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A developmental profile of tocol accumulation in oat seeds

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ABSTRACT

Oats are a good source of many vitamins and minerals, including compounds that collectively constitute vitamin E, the tocols. Tocols are comprised of two subgroups, tocopherols and tocotrienols. Due to their potent antioxidant properties, attention has been given to the health benefits of tocols in oats. However, little is known about developmental aspects of their accumulation. Moreover, aside from their role in protecting membranes from oxidative damage, their possible physiological roles *in planta* have largely gone uninvestigated. In this study, we quantified tocol accumulation at five time points during oat seed development, and also compared tocol concentration and composition in the whole seed both to endosperm and embryos at a late stage of seed development. The temporal pattern of accumulation of the tocol subgroups was different. Both tocotrienols and tocopherols steadily increased over the course of seed development; however, tocotrienol progression was sigmoidal and tocopherol linear. By the end of seed development, tocotrienols were the most abundant tocol both in whole seed and in seed with embryos removed. In contrast, embryos contained higher concentrations of tocopherols than tocotrienols late in seed development. Differences in both temporal and spatial distribution during seed development suggest distinct roles for tocotrienols and tocopherols.

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1. Introduction

Oat (*Avena sativa* L.) seeds contain a wide spectrum of bioactive compounds such as avenanthramides, tocopherols (T), and tocotrienols (T3) (recently reviewed by Behall and Hallfrisch, 2011; Mene-Saffrane and DellaPenna, 2010). The latter two compounds collectively are called tocols or tocochromanols, and represent vitamin E. They are powerful antioxidants synthesized exclusively in photosynthetic organisms, and not only are they essential for humans but they also have been associated with numerous other positive health benefits (reviewed by Galli and Azzi, 2010). Tocols are naturally present in four different forms (α, β, γ, and δ), that are distinguished by the number and position of methyl groups in the chromanol ring.

The vitamin E activity varies not only among tocol forms but also with the particular conditions of the cellular environment *in vivo*, and with experimental conditions *in vitro* (Kamal-Eldin and Appelqvist, 1996; Yoshida et al., 2003). The α-homologues and the β-tocopherol appear to exert the greatest liposomal membrane antioxidant activity (Yoshida et al., 2003). However, α-tocopherol has higher bioavailability since the other forms have less affinity for the α-tocopherol transfer protein (Hosomi et al., 1997) and the concentrations of all tocol forms in plasma are determined by this affinity (Dormann, 2003; Traber et al., 1994). This, coupled with the observation that the different homologues are not interconvertible in humans, makes the form ingested crucial, as recognized by the US National Academy of Sciences which indicates that only α-tocopherol should be regarded as vitamin E (FNB, 2000).

In plants, tocols are synthesized in plastids and chloroplasts of seeds and leaves, respectively, and their main role is to serve as a fat-soluble antioxidant, preventing lipid oxidative damage. Despite their importance, the pattern of tocol accumulation in seeds, including those of oats, is poorly understood. The objective of this study was to profile tocol accumulation during oat seed development and to compare tocol profiles in different parts of the seed. These findings advance our understanding of the regulation of tocol synthesis and accumulation in developing oat seeds.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of oat (*A. sativa* L.) cultivar Ogle-C (derived from a single plant reselection from the cultivar 'Ogle') were germinated in trays filled with potting mix in a growth chamber in short-day conditions

Abbreviations: daa, Days after anthesis; MS, Mature seed; T, Tocopherols; T3, Tocotrienols; WS, Whole seed.

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(11 h light at 20 °C, 13 h dark at 16 °C) to promote vegetative growth. After 4 weeks, developing plants were transplanted to cones containing two parts soil-one part potting mix. Four independent trays of 20 cones constituted biological replicates. Plants were grown to maturity in long-day photoperiod conditions (16/8 h light/dark) and 21 °C day/16 °C dark temperatures.

Individual florets were tagged at the onset of anthesis, and developing dehulled seeds were collected at 7, 14, 21, and 28 days after anthesis (daa), and at maturity (approximately 35-daa). When possible (7, 14, 21 and 28-daa), embryos were excised from some seeds at the time of harvest to obtain endosperm for analysis. The 28-daa embryos were also saved for analysis. Embryos were carefully separated from the adjacent endosperm with a scalpel, with the aid of a dissecting scope. All samples were frozen in liquid nitrogen and stored in cryovials at −80 °C until used for tocol analysis. Pools of whole seeds and embryo-free seeds at each developmental stage from each replicate tray were used for tocol quantification, as were embryos from the 28-daa time point. To obtain tocol concentrations on a dry weight basis, additional seed samples for each stage were placed in an oven at 80 °C for 72 h to dry. Dry seed weight was subtracted from starting fresh weight to calculate their water content (64.9%, 54.6%, 47.7%, 36.3%, and 13.1% for 7, 14, 21, 28-daa, and MS stages, respectively).

2.2. Tocol extraction, identification and quantification

Extraction and quantification was performed on the four biological replicates by a method of Panfili et al. (2003) as adapted by Peterson et al. (2007). No technical replicates were conducted on biological replicates. Briefly, for embryo-free seed and whole seed, approx. 0.5 g samples (0.1 g for embryos) were ground in 2.0 mL stainless steel vials using three aluminum beads with a Biospec Bead Beater (Bartlesville, OK). The samples were shaken 3 × 30 s with 30 s intervals. After grinding, the vials were inverted into 15 ml BD Falcon tubes and centrifuged at 1000 g for 5 min to ensure that the ground tissue was completely expelled from the grinding tubes and into the Falcon tubes for tocol extraction, which was completed by adding 0.5 ml 10M KOH, 0.5 ml 95% ethanol, 0.5 ml 0.15M NaCl and 1.25 ml of a 60 g/L solution of pyrogallol (in ethanol) and shaken in a water bath at 70 °C for 30 min, vortexing every 10 min. The tubes were cooled on ice and an additional 3.75 ml of 0.15M NaCl was added. This suspension was extracted twice with hexane/ethyl acetate (9:1 v/v) by vortexing and centrifuging at 1000 g for 5 min and transferring the supernatant to a glass test tube. The combined organic phase was reduced to dryness in a Thermo-Savant SPD1010 speed-vac system (Asheville, NC) at 45 °C. The dried extract was resuspended in either 0.5 or 1.0 ml hexane depending on the amount of tissue available and centrifuged to remove particulates prior to analysis by HPLC. For HPLC analysis, each sample was analyzed with a Shimadzu LC-5a HPLC (Kyoto, Japan) using a 4.6 × 250 mm, 5 μm Adsorbosil silica column (Grace Co., Deerfield, IL) with an isocratic mobile phase of 2% ethyl acetate and 2% dioxane in hexane at a flow rate of 2.0 ml/min. Fluorescence detection was employed using a Shimadzu RF-10A spectrofluorometer with excitation at 295 nm and detection at 330 nm. Peaks were integrated and compared to tocol standards (Matreya LLC, Pleasant Gap, PA). Tocotrienols were quantitated using the standard curve developed for the corresponding tocopherol (Fig. 1 and Supplementary file 1).

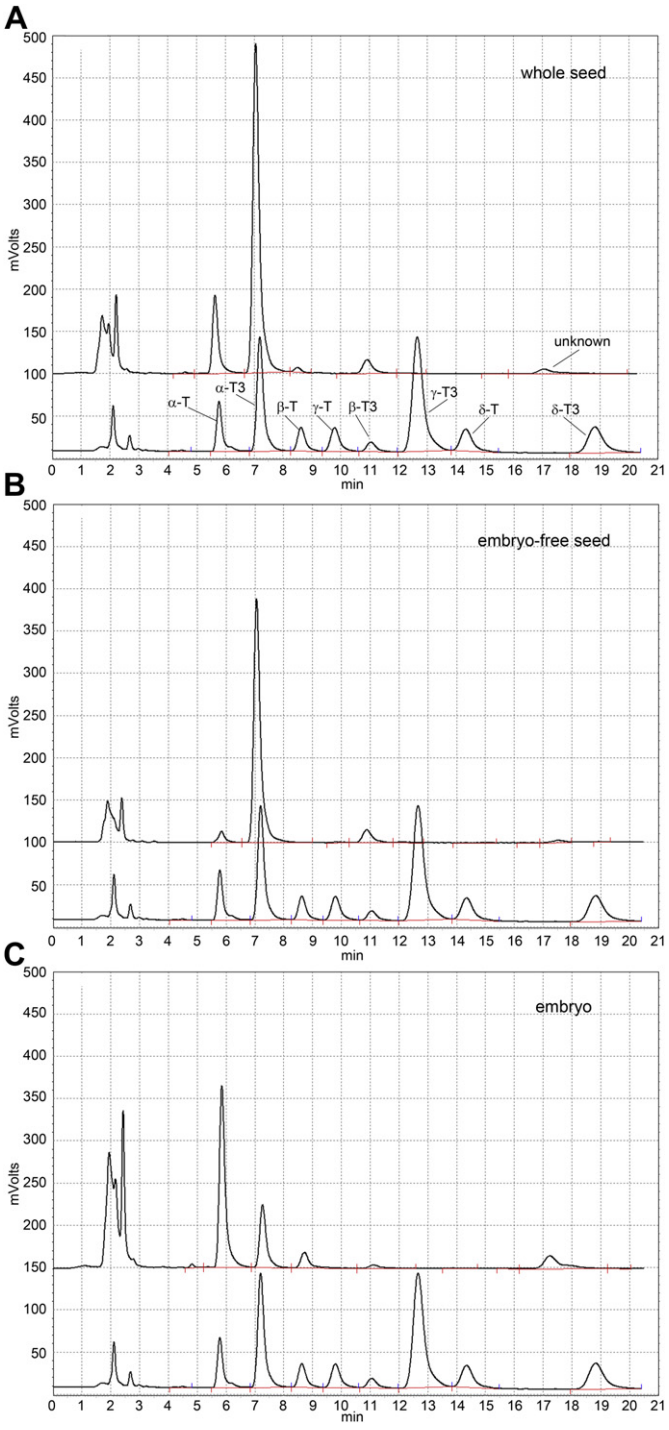

Fig. 1. Example of HPLC chromatograms showing the separation of the different tocol forms (upper lines) and reference standards (lower line) from (A) the whole seed, (B) embryo-free seed, and (C) dissected embryo. For comparison, all chromatograms correspond to the 28-daa stage. Separation was performed in a 5 μm Adsorbosil silica column with mobile phase of 2% ethyl acetate and 2% dioxane in hexane at a flow rate of 2.0 ml/min. A magnified image for minor tocols is shown in Supplementary file 1.

3. Results and discussion

For this study, plants of oat cv. Ogle were grown in controlled environmental conditions in a growth chamber. Developing whole seeds (WS) were collected at five stages: 7, 14, 21, and 28 days after anthesis (daa), and at maturity (mature seed, MS). In addition, embryo-free seeds were also obtained by cleanly excising embryos with a scalpel under a dissecting scope when feasible (7, 14, 21, and 28-daa). Thus, these embryo-free seeds comprise tissues from both endosperm and outer seed layers. Whole seeds, embryo-free

seeds, and 28-daa whole embryos were used for tocol analysis. All values are reported on a dry weight basis.

Results revealed that in WS at 7- and 14-daa tocotrienols and tocopherols were present in comparable amounts (t-test, $P > 0.05$), whereas tocotrienols predominated at the later developmental stages (t-test, $P < 0.001$) (Fig. 2A). While tocopherol and tocotrienol concentrations remained relatively constant during the first two stages, both increased in the last phases of seed development, although following different patterns. Tocopherol accumulated uniformly during the course of seed development. However, tocotrienol concentrations increased rapidly after 14-daa, reaching a maximum near maturity (46.6 μg/g at 28-daa). The pattern of tocotrienol accumulation resembled a sigmoidal curve, versus the linear progression of tocopherols (Fig. 2A). Of the individual tocols, α-tocotrienol was the most abundant at all developmental stages, followed by α-tocopherol (Fig. 2B and C). For instance, at the end of seed development (MS stage), the α-tocotrienol concentration was 42.8 ± 3.2 μg/g, followed by α-tocopherol (12.5 ± 1.0 μg/g), representing about 71% and 21% of total tocols, respectively. Small amounts of β-tocotrienol (3.0 ± 0.3 μg/g) and β-tocopherol (1.1 ± 0.1 μg/g) were also detected in the MS. Only traces (<1 μg/g) of the γ and δ forms were found, in contrast to seeds of Arabidopsis, corn and some other plants in which γ-tocopherol represents the main form (Chander at al., 2008; DellaPenna, 2005). These results are consistent with previous studies on dehulled mature whole oat seeds (Peterson and Qureshi, 1992; Peterson et al., 2007). Although we detected a higher total tocol concentration in MS than in these previous reports (60.0 ± 4.5 μg/g vs. the 19–41 μg/g), this is not surprising because genotype, environment, and extraction method have all been shown to influence tocol concentrations in oats (Peterson and Qureshi, 1992; Peterson et al., 2007), barley (Peterson and Qureshi, 1992), wheat (Hidalgo et al., 2009), and maize (Chander et al., 2008). Little is known about developmental changes in tocol profiles in seeds of other small grain cereals. As for oat, in barley tocotrienols are also the main tocol (*Hordeum vulgare* L.) (Falk et al., 2004). However, in contrast to our results, developing barley seeds rapidly accumulate tocotrienols and reach a plateau earlier in development than what we observed in oat. But tocopherols accumulated more uniformly in barley, in a manner similar to what we observed for oat. To our knowledge, no comparable temporal analyses of tocol accumulation have been performed on seeds of other plant species.

Embryo-free seed, which is mainly endosperm, represents most of the developing oat seed; its contribution to fresh seed weight ranged from 97.8% (7-daa) to 84.4% (28-daa), which explains the observation that comparable concentrations of tocols were found in the embryo-free seed and WS (Fig. 2 and Table 1). In the embryo-free seed, total tocol concentrations at 7- and 14-daa were 6.5 ± 0.8 μg/g and 9.9 ± 0.8 μg/g, respectively (Table 1), which in the later stages rose to 22.5 ± 2.6 μg/g and 34.0 ± 4.5 μg/g for 21- and 28-daa, respectively, following a pattern of accumulation similar to that observed for WS. With few exceptions, the predominant tocol forms at all stages were, in order of abundance, α-tocotrienol, α-tocopherol, and β-tocotrienol, with α-tocotrienol constituting 87% of total tocols at 28-daa. Using different tocol extraction methods, Peterson (1995) reported comparable tocol concentrations for endosperm of mature seeds from field-grown plants, with 32.1–37.5 μg/g α-tocotrienol and 3.4–3.6 μg/g α-tocopherol, representing 83% and 8% of total tocols, respectively (versus the 86.9 and 4.0% that we found at 28-daa), and smaller amounts of β-tocotrienol. While both embryo-free seed and the WS initially contain roughly equal ratios of tocopherols and tocotrienols: 42–58% at both 7- and 14-daa for WS, and 53-47% for embryo-free seeds, as the seed progresses to maturity, α-tocotrienol increases proportionally until tocotrienols comprise about

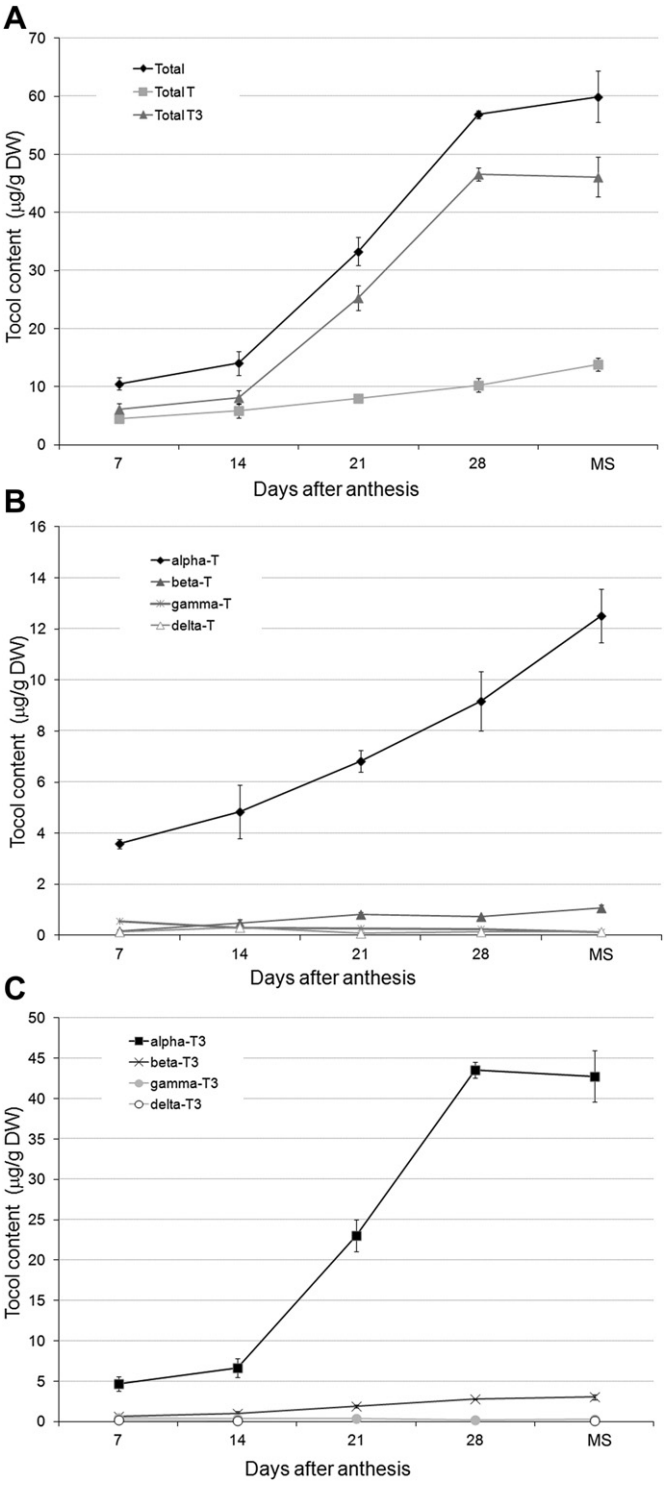

Fig. 2. Accumulation of tocols in whole oat seeds during. A) total tocopherols (T), tocotrienols (T3) and total tocols at five oat seed maturity stages. B) quantification of alpha − (α), beta − (β), delta − (δ), and gamma − (γ) tocopherol. C) quantification of alpha − (α), beta − (β), delta − (δ), and gamma − (γ) tocotrienol. Data are the means of measurements of four biological replicates. Bars represent the SE of the mean.

82% (WS) and 95% (embryo-free seeds) of total tocols at late stages (Fig. 3).

Embryos were the last seed component analyzed. Due to the small size of embryos at the earlier stages of seed development (on average, 0.6, 1.5 and 3.1 mg fresh weight for 7-, 14-, and 21-daa,

Table 1
Accumulation of individual tocopherols and tocotrienols in the embryo-free seed during seed development.

Embryo-free seed	μg/g ± SE of dry weight										
	α-T[a]	α-T3[b]	β-T	β-T3	γ-T	γ-T3	δ-T	δ-T3	Total T	Total T3	Total
7-daa	2.6 ± 0.3	2.7 ± 0.6	0.1 ± 0.0	0.4 ± 0.0	0.7 ± 0.2	Nd	Nd	Nd	3.4 ± 0.3	3.1 ± 0.7	6.5 ± 0.8
14-daa	1.8 ± 0.2	6.7 ± 0.6	0.1 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	Nd	Nd	2.3 ± 0.2	7.5 ± 0.7	9.9 ± 0.8
21-daa	2.1 ± 0.6	17.7 ± 2.4	0.6 ± 0.0	1.5 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	Nd	Nd	3.0 ± 0.8	19.5 ± 2.7	22.5 ± 2.6
28-daa	1.4 ± 0.2	29.6 ± 4.1	Nd	2.5 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	Nd	1.7 ± 0.1	32.3 ± 4.4	34.0 ± 4.5
Embryo only											
28-daa	83.0 ± 6.6	30.4 ± 3.7	10.2 ± 1.5	3.4.±0.3	Nd	Nd	Nd	Nd	93.2 ± 8.0	33.8 ± 3.5	127.0 ± 6.0

daa: Days after anthesis.
Nd: Non-detected.
[a] T: Tocopherols.
[b] T3: Tocotrienols.

respectively), as well as the difficulty in cleanly separating them from the rest of the seed at the MS stage, the only embryos used for tocol analysis in this study were from the 28-daa stage, where embryos averaged 7.1 mg fresh weight, and could still be removed cleanly. Embryo size clearly differs from that of the endosperm, whose fresh weight was 26.5, 35.2, 46.0, and 34.9 mg for 7-, 14-, 21-, and 28-daa, respectively. Results show that, in sharp contrast to embryo-free seeds and WS, these embryos accumulated three times the concentration of tocopherols relative to tocotrienols (93.2 ± 8.0 vs. 33.8 ± 3.5 μg/g), for a total tocols of 127.0 ± 6.0 μg/g (vs. 34.0 ± 4.5 μg/g in embryo-free seeds) (Table 1). However, the small contribution of the embryo to seed mass greatly dilutes the overall effect of this difference. Indeed, only about 35–40% of the whole seed tocols were found in 28-daa embryos. As for WS and embryo-free seeds, in the embryo the α-forms were predominant at that stage (83.0 ± 6.6 and 30.4 ± 3.7 μg/g for α-tocopherol and α-tocotrienol), representing 65.4% and 23.9% of the total tocols respectively. For the β-homologues, β-tocopherol was also more abundant than the tocotrienol form (10.2 ± 1.5 and 3.4 ± 0.3 μg/g, respectively). Overall, tocopherols represented roughly 75% of total tocols in 28-daa embryos. Our results agree with those of Peterson (1995) who reported that α-tocopherol is the most prevalent form in the germ fraction, although that study focused on embryos removed from mature seeds. However, our results differ for embryo tocol composition compared to those reported in Peterson (1995). That study reported the absence of α-tocotrienol and β-tocopherol in the embryo, whereas we found that these two tocols were the second and third most abundant in 28-daa embryos. Further, Peterson (1995) reported high concentrations of γ-tocopherol in the embryo (20.2 μg/g), versus the trace amount of this tocol that we detected. Our embryo excision method is quite precise, being adapted from a microdissection method to obtain intact immature Brachypodium embryos, including the scutellum, for tissue culture. Although minor contamination of embryos with endosperm that might adhere to the scutellum in our dissection might occur, this is not a likely cause for the differences between the two studies. For instance, it is unlikely to account for the significant amounts of α-tocotrienol we detected in embryos. Additionally, Peterson's (1995) analysis was performed on embryos scraped from mature seeds with a spatula while ours was conducted on embryos cleanly excised from immature seeds 28 daa. It is certainly possible that the tocol concentration may change as the embryo undergoes final maturation to MS. We observed a slight but not significant decrease in α-tocotrienol concentration in WS in the transition from 28-daa

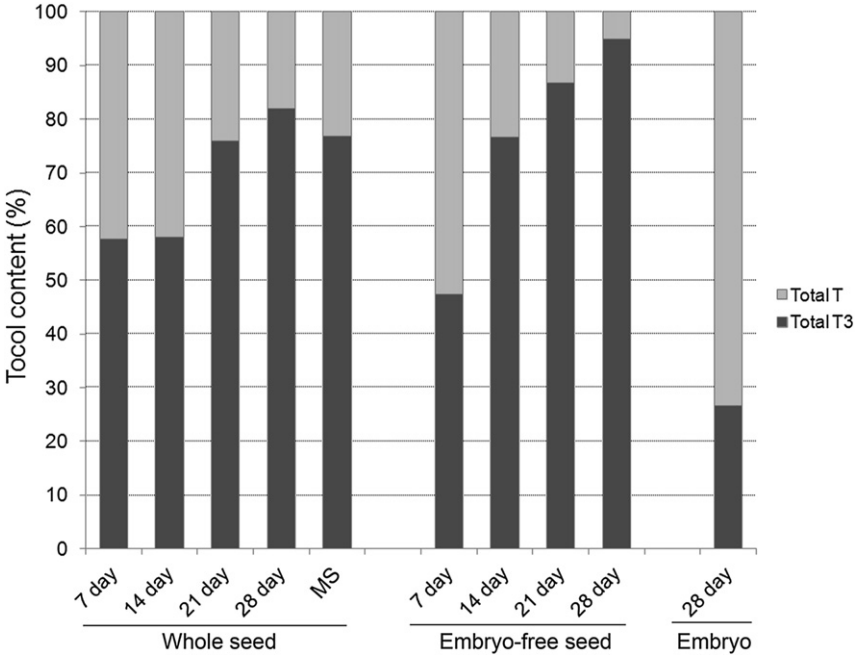

Fig. 3. Distribution of tocotrienols and tocopherols at different stages of oat seed development. Embryo-free and whole seeds were analyzed at 7, 14, 21, 28-daa. Whole seed tocols were also measured in mature seeds. Embryo tocol measurements were taken solely at 28 days after anthesis.

to MS (Fig. 2C), and this difference might be due to decay of this compound in the embryo between 28 daa and seed maturity, not being more noticeable because of the smaller size of the embryo vs. WS. There is no obvious explanation for the difference in embryo γ-tocopherol content reported in the Peterson analysis (1995) and ours. Besides the different seed stages from which measurements were obtained, the possibility exists that the values reported in Peterson (1995) for γ-tocopherol are incorrect. This proposal is based on a recently completed analysis of over 1000 experimental oat genotypes, which detected no γ-tocopherol in the MS of any of the lines (unpublished results).

In other cereal grain seeds, tocotrienols were located mainly in the endosperm of wheat and corn, while tocopherols were the main fraction of their embryo tocols (Grams et al., 1970; Hidalgo and Brandolini, 2008), which is similar to our results. The fact that plants produce different forms of tocols that differentially accumulate in different plant tissues suggests that they may serve various physiological functions. For example, an association between γ-tocopherol levels and barley germination has been reported (Desel and Krupinska, 2005). The temporal and spatial dynamics of tocol accumulation described here provides a basis for further investigating this issue.

4. Conclusions

We evaluated the temporal pattern of tocol accumulation in developing oat seeds. Tocotrienols and tocopherols showed different patterns of accumulation. Tocotrienols were the main fraction in whole seeds and the endosperm; their accumulation followed a sigmoidal curve, slowly accumulating over the first stages of seed development, quickly increasing in mid-development, and reaching a maximum at seed maturation. In contrast, tocopherols were more abundant in the embryo in the developmental stage studied (28-daa). In endosperm and whole seed, tocopherols exhibited a stable increase in concentration during the course of seed development. The tocopherol pathway has been broadly studied and characterized in Arabidopsis (Mene-Saffrane and DellaPenna, 2010). However, in seeds of oats and other monocots, tocotrienols rather than tocopherols are the main forms of tocols.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2012.10.001.

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