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Tocopherol and tocotrienol accumulation during development of caryopses from barley (*Hordeum vulgare* L.)

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Abstract

Tocotrienols are lipophilic antioxidants belonging to the tocochromanols, better known as vitamin E. Although present in cereal grains in high quantities not much is known about their function in plants. In a detailed study the temporal and spatial accumulation of tocotrienols and tocopherols during grain development in two barley cultivars was analyzed. Tocochromanols and lipids accumulated in parallel until 80% of the final dry weight of the kernels was reached. Later on the tocochromanol content did not change while the lipid content decreased. Generally, only about 13% of the tocochromanols were found in the germ fraction, whereas the pericarp fraction contained about 50% and the endosperm fraction about 37% of the tocochromanols. Altogether, about 85% of the tocochromanols were tocotrienols in both cultivars. In case of the tocopherols about 80% were found in the germ fraction and the remaining 20% in the pericarp fraction. Tocotrienols were almost equally present in the pericarp and the endosperm fraction. Individual forms of tocopherols and tocotrienols accumulated with different kinetics during barley grain development. The differences in distribution and accumulation indicate different functions of the individual tocochromanols during grain development.

Keywords: *Hordeum vulgare* L.; Poaceae; Barley; High performance liquid chromatography; Tocopherols; Tocotrienols; Caryopsis

1. Introduction

Tocochromanols, better known as vitamin E, have well known antioxidative properties and are important lipophilic quenchers of reactive oxygen species and lipid radicals (Kamal-Eldin and Appelqvist, 1996). The tocochromanols consist of a chromanol ring system and a polyprenyl side chain which is saturated in tocopherols and threefold unsaturated in tocotrienols (Munné-Bosch and Alegre, 2002). The number and the position of methyl groups at the chromanol ring system defines the δ-, γ- or β-, and α-form (Kamal-Eldin and Appelqvist, 1996).

In nearly all green plant tissues α-tocopherol is the major vitamin E form (Fryer, 1992; Hess, 1993). Due to its dominant occurrence and its higher biological activity in humans, α-tocopherol has been the main focus of research (Shintani and DellaPenna, 1998). In non-green plant parts, especially seeds and fruits, either γ tocopherol is preferentially found instead (Hess, 1993) or, as in seeds of plants from various monocot and dicot plant families, tocotrienols can be the dominant vitamin E form (Ong, 1993; Lehmann et al., 1994; Ivanov and Aitzetmüller, 1995; Falk et al., 2003). The reason for the preferential occurrence of γ-tocopherol and tocotrienols in seeds is not clear, so far. It is known that the effectiveness of the individual tocochromanols as

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antioxidants depends on the surrounding conditions when tested in vitro (Kamal-Eldin and Appelqvist, 1996). Therefore, in seeds the requirements for antioxidants might differ from leaves. On the other hand it has to be kept in mind, that possible non-antioxidant roles of the tocochromanols like cell signalling have gained attention recently (Azzi and Stocker, 2000; Munné-Bosch and Falk, 2004).

Cereal grains are a major source for tocotrienols (Grams et al., 1970; Holasová, 1997; Peterson and Wood, 1997) though they do not have a high oil content. Indeed, their oil content does not coincide with their tocochromanol content. Though oat kernels contain double the amount of oil compared to barley caryopses, their tocochromanol content is generally only half of the tocochromanol content of barley kernels (Peterson and Qureshi, 1993). However, high oil oat genotypes also have an increased tocochromanol content (Peterson and Wood, 1997). Several reports demonstrated that tocopherols are preferentially localized in the germ, whereas the tocotrienols are found in the other parts of the kernel (Morrison et al., 1982; Balz et al., 1992; Holasová et al., 1995; Holasová, 1997).

For a better understanding of the function of tocopherols and tocotrienols in cereal grains the temporal and spatial accumulation of tocochromanols during development of barley kernels was investigated. A spring (cv. 'Carina') and a winter cultivar (cv. 'Nikel') were grown in a field trial and the accumulation of lipids, protein, tocopherols, and tocotrienols in kernels was recorded. Furthermore, the accumulation of the individual tocochromanols in the germ, pericarp, and endosperm fraction was analyzed.

2. Results

2.1. Kernel development

Since environmental factors may influence the tocochromanol content of barley kernels, the climatic conditions during kernel development of the two barley cultivars were recorded (Fig. 1). Kernel ripening of the winter cultivar 'Nikel' occurred from early June until the end of July while kernel ripening of the spring cultivar 'Carina' started about one month later. Sunshine duration as an indicator of the light conditions in the field varied irregularly during the sampling period but was in sum about the same during kernel development for both barley cultivars. However, the temperature fluctuated between 8 and 15 °C from end of May until end of June. From the beginning of July until the end of the sampling air temperature increased to values between 12 and 22 °C. Overall, the mean air temperature was more than 1 °C higher during kernel ripening of the cultivar 'Carina'. Two periods of heavy rainfall were observed from 07.07.01 to 15.07.01 and 04.08.01 to 12.08.01, respectively.

To compare the developmental stages of the caryopses of both cultivars the fresh weight as well as the water, lipid, and protein contents were analyzed during kernel growth and ripening (Fig. 2). Sampling started after embryogenesis had completed and continued during the phase of seed filling (milky stage until soft dough) and maturation. Since weight and water content of the first sample of the winter cultivar 'Nikel' were very similar to the third sample of the spring cultivar 'Carina', these samples were regarded as compara-

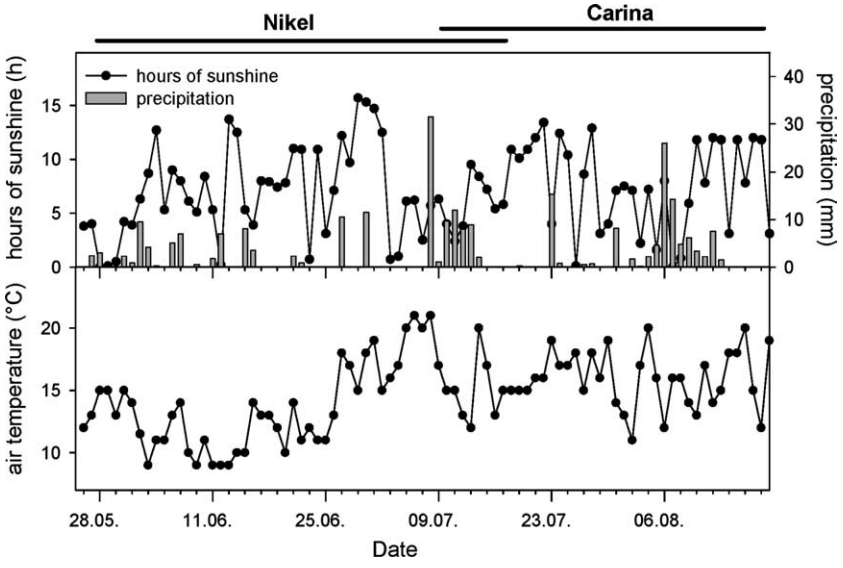

Fig. 1. Climatic conditions measured as hours of sunshine, rain precipitation, and mean air temperature during the field trial in summer 2001 near Kiel, Germany. Periods of kernel development of the barley (*Hordeum vulgare* L.) winter cultivar 'Nikel' and spring cultivar 'Carina' are indicated by black bars at the top of the figure.

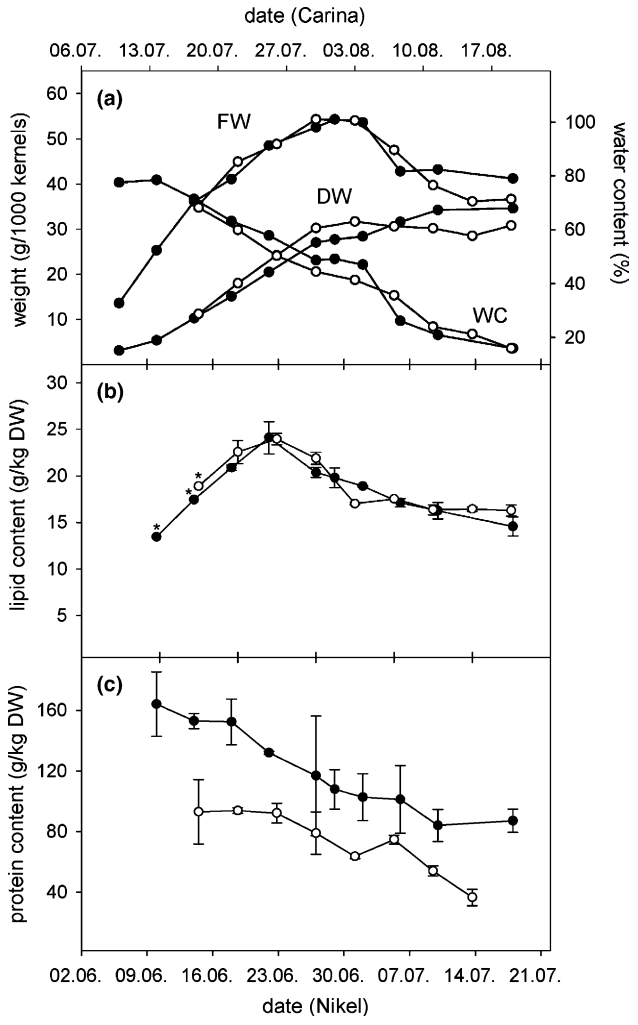

Fig. 2. Comparison of the growth and ripening of caryopsis of the cultivars 'Nikel' (•) and 'Carina' (○). (a) Fresh weight (FW), dry weight (DW), and water content of the caryopses, (b) lipid content and (c) protein content of the kernels during development are given. Sampling dates of the cultivar 'Carina' are placed on top of the figure. Corresponding sampling dates of the cultivar 'Nikel' are indicated at the bottom of the figure. The weight and water content of the first sampling date of the spring cultivar 'Carina' was very similar to those of the third sampling date of the winter cultivar 'Nikel' and thus regarded as comparable development stages. Single measurements are indicated by an asterisk.

ble developmental stages. Fresh weight increased in both cultivars to a maximum value of about 52 g/1000 kernels at the soft dough stage and then declined to values of about 40 g/1000 kernels. A maximum dry weight of about 31 g/1000 kernels and 34 g/1000 kernels was obtained in the cultivars 'Carina' and 'Nikel', respectively (Fig. 2(a)). The water content declined steadily in caryopses of both cultivars from about 80% at the beginning of development to about 18% at the last time of sampling.

The changes in lipid content during development of the caryopses were similar in both cultivars (Fig. 2(b)). The lipid content increased during the first weeks of development to a maximum of about 24 g/kg DW at late milky stage of the grains which occurred one week before the maximum weight of the kernels was reached. During further development the lipid content decreased steadily to about 16 g/kg DW at harvest time. The protein content of caryopses on a dry weight basis decreased during kernel development in both cultivars (Fig. 2(c)). In the cultivar 'Carina' the protein content of the kernels was regularly about 50 g/kg DW lower compared to the kernels from 'Nikel'.

Altogether only minor differences in kernel development were observed between both barley cultivars, thus indicating that differences in climatic conditions only had a minor influence on kernel development in this field trial.

2.2. Tocochromanol accumulation during kernel development

The accumulation of tocochromanols on a dry weight basis was measured for both cultivars during development of the caryopses (Fig. 3). Generally, the maximum tocochromanol and lipid level was reached early at the milky stage of the grains before the maximum dry weight of the caryopsis was reached (Fig. 2(a)). While the tocochromanol level slightly decreased afterwards in 'Nikel', no decrease was observed in 'Carina' until kernel maturation. About 85% of the tocochromanols were tocotrienols in both cultivars. The reason for the rapid transient decrease in tocochromanols in 'Nikel' at the 27.06.2001 (Fig. 3(a)) is unknown and could not be related to any parameter taken. While the protein content was higher in the cultivar 'Nikel' the final tocochromanol content was almost 70% higher in the spring cultivar 'Carina'.

The higher tocochromanol content in the cultivar 'Carina' could either result from a generally higher content of tocopherols and tocotrienols, respectively, or from the accumulation of specific tocochromanols. A detailed analysis revealed that the patterns of accumulation differed among the individual tocochromanols (Fig. 4). The α-form was dominant among the tocopherols and tocotrienols in both cultivars. Among the tocotrienols β- and γ-tocotrienol were almost equally present whereas among the tocopherols only γ-tocopherol was detectable in considerable amounts. The level of α-tocotrienol increased steadily in 'Carina' almost until the last sampling whereas in 'Nikel' the content did not increase after the maximum was reached at the late milky stage. Furthermore, in 'Carina' the proportion of δ-tocotrienol was at least double as high and the γ-tocopherol concentration at least threefold higher as in the cultivar 'Nikel'. The δ-tocotrienol content increased transiently before the maximum tocotrienol content was reached only in the cultivar 'Carina'.

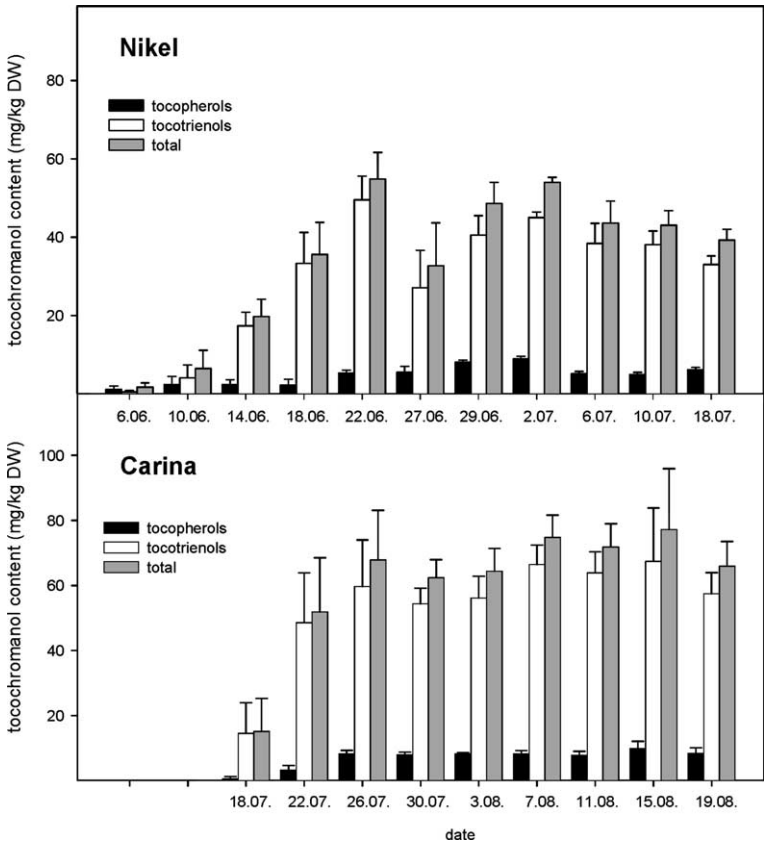

Fig. 3. Tocochromanol accumulation during kernel development of the barley cultivars 'Carina' and 'Nikel'. Total tocochromanol contents were calculated as sum of all individual tocopherols and tocotrienols determined by HPLC analysis. The sampling dates of both cultivars were arranged as in Fig. 2 to represent comparable developmental stages.

2.3. Tissue-specific distribution of tocochromanols

To better understand the differences in the accumulation of the individual tocochromanols during kernel development, the distribution of tocochromanols in different parts of the kernel was analyzed. For this purpose kernels of the cultivar 'Carina' were hand-dissected starting at the late milky stage. Three fractions were obtained from the kernels, the germ fraction consisting of the embryo and the scutellum, the pericarp fraction, and the endosperm fraction. In early milky stages (before the 18.07.2001) the endosperm could easily be squeezed out, but the peripheral aleurone cell layer of the endosperm was bound to the pericarp fraction. When a water content of about 50% in the kernel was reached the aleurone layer was present in the endosperm fraction, but with a water content below 50% (after the 26.07.2001) the aleurone layer remained attached to the pericarp again. Therefore, after the soft dough stage (30.07.2001–3.08.2001) only the germ fraction was analyzed further on. Due to some adherence of endosperm at later stages the germ fraction had to be regarded as partly contaminated.

Striking differences in the composition and pattern of tocochromanol accumulation in the three fractions were observed (Fig. 5). In the germ fraction almost exclusively tocopherols were accumulating. The pericarp fraction contained only a small amount of tocopherols and mainly tocotrienols while the endosperm fraction contained almost exclusively tocotrienols.

In the germ fraction the γ-tocopherol content reached its final level early during development at the same time when the highest lipid content was reached (Fig. 2) and did not change further during ripening. In contrast to γ-tocopherol, the content of α-tocopherol increased continuously until kernel maturity, reaching a more than fourfold higher concentration than the other tocochromanols in all kernel fractions (Fig. 5). The other tocopherols were present in the germ fraction only at very low levels (<5 mg/kg FW). The occurrence of tocotrienols in the germ fraction at later stages was most likely caused by contamination with adjacent endosperm.

In the pericarp fraction α-, β-, and γ-tocotrienols were present at similar levels in early stages of kernel development until the 22.07.2001 (late milky stage). Later on the β-tocotrienol content ceased to rise whereas the α- and γ-tocotrienol contents continued to increase. However, α-tocotrienol began to accumulate at higher rates resulting in an almost twofold higher level at the end of the sampling period when the kernels reached

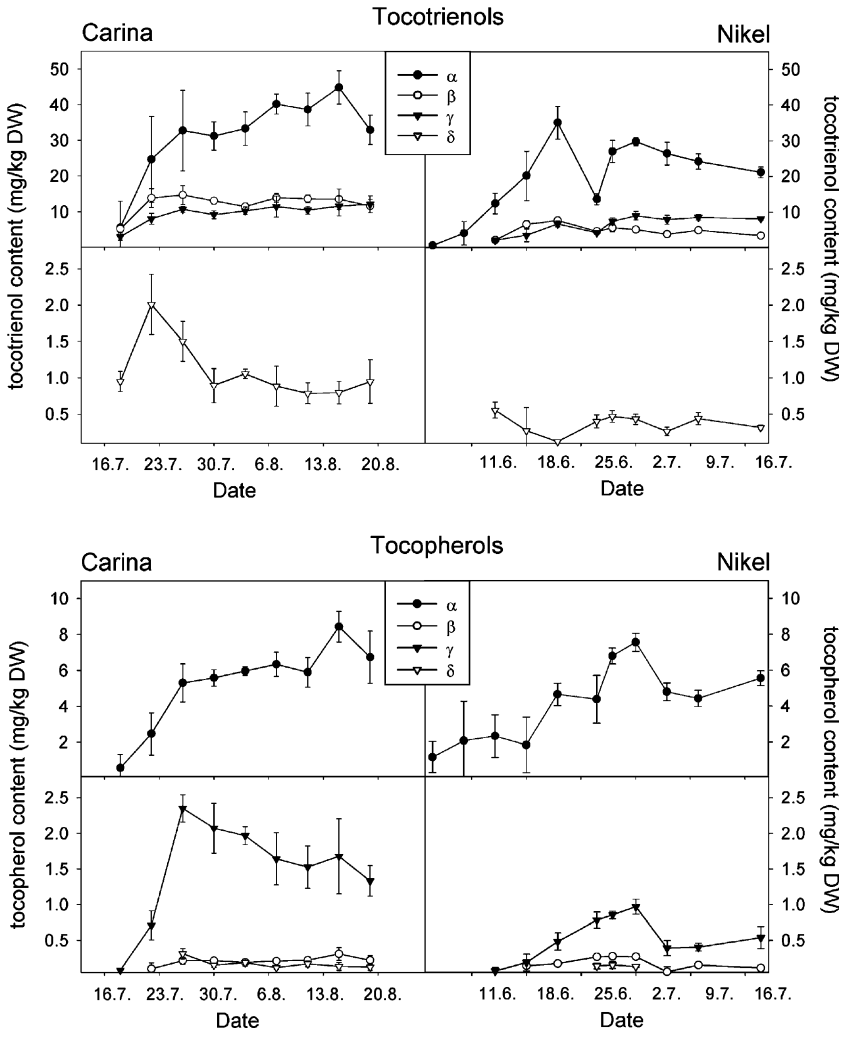

Fig. 4. Accumulation of individual tocopherols and tocotrienols during kernel development of the barley cultivars 'Carina' and 'Nikel'. Contents of individual tocochromanols were determined by HPLC analysis. For details, see Section 4.

their maximum fresh weight (Fig. 2). δ-Tocotrienol was present in the pericarp fraction at a low level throughout the sampling period. Tocopherols (mainly α-tocopherol) became detectable when the β-tocotrienol concentration began to decrease in the pericarp fraction.

In the endosperm fraction α-tocotrienol was dominant followed by β- and γ-tocotrienol. All tocotrienols were present at almost constant levels throughout the sampling period. The tocotrienol level was only about half of that which was found in the pericarp fraction at the end of the sampling period.

The results show that tocopherols and tocotrienols were distributed in a tissue specific manner in barley kernels, and individual tocochromanols were accumulating with different kinetics during barley grain development.

The contribution of each fraction to the total tocochromanol content of the whole kernel was calculated for both cultivars. Calculation was performed at a stage when the maximum fresh weight of the kernels and tocochromanol concentration was reached (soft dough stage) and water content was just below 50% (data from 2.07.2001 and 3.08.2001 for 'Nikel' and 'Carina', respectively). The pericarp fraction contributed 25.8 ± 0.9/27.9 ± 1.9% ('Carina'/'Nikel') on average, the endosperm fraction contributed 58.6 ± 2.2/59.5 ± 1.7% and the germ fraction represented 8.5 ± 1.5/6.7 ± 0.1% of the kernel. For statistical reasons the sum of mean values of all fractions is not 100%.

Generally, in both cultivars the tocopherol content was more than three times higher in the germ fraction compared to the pericarp fraction accounting for about 80% of the total tocopherols (Fig. 6). The remaining tocopherols were present in the pericarp fraction with only minor amounts of tocopherol detectable in the endosperm fraction. β- and δ-Tocopherol were detectable in the germ exclusively. Differences in the tocopherol contents between both cultivars were due

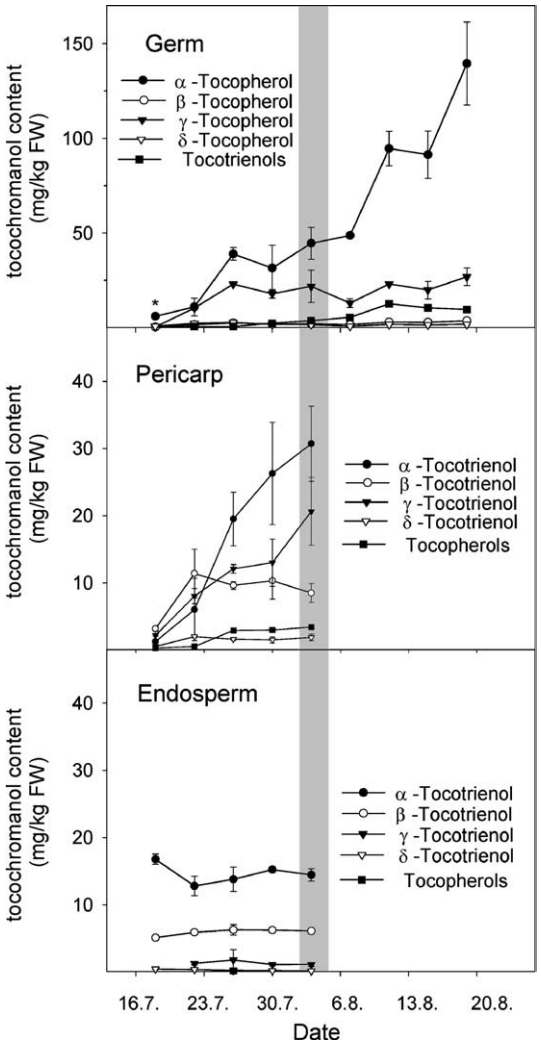

Fig. 5. Concentration of individual tocopherols and tocotrienols in hand-dissected barley fractions of cultivar 'Carina' during kernel development. Tocochromanol contents were measured by HPLC analysis. The sample taken for a detail analysis of absolute amounts of individual tocochromanols (see Fig. 6) is marked by shading. Single measurements are indicated by an asterisk. For details, see Section 4.

to the α- and γ-tocopherol level of the germ fraction being almost twice as high in the cultivar 'Carina' compared to 'Nikel'. On the other hand, tocotrienols were hardly detectable in the germ fraction (below 1% of total tocotrienols) but almost equally present in the pericarp and endosperm fraction with about 1138 ± 88 and 930 ± 28 μg/1000 kernels in the cultivars 'Carina' and 680 ± 71 and 502 ± 74 μg/1000 kernels in 'Nikel', respectively (Fig. 6). γ-Tocotrienol was preferentially present in the pericarp fraction in both cultivars. From the data presented in Fig. 6 it was calculated that about 13–14% of the tocochromanols are present in the germ fraction, whereas the pericarp fraction contributed about 49–51% and the endosperm fraction 35–38% of the tocochromanols of the barley kernel at that developmental stage.

3. Discussion

A spring and a winter cultivar of barley were grown in the fields for an analysis of the accumulation of tocochromanols during kernel development. The pattern of grain development was the same for both cultivars and followed the classical scheme (Kirby and Appleyard, 1984). While the lipid content was almost identical in both cultivars, protein content was lower in case of the cultivar 'Carina' which however had a much higher total tocochromanol content. The higher tocochromanol content could either be caused by the genotype or by environmental effects. Indeed, significant genotype differences among several oat and barley varieties with regard to their tocochromanol content were reported, but differences due to growth location were significant for oat only (Peterson and Qureshi, 1993). Although environmental effects can not be ruled out, it is unlikely that the differences observed between cv. 'Carina' and cv. 'Nikel' are due to the minor differences in temperature which occurred in the fields during the sampling period.

Though lipid content and tocochromanol accumulation coincided during early kernel development these two parameters did not correlate at later stages of development (Figs. 2(b) and 3). At the late stage of grain development the lipid content decreased whereas the tocochromanol content did not. One possible major function of the lipophilic tocochromanols in seeds may be the protection of stored lipids from oxidation. However, attempts to verify a positive correlation between the accumulation of tocopherol and tocotrienol and that of lipids or polyunsaturated fatty acids in seeds gave inconsistent results (Kamal-Eldin and Andersson, 1997; Dolde et al., 1999; Goffman and Becker, 2002). An explanation for the discrepancies might be the different subcellular location of tocochromanols and lipid bodies in plant cells. Generally, storage lipids are found in oil bodies derived from the endoplasmatic reticulum (Huang, 1996), whereas tocochromanols are synthesized and accumulate in plastids (Schultz et al., 1991). Internal or external factors may affect the accumulation of both, tocochromanols and lipids, resulting in a spurious correlation during early kernel development (Dolde et al., 1999).

The pattern of tocochromanol accumulation in the kernels differed individually. Tocotrienols were the dominant tocochromanol type in kernels of both cultivars and α-tocotrienol was present at a two- to threefold higher concentration than all the other tocotrienols (Fig. 4). Most tocotrienol forms were present at their highest concentration during grain filling before the maximum weight of the kernels was reached. Both cultivars mainly differed in their final α-tocotrienol content and in the pattern of δ-tocotrienol accumulation. The δ-tocotrienol content increased transiently in kernels of

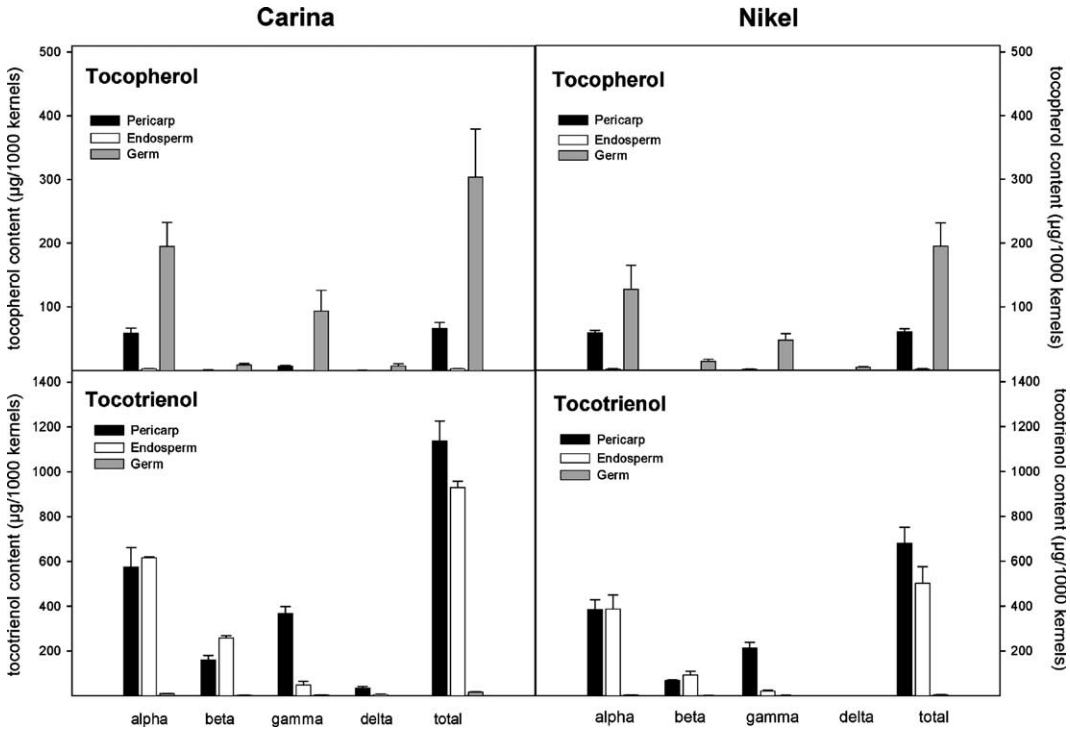

Fig. 6. Amounts of individual tocopherols and tocotrienols in hand-dissected barley fractions at the soft dough stage when the maximum fresh and dry weight was reached. Samples were taken at the 2.07.2001 for the cv. 'Nikel' and at the 3.08.2001 for the cv. 'Carina' (see Fig. 2). Ten kernels were fractionated and extracted for the analysis of the pericarp and endosperm fraction, respectively, whereas twenty kernels were analyzed for the germ fraction. Data are based on three independent measurements. For details, see Section 4.

the cultivar 'Carina' before the maximum tocotrienol content was reached. Since δ-tocotrienol is a precursor of β-tocotrienol and not of the other tocotrienols (Eckhardt, 2003; Dörmann, 2003), the transient rise in the δ-tocotrienol accumulation could be connected to the higher β-tocotrienol level in kernels from 'Carina' compared to 'Nikel'. The cultivars differed also in the pattern of γ-tocopherol accumulation. Since γ-tocopherol is a precursor of α-tocopherol differences in γ-tocopherol accumulation could be responsible for the differences in final α-tocopherol levels in the kernels of the two cultivars, too.

The functions of tocopherols and tocotrienols in cereal kernels is certainly related to the distribution of these compounds in the kernel. Tocopherols were mainly present in the germ fraction whereas the tocotrienols were present in the pericarp and endosperm fraction, respectively. Similar observations were made in corn, barley, oat, and wheat, (Grams et al., 1970; Morrison et al., 1982; Balz et al., 1992; Peterson, 1995). Tocotrienols are present in the endosperm at a rather constant level from the late milky stage until the soft dough stage indicating a correlation between starch accumulation and tocotrienol accumulation. However, tocotrienol function seems not to be restricted to the accumulation of starch since they are also found in palm oil which mainly derives from the mesocarp of the fruit (Ebong et al., 1999), in rubber latex of *Hevea brasiliensis* (Ong, 1993), or the germ of chilean hazelnut (*Gevuina avellana* Mol.) (Bertoli et al., 1998). Due to their lipophilic properties it is likely that tocotrienols are part of the lipid fraction of starch granules which in barley constitute almost 20% of the total kernel lipids in barley and which account for about 1% of the total kernel weight (Kaukovirta-Norja et al., 1997). Based on our data the contribution of the aleurone layer to the tocotrienol content of the kernels cannot be determined. While at the early stage of development the aleurone layer was present in the endosperm fraction, it was attached to the pericarp and endosperm fraction later on. The preferential synthesis of β-tocotrienol at the late milky stage suggests a special function for this tocochromanol in the pericarp fraction. As mentioned before, β-tocopherol and β-tocotrienol are not usual intermediates and are not present in all plants and all plant parts.

The presence of tocotrienols and individual tocopherols in seeds suggests that they fulfil specific functions which differ from the function of tocopherols in leaves. This would implicate that such seeds have a demand for specific antioxidants during desiccation. In orthodox seeds including cereal grains significant changes of the redox status occur during development and maturation (Finnie et al., 2002; De Gara et al., 2003). Catalase, ascorbate content, ascorbate peroxidase, and other

redox enzymes were shown to decrease during the desiccation phase. In contrast, the ascorbate system is little affected in recalcitrant seeds (De Tullio and Arrigoni, 2003). The presence or absence of ascorbate might be important for the regulation of the ethylene, gibberellins, and abscisic acid synthesis during seed development. A central enzyme of the tocochromanol and plastoquinone pathway, the 4-hydroxyphenylpyruvate dioxygenase, is also depending on ascorbate (Garcia et al., 1999). This might be one reason for the early accumulation of tocochromanol during barley kernel development. On the other hand this suggests that the tocochromanols which are enriched at later stages of kernel development are rather stable. Possibly the accumulation of tocotrienols and specific tocopherols is related to an increased stability under the conditions of the dry seed.

With the presented data about the temporal and spatial accumulation of tocopherols and tocotrienols during barley grain development in mind it now becomes clear that the different tocochromanols most likely fulfill different functions in the barley kernel. It certainly is of importance that tocopherols are accumulating in that part of the grain which survives and builds up the new plant, while the tocotrienols are located in the endosperm and pericarp, which finally die before and during germination, respectively. It will be important to identify the exact localization of the tocotrienols in the cells of the pericarp and endosperm to better understand what makes the tocotrienols special. If the tocotrienols are present as antioxidants it will be necessary to identify what they are supposed to protect.

4. Experimental

4.1. Plant material

A winter (cv. Nikel) and a spring cultivar (cv. Carina) of barley (*Hordeum vulgare* L.) were grown in the fields of the 'Norddeutsche Pflanzenzucht Hans-Georg Lembke KG' at Hohenlieth, near Kiel, Germany, during winter 2000 to summer 2001. Fertilization and plant protection were performed to ensure optimal plant growth. Temperature and rainfall data were measured in the near vicinity of the field while data of sunshine duration were obtained from the meteorological station of the 'Deutscher Wetterdienst' in Kiel, Germany.

Sampling started after embryogenesis of the kernels was completed and covered the phases of seed filling (milky stage, about 10–12 days after anthesis (DAA), until soft dough) and maturation (about 48 DAA) (Kirby and Appleyard, 1984). Whole plants were sampled about every fourth day at 2 pm, were transferred to the laboratory within one hour after collection, and processed immediately. Generally, kernels were sampled from 10 to 20 plants and pooled for further analysis. Replications were done with different pools.

Fresh and dry weights of the dehulled caryopses were determined by weighing before and after drying for 72 h at 80 °C. Dehulled kernels of different developmental stages were dissected by hand into a germ (including the scutellum), pericarp (including parts of the aleurone layer in early and late stages), and endosperm fraction. Samples were immediately frozen in liquid nitrogen after dissection and stored at −70 °C until analysis.

4.2. Analysis of lipid and protein content

The lipid content was determined after grinding and extracting the lipophilic contents with light petroleum ether (boiling point 40–60 °C) for 24 h. The extract was evaporated under nitrogen atmosphere and the weight of the residue was determined. Reextractions were performed until the weight of the residue was constant. When not indicated otherwise, samples were prepared and analyzed twice.

The protein content was measured by using the Bio-Rad DC Protein Assay (BioRad, Munich, Germany) based on the protein determination by Lowry et al. (1951). The protein content was determined twice for each sample.

4.3. Analysis of tocochromanols

The eight tocochromanol forms (α-, β-, γ-, and δ-tocopherol/-tocotrienol) were analyzed as described by Falk et al. (2003). Briefly, 100–200 mg of plant material were ground with liquid nitrogen and extracted with twice the volume of *n*-heptane/2-propanol (99.5 + 0.5) at −20 °C for at least 24 h, which proofed to be sufficient for a complete extraction of tocochromanols (data not shown). After centrifugation at 15,000 *g* the clear supernatant was taken for analysis by isocratic HPLC.

20 μl of the tocochromanol sample were chromatographically analyzed using a LiChrosphere® Si 100 (5 μm) column (10 × 250 mm) with *n*-heptane/2-propanol (99.5 + 0.5) as elutant at a flow rate of 1.0 ml/min. Tocochromanols were detected and quantified using a fluorescence detector (RF10AXL, Shimadzu, Japan) set to $\lambda_{excitation}$ = 290 nm and $\lambda_{emission}$ = 328 nm. To calibrate the system and verify the identity of individual peaks tocopherol and tocotrienol standards purchased from Merck (Darmstadt, Germany) were used.

The tocochromanol content of whole kernels was measured at least five times and those of kernel subfractions at least twice when not indicated otherwise. The total tocochromanol content was calculated by adding up all individual tocopherol and tocotrienol contents in the subfractions.

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