
OTHER SKILLS

LANGUAGES:	Spanish: native language German: fluent English: fluent
PROGRAMMING LANGUAGES	
	LaTeX R-Program SAS

Stuttgart, ________________________

Signature ________________________

Declaration



Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

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is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Place, Date	Signature