

# Factors Affecting Tocopherol Concentrations in Soybean Seeds

Constanza S. Carrera<sup>\*,†,§,#</sup> and Philippe Seguin<sup>†</sup>

<sup>†</sup>Department of Plant Science, McGill University, Macdonald Campus, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec H9X 3V9, Canada

<sup>§</sup>Instituto de Fisiología y Recursos Genéticos Vegetales (IFRGV), Centro de Investigaciones Agropecuarias (CIAP), Instituto Nacional de Tecnología Agropecuaria (INTA), Camino a 60 Cuadras Km 5 1/2, X5020 ICA Córdoba, Argentina

<sup>#</sup>Consejo Nacional de Investigaciones Científicas y Técnicas, Córdoba, Argentina

**ABSTRACT:** Soybean seeds contain several health-beneficial compounds, including tocopherols, which are used by the nutraceutical and functional food industries. Soybean tocopherol concentrations are, however, highly variable. Large differences observed in tocopherol concentrations among soybean genotypes together with the relatively simple biosynthetic pathway involving few genes support the feasibility of selecting for high-tocopherol soybean. Tocopherol concentrations are also highly influenced by environmental factors and field management. Temperature during seed filling and soil moisture appear to be the main factors affecting tocopherol concentrations; other factors such as soil fertility and solar radiation also affect concentrations and composition. Field management decisions including seeding date, row spacing, irrigation, and fertilization also affect tocopherols. Knowledge of factors affecting soybean tocopherols is essential to develop management strategies that will lead to the production of seeds with consistent target concentrations that will meet the needs of the nutraceutical and functional food industries.

**KEYWORDS:** *Glycine max* L., tocopherols, genotypes, environment, seeds, field management, nutraceuticals

## ■ INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] seeds contain several high-value health-beneficial compounds including tocopherols.<sup>1</sup> These compounds are natural lipophilic antioxidants that are synthesized only by photosynthetic organisms, but are important for humans as they have been reported to help in preventing cancer, cardiovascular diseases, and neurodegenerative diseases, such as Alzheimer's and Parkinson's, and to enhance immune function.<sup>2,3</sup> Although all tocopherols are absorbed equally,  $\alpha$ -tocopherol ( $\alpha$ -toc) is the most active in terms of vitamin E activity in the human body and, thus, the one for which interest is the greatest.<sup>4</sup>  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherol ( $\beta$ -,  $\gamma$ -, and  $\delta$ -toc) exhibit 60, 90, and 98–99% less vitamin E activity, respectively, compared to  $\alpha$ -toc.<sup>5</sup>

Soybean has been an essential part of the diet of many Asian populations for centuries, but its use as a food in Western countries is more recent.<sup>1</sup> Part of this increased interest for soy foods is in part due to their potential role in preventing and treating chronic diseases. The demand for vitamin E is not limited only to human applications (nutrition, as well as pharmaceutical and cosmetic) but also to animal nutrition, the feeding of vitamin E being reported to improve meat quality.<sup>6</sup> Currently synthetic  $\alpha$ -toc comprises 85–88% of the worldwide vitamin E market, the remaining being from natural sources that are almost exclusively derived from soybean oil processing and are known to be more biopotent than synthetically produced ones.<sup>7</sup> Increasing natural  $\alpha$ -toc production and supply by developing high  $\alpha$ -toc oilseed crops could contribute to reducing the production cost, which has been reported to be lower compared to synthetic sources.<sup>8</sup>

This demand for tocopherols has contributed to the enhancement of efforts in the development of specialty food

grade soybeans with novel chemical profiles, although in most soybean-producing countries (e.g., the United States, Brazil, and Argentina) the vast majority of the focus remains on commodity soybean with high protein and oil concentrations. Soybean production for the small but growing value-added nutraceutical and functional food markets is an opportunity for breeders and agricultural producers. Isoflavones have been the main focus of research and product development in this area in recent years.<sup>9–11</sup> However, recent studies conducted by seed companies illustrate their interest in developing high-tocopherol oilseed crops to meet more efficiently specific end usages.<sup>12–15</sup> Little is known, however, regarding factors affecting the concentration and synthesis of other health-beneficial compounds, especially tocopherols, despite their large market potential. Understanding the genetic, environmental, and agronomic factors affecting tocopherol concentrations is essential to develop genotypes and management strategies that will lead to the production of soybeans with stable and targeted concentrations that meet the requirements of these new markets. This paper thus reviews tocopherol synthesis and biological functions in plants and genetic and environmental factors affecting tocopherol concentrations and composition in soybean seeds.

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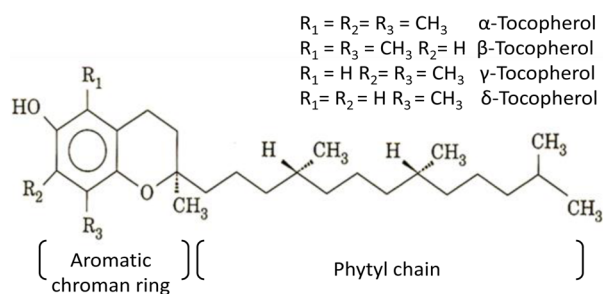
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## ■ SYNTHESIS AND BIOLOGICAL FUNCTIONS OF TOCOPHEROLS IN PLANTS

Soybean oil is a major source of natural tocopherols; it contains an appreciably higher concentration of total tocopherol (tot-toc) compared to other vegetable oils such as canola (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), maize (*Zea mays* L.), or flax (*Linum usitatissimum* L.).<sup>16</sup> The relative proportions of the four forms found in soybean oil are 4–10, 1–3, 60–66, and 24–29% for  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -toc, respectively.<sup>17</sup> The basic structure of the tocopherols is characterized by a polar aromatic chromanol head and a hydrophobic phytyl tail of 16 saturated carbon atoms.<sup>18</sup> The amount and position of the methylation on the aromatic ring determine the different tocopherol forms (Figure 1) and also their ability to quench free radicals.<sup>19</sup> The



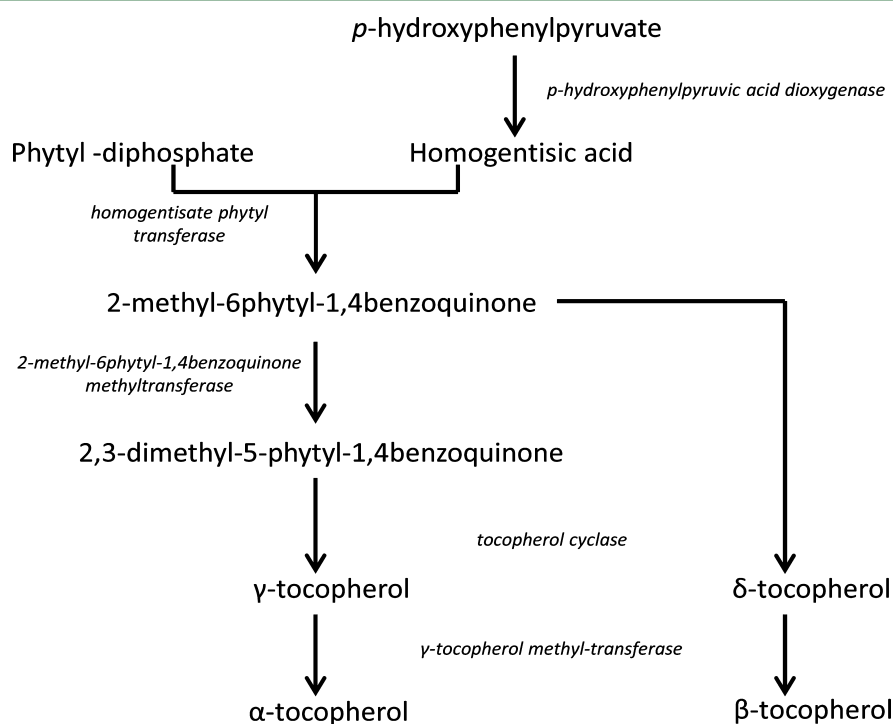
**Figure 1.** Structural differences of the four types of tocopherol forms found in soybeans.

first step of the tocopherol biosynthetic pathway (Figure 2) is the synthesis of homogentisic acid (HGA, the tocopherol aromatic headgroup) from *p*-hydroxyphenylpyruvic (HPP), which is catalyzed by HPP acid dioxygenase. Then the homogentisate phytyl transferase catalyzes the condensation of the HGA with the phytyl diphosphate to generate 2-methyl-

6-phytyl-1,4-benzoquinone (MPBQ). A new methylation of this compound by the MPBQ methyltransferase forms 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ), which the tocopherol cyclase converts to  $\gamma$ -toc. This enzyme can also convert the MPBQ directly into  $\delta$ -toc. Finally, the methylation in the sixth position of the aromatic ring of  $\delta$ - and  $\gamma$ -toc leads to  $\beta$ - and  $\alpha$ -toc, respectively, with both reactions being catalyzed by  $\gamma$ -toc methyl-transferase ( $\gamma$ -TMT).<sup>20</sup>

Tocopherols have several important functions in plants. They were found to be essential for seed longevity and protecting lipids from oxidation during germination and early seedling growth.<sup>20</sup> They increase oil stability by reacting with fatty acid peroxy radicals, the primary products of lipid peroxidation, and intercepting the chain reaction process, which is perpetuated by fatty acids.<sup>21,22</sup> Optimal antioxidant activity that increases oil oxidative stability is detected at concentrations ranges of 100–250, 250–500, and 500–1000  $\mu\text{g g}^{-1}$  for  $\alpha$ -,  $\gamma$ -, and  $\delta$ -toc, respectively,<sup>23–26</sup> beyond which tocopherols could exhibit prooxidant activities in oils. In vitro studies comparing the antioxidant activity of tocopherols at their optimal concentrations revealed that  $\alpha$ -toc was 3–5 times more potent than  $\gamma$ -toc and 16–32 times more potent than  $\delta$ -toc.<sup>27</sup> Tocopherols are localized in the plastids, the only site for their synthesis. There they protect chloroplasts against oxidative stress, allowing photosynthetic function in thylakoid membranes.<sup>28</sup> They are essential to maintaining the structure of these membranes to which they are anchored, stabilizing polyunsaturated fatty acids within lipid bilayers by protecting them from lipoxygenase attack.<sup>2</sup> They also control the lifetime of reactive oxygen species, due to their quenching and scavenging of singlet oxygen activity.<sup>29</sup>

In the past 10 years several studies conducted on plants with tocopherol deficiencies (mutants and some transgenic lines overexpressing genes involved in tocopherol synthesis) highlighted new emerging biological functions of tocopherols in



**Figure 2.** Simplified diagram of the biosynthetic pathway of tocopherols. (Adapted from Sattler et al.)<sup>20</sup>

plants related to physiological processes. Sattler et al.<sup>20,30</sup> found defects in germination. Maeda et al.<sup>31,32</sup> observed alterations in carbohydrate export in leaf, accompanied by reduced plant growth,<sup>33</sup> and accelerated leaf senescence.<sup>34</sup> Other evidence suggests that tocopherols may also serve important functions in signal transduction pathways and the regulation of gene expression in diverse processes, including photoassimilate export and plant defense responses.<sup>28,35</sup>

## GENETIC FACTORS AFFECTING TOCOPHEROL CONCENTRATIONS

### Genotypic Contribution to Tocopherols Variation.

There is ample evidence in the literature supporting that genotype, environment, and genotype by environment interactions all contribute to determining seed tocopherol concentrations.<sup>36–40</sup> However, quantification of the relative contribution of these sources of variation to tocopherol concentration and composition is scarce, being limited to a few papers.<sup>36,39,40</sup> Dolde et al.<sup>36</sup> reported that genetic factors were the most important source of tot-toc variation in soybeans, accounting for 81% of its variation, environmental factors and the genotype by environment interaction having lesser impacts, 3 and 12%, respectively. In line with those results, Whent et al.<sup>40</sup> observed that genotype contributed the most to variation in  $\alpha$ -toc (57%),  $\gamma$ -toc (70%),  $\delta$ -toc (43%), and tot-toc (69%) concentrations. The second most important source of variation for individual tocopherol forms was the environment, followed by the genotype by environment interaction; noticeably, no significant contribution of the environment to tot-toc variability was found in that study. Contrasting results emerged from Carrera et al.,<sup>39</sup> who found that although the genotype and genotype by environment interaction were significant sources of variation, the environment accounted for most of the total variation in the concentration of all individual tocopherols as well as tot-toc (84, 38, 84, and 41% for  $\alpha$ -,  $\gamma$ -,  $\delta$ -, and tot-toc, respectively). Although the aforementioned studies were all conducted in multiple environments, the difference between them is that Dolde et al.<sup>36</sup> and Whent et al.<sup>40</sup> analyzed results from soybeans grown in five relatively close locations (from 36° 15' to 41° 46') and two nearby locations (from 38° 21' to 38° 54' northern latitude), respectively, both studies including only one sowing date and one year of evaluation. In contrast, Carrera et al.<sup>39</sup> analyzed results from a wider range of more distant environments including eight locations (covering a latitudinal range from 24° 54' to 38° 19' S), two sowing dates per site, and two years. Despite the differences observed between studies, the significant contribution of the genotypic effect to variation in soybean tocopherol concentrations suggests that soybean tocopherol concentrations is in part genetically determined, which in turn can be exploited by plant breeders.

**Differences in Tocopherol Concentrations among Genotypes.** Large differences in tocopherol concentrations among soybean genotypes have been documented in several studies conducted in contrasting regions.<sup>17,18,36–38,41,42</sup> Reported concentrations ranged between 7 and 436  $\mu\text{g g}^{-1}$  oil for  $\alpha$ -toc, between 2 and 64  $\mu\text{g g}^{-1}$  oil for  $\beta$ -toc, between 40 and 1559  $\mu\text{g g}^{-1}$  oil for  $\gamma$ -toc, and between 18 and 1334  $\mu\text{g g}^{-1}$  oil for  $\delta$ -toc, whereas mean tot-toc concentrations varied between 78 and 2195  $\mu\text{g g}^{-1}$  oil (Table 1). When expressed on a seed basis  $\alpha$ -toc concentration ranged from 2 to 127  $\mu\text{g g}^{-1}$  seed,  $\beta$ -toc from 2 to 10  $\mu\text{g g}^{-1}$  seed,  $\gamma$ -toc from 14 to 362  $\mu\text{g g}^{-1}$  seed,

**Table 1. Range and Mean (in Parentheses) of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -Tocopherol and Total Tocopherol Concentrations in Seeds of Soybean Genotypes from Different Studies Conducted in Contrasted Regions**

country	no. of genotypes evaluated	no. of environments <sup>a</sup>	tocopherols ( $\mu\text{g g}^{-1}$ oil)					total	reference
			$\alpha$	$\beta$	$\gamma$	$\delta$	total		
USA	14	1	44–158 (96)	2–29 (11)	850–1559 (1048)	254–477 (372)	1363–2195 (1526)	Dolde et al. <sup>36</sup>	
USA	20	3	85–174 (130)		631–847 (710)	265–423 (330)	1041–1359 (1171)	Scherder et al. <sup>42</sup>	
Brazil	89	1	12–191 (69)	6–64 (24)	174–580 (717)	304–1334 (318)	561–1983 (1128)	Carrão-Panizzi and Erhan <sup>17</sup>	
Argentina	6	23	114–139 (129)	38–52 (44)	940–1080 (990)	308–397 (348)	1429–1578 (1511)	Carrera et al. <sup>41</sup>	
Canada	79 <sup>b</sup>	6	7–436 (67)		40–1024 (331)	18–395 (117)	78–1297 (516)	Shaw et al. <sup>38</sup>	
tocopherols ( $\mu\text{g g}^{-1}$ seed)									
country	no. of genotypes evaluated	no. of environments <sup>a</sup>	$\alpha$	$\beta$	$\gamma$	$\delta$	total	reference	
worldwide	97	41	2–127 (20)	2–10 (4)	14–362 (167)	9–149 (77)	29–363 (263)	ILSI <sup>43</sup>	
Japan	1109 <sup>c</sup>		5–43 (25)		27–69 (54)	11–54 (29)	80–128 (108)	Ujite et al. <sup>18</sup>	
Canada	20	6	9–33 (17)		153–193 (169)	79–121 (97)	251–313 (282)	Seguin et al. <sup>9</sup>	

<sup>a</sup>Location, crop year, and sowing date combinations. <sup>b</sup>Recombinant inbred lines (RIL) derived from the cross OAC Bayfield × OAC Shire. <sup>c</sup>Germplasm: 909 cultivated and 200 wild type accessions from the Japanese gene bank.

and  $\delta$ -toc from 9 to 149  $\mu\text{g g}^{-1}$  seed, whereas tot-toc ranged from 29 to 363  $\mu\text{g g}^{-1}$  seed (Table 1).

Special attention has been given to differences between genotypes in terms of  $\alpha$ -toc, being the most bioactive form of vitamin E. In Canada, Seguin et al.<sup>37</sup> reported a 4-fold variation for  $\alpha$ -toc among 20 genotypes sown in multiple environments. Even higher differences were reported by Ujiie et al.<sup>18</sup> and Carrão-Panizzi and Erhan,<sup>17</sup> who observed 9- and 16-fold differences in  $\alpha$ -toc among 1109 germplasm accessions from the Japanese gene bank and 89 Brazilian soybean genotypes, respectively. Analyzing samples of 97 soybean genotypes obtained from multiple controlled field trials over 41 sites across the world, the International Life Sciences Institute (ILSI) released an online comprehensive crop composition database that suggests a possible 66-fold variation for  $\alpha$ -toc.<sup>43</sup> The large differences in tocopherol concentrations seen between genotypes are supported by a substantial body of work, suggesting that selection for high tocopherol and in particular for high  $\alpha$ -toc concentrations could be possible.

**Selection and Breeding for Tocopherol Concentrations.** Although soybean oil is one of the richest sources of tocopherols, it is relatively poor in  $\alpha$ -toc, this form representing <10% of tot-toc in seed tissues.<sup>3,18</sup> Thus, there is a growing interest from breeders to increase the concentration of  $\alpha$ -toc, driven also by the fact that as demonstrated by Maras et al.,<sup>44</sup> the majority of adults fail to meet the current recommendations for vitamin E intake in the average diet. The development of new  $\alpha$ -toc-rich cultivars would contribute to an improved intake of vitamin E. Unlike complex quantitative traits, such as yield, the heritability of seed tocopherol appears to be relatively high as observed not only in soybean<sup>38,45</sup> but also in maize.<sup>46</sup>

Dwiyanti et al.<sup>45</sup> estimated that the broad-sense heritability value for  $\alpha$ -toc concentration was 65% in 140 F2 seeds grown in a greenhouse, whereas Shaw et al.,<sup>38</sup> using 79 recombinant inbred lines (RILs) grown in 6 environments, estimated that the broad-sense heritabilities for  $\alpha$ -,  $\gamma$ -, and  $\delta$ -toc were 38, 47, and 35%, respectively. Analyzing 155 maize inbred lines in two environments Yang et al.<sup>46</sup> found that the broad-sense heritability of  $\alpha$ -toc was 78%, 95% for  $\gamma$ -toc, 94% for  $\delta$ -toc, and 92% for tot-toc. These results are thus encouraging, further supporting the feasibility of selecting for high-tocopherol soybean.

**Genetic Engineering as a Means of Increasing Tocopherol Concentrations.** Overall, the tocopherol biosynthesis pathway is relatively simple (Figure 2), involving a few key enzymes encoded by a small number of genes. The key enzymes homogentisate phytyl transferase (which generates MPBQ), MPBQ methyltransferase (which forms DMPBQ from MPBQ), tocopherol cyclase (the enzyme responsible for  $\gamma$ - and  $\delta$ -toc synthesis), and  $\gamma$ -TMT (the key enzyme in  $\alpha$ -, and  $\beta$ -toc synthesis) are encoded in *Arabidopsis* by the genes VTE2, VTE3, VTE1, and VTE4, respectively.<sup>20</sup> This represents an opportunity for metabolic engineering. For instance, the  $\gamma$ -TMT gene was cloned and overexpressed using a seed-specific promoter in *Arabidopsis* seeds. This resulted in transgenic seeds with a >80-fold increase in  $\alpha$ -toc concentrations at the expense of  $\gamma$ -toc, without altering tot-toc concentration in the seeds.<sup>2</sup> Savidge et al.<sup>12</sup> and Collakova and DellaPenna<sup>47</sup> obtained 60 and 40% higher seed tot-toc concentration than wild type control plants, respectively, as a result of overexpressing the gene encoding homogentisate phytyl transferase activity in *Arabidopsis*. Collakova and DellaPenna,<sup>47</sup> crossing two transgenic *Arabidopsis* lines, one constitutively overexpressing the

gene encoding  $\gamma$ -TMT and the other overexpressing the gene encoding homogentisate phytyltransferase activity, resulted in transgenic seeds with a 12-fold increase in vitamin E activity relative to the wild type control plants. However, seed-specific expression of genes encoding MPBQ methyltransferase and  $\gamma$ -TMT (VTE3 and VTE4, respectively) alone or combined did not produce significant changes in tot-toc concentration in transgenic *Arabidopsis*.<sup>2</sup>

In transgenic soybean, overexpressing the VTE3 gene led to increases in seed  $\gamma$ - and  $\alpha$ -toc concentrations with concomitant decreases of  $\beta$ - and  $\delta$ -toc as shown by Van Eenennaam et al.<sup>13</sup> On the other hand, these authors found that soybean lines transformed to overexpress the VTE4 gene accumulated tocopherol mainly as  $\alpha$ -toc (approximately 75% of tot-toc compared to the 10% in the wild type control), exhibiting a corresponding decrease in  $\gamma$ -toc;  $\delta$ -toc also decreased, leading to higher  $\beta$ -toc concentrations. Finally, the simultaneous expression of both genes led to production of seeds with higher concentrations of  $\alpha$ -toc (>90% of tot-toc compared to the 10% of the wild type control). Interestingly, the change in the proportion of tocopherol forms favoring  $\alpha$ -toc concentrations resulted in no net change in tot-toc levels. The overexpression of multiple genes encoding for key enzymes in the tocopherol biosynthesis pathway was previously demonstrated to increase tot-toc concentration in *Arabidopsis* seeds,<sup>12,47</sup> which could lead to the production of even higher  $\alpha$ -toc concentrations in soybean seeds.<sup>20</sup>

Despite the potential genetic engineering offers to increase the concentrations of both  $\alpha$ -toc and tot-toc, the technology remains controversial. Moreover, the difficulties of soybean transformation and legal obstacles to transgenic soybean in some countries still limit this approach to increase soybean nutraceutical value.<sup>48</sup> Hence, the development of high-tocopherol soybeans will preferably be achieved via traditional plant breeding. However, the use of marker-assisted selection is a powerful alternative in the design of an efficient and cost-effective breeding strategy. Indeed, markers specific to tocopherol biosynthesis could be used for developing soybean genotypes with high tocopherol concentrations, because they are stable and unaffected by environmental factors.<sup>49</sup>

**Molecular Markers Associated with Tocopherol Concentrations.** At present, ample information is available in the literature on molecular markers mapping identifying chromosomal segments with desirable genes controlling tocopherol concentrations not only in soybean<sup>48,50</sup> but also in other crops such as maize,<sup>51,52</sup> winter rapeseed (*Brassica napus* L.),<sup>53</sup> and sunflower.<sup>54</sup> Quantitative trait loci (QTL) analysis of 144 RILs derived from a cross between Chinese (Hefeng 25, low in  $\alpha$ -toc) and Canadian (OAC Bayfield, high in  $\alpha$ -toc) soybean genotypes revealed several QTL associated with individual tocopherols as well as tot-toc (4, 8, 4, and 5 QTL with  $\alpha$ -,  $\gamma$ -,  $\delta$ -, and tot-toc, respectively).<sup>48</sup> However, the causal genes associated with these QTLs still have to be identified. Analyzing QTL of populations from a cross between a high and a low  $\alpha$ -toc genotype (KAS and Ichihime, respectively) Dwiyanti et al.<sup>50</sup> identified a gene encoding  $\gamma$ -TMT with higher expression levels in the genotype KAS. The expression level was correlated with an increase in  $\alpha$ -toc concentration in the seeds of this genotype. These findings provide insights on the genetic control of tocopherol biosynthesis in soybean seeds, which in turn is helpful for breeding programs aimed at developing new soybean genotypes with high  $\alpha$ -toc content.

Despite the importance of genetic factors in determining tocopherol concentrations, it is essential not to overlook the differential response of genotypes to changing environmental conditions, which is illustrated by the genotype by environment interaction<sup>55</sup> and which may hinder breeding efforts. Also, the correlation between tocopherols and other agronomically important seed characteristics should not be ignored while evaluating and selecting genotypes with high tocopherol concentrations.

**Correlations between Tocopherol Concentrations and Important Agronomic and Seed Quality Characteristics.** Selection of soybean genotypes with high tocopherol concentrations could lead to the possibility of decreasing other aspects of nutritional composition and/or agronomic performance such as seed yield (because they can be closely associated with unfavorable genes).<sup>51</sup> Therefore, analyzing and understanding the association between tocopherol concentrations and other agronomic characteristics will ensure that selection for high tocopherol concentrations will not be made at the expense of other important traits.

Although knowledge of the correlation between tocopherol concentrations and important agronomic characteristics (e.g., seed number and weight) is crucial for breeding programs, information available on this particular aspect remains scarce and is limited to two papers utilizing multiple-environment field trials.<sup>37,38</sup> Some supporting evidence of negative correlations between seed yield and  $\gamma$ - and tot-toc concentrations, as well as between 100-seed weight and  $\delta$ -,  $\gamma$ - and tot-toc, was reported by Seguin et al.<sup>37</sup> Shaw et al.<sup>38</sup> reported only a negative correlation between 100-seed weight and  $\gamma$ -toc across environments; these authors also reported a negative correlation between seed yield and  $\alpha$ -toc concentration, but their results were not consistent across environments.

In terms of correlation with important seed composition characteristics, Seguin et al.<sup>37</sup> and Shaw et al.<sup>38</sup> reported that  $\delta$ -,  $\gamma$ -, and tot-toc concentrations were negatively correlated with crude protein concentration and positively correlated with oil concentration, but no correlation between these major seed components and  $\alpha$ -toc was observed. These findings are in contrast with Whent et al.<sup>40</sup> and Carrera et al.,<sup>56</sup> who studied correlations between several seed chemical compounds in multiple environments and found that oil concentration exhibited a strong negative correlation with  $\delta$ - and tot-toc and a positive one with  $\alpha$ -toc concentration. There is supporting evidence<sup>37,40,56</sup> of the negative correlation between  $\alpha$ - and  $\delta$ -toc concentrations, possibly due to the competition for MPBQ, the common precursor from which both are synthesized.<sup>20</sup> This negative correlation between both forms is then reflected in the way they correlate with other compounds. For instance, total isoflavones has been shown to be negatively correlated with  $\alpha$ -toc and positively with  $\delta$ -toc concentration.<sup>40,56</sup> With respect to fatty acids, it is documented that the oleic to linolenic acid ratio (considered a general indicator of oil quality) exhibits a positive relationship with  $\alpha$ -toc and a negative one with  $\delta$ -,  $\gamma$ -, and tot-toc.<sup>40,56,57</sup> Research aimed at studying the correlations among the main seed chemical components might provide useful information for breeding and selection programs.

The presence of positive correlations among several seed composition traits including tocopherols indicates that selection for several desirable traits could be done concurrently.<sup>9</sup> Combining this information with the identification of environments in which such positive correlations are expressed would

allow the production of soybeans with desirable compositional profiles more efficiently and cost-effectively than improving components individually.<sup>56</sup>

## ■ ENVIRONMENTAL AND AGRONOMIC FACTORS AFFECTING TOCOPHEROL CONCENTRATIONS

The nutritional composition of soybean seeds is often greatly affected by the growing environment, especially during the filling period (stages R5–R7<sup>58</sup>) when seed component accumulation takes place. Some of the environmental factors that have been shown to affect the composition and quality of soybean seeds the most include air temperature, soil moisture, soil fertility, and solar radiation. Environmental conditions to which soybeans are exposed during plant growth are also modified by crop and field management (i.e., seeding date, row spacing, irrigation, fertilization), the effects of which will be reviewed along with the environment factors they affect the most.

**Air Temperature.** It is well-known that tocopherol concentrations are very sensitive to air temperature during seed development. Studies conducted under controlled-environment chambers,<sup>36,59–61</sup> as well as in multi-environment field trials,<sup>17,56,62</sup> have demonstrated that high temperatures considerably affect the contribution of each individual tocopherol to tot-toc concentration in soybean seeds. For instance, in a greenhouse experiment plants grown at 28 °C during the entire seed development period have seeds with 106% greater  $\alpha$ -toc and 53% lower  $\delta$ -toc concentrations compared to the control plants grown at 23 °C.<sup>61</sup> In controlled-environment chambers, Chennupati et al.<sup>60</sup> observed 675% more  $\alpha$ -toc and 66% less  $\delta$ -toc when plants were exposed to 29 °C during the seed-filling period compared to control plants grown at 19 °C during all development stages. These findings were also corroborated under field conditions by Britz et al.,<sup>62</sup> who found that under warm environments the ratio of  $\alpha$ -toc/tot-toc was highest (implying an increased proportion of  $\alpha$ -toc). Carrera et al.<sup>56</sup> also observed higher  $\alpha$ -toc concentrations and lower  $\delta$ - and tot-toc concentrations in warm environments (22.5–25.0 °C) compared to cooler ones (17.5–22.4 °C). Moreover, these authors reported across 76 contrasted environments that  $\alpha$ - and  $\delta$ -toc were linearly related to temperature,  $\alpha$ -toc increasing by 17.5  $\mu\text{g g}^{-1}$  oil and  $\delta$ -toc decreasing by 35.2  $\mu\text{g g}^{-1}$  oil per degree Celsius increase in temperature during the seed-filling period. The simultaneous increase of  $\alpha$ -toc and decrease of  $\delta$ -toc in warm environments might be due to the temperature effect on the key enzyme  $\gamma$ -toc methyl transferase (which methylates both  $\gamma$ - into  $\alpha$ - and  $\delta$ - into  $\beta$ -toc) or the gene encoding it.<sup>20</sup> Further research is needed to corroborate this hypothesis because to our knowledge there is currently no information on the effect of high temperature on key enzymes and/or the expression of genes involved in tocopherol synthesis. Temperature has already been demonstrated to affect the synthesis and thus accumulation of certain important seed components including fatty acids<sup>63</sup> and isoflavones.<sup>64</sup>

With regard to  $\gamma$ - and tot-toc concentrations, the literature to date is contradictory and inconclusive. Almonor et al.<sup>59</sup> observed an increase of both  $\gamma$ - and tot-toc concentrations when the temperature increased 12 °C (from 15.5 to 27.5 °C) just after pod initiation (before R5); Britz and Kremer<sup>61</sup> reported that tot-toc remained relatively constant, with only very minor increases in genotypes grown at 28 °C compared to genotypes grown at 23 °C after flowering. Two recent

studies<sup>56,60</sup> reported a decrease in  $\gamma$ - and tot-toc concentrations with increasing temperature during the seed-filling period. Differences among the aforementioned studies might be associated with differences in timing of stress onset, intensity, and duration. Tocopherols are a constituent of the oil fraction in the seed, and it is well documented that oil concentration exhibits a curvilinear relationship with a quadratic response to temperature during seed filling.<sup>65–67</sup> Tot-toc accumulation paralleled oil deposition during seed filling,<sup>59</sup> and it has also been documented that late rather than early onset of stress could have a greater impact on oil concentration.<sup>65,68</sup> It seems likely that the apparent discrepancies observed among the different studies in the response of  $\gamma$ -toc concentrations to increasing temperature could be suggesting a curvilinear relationship with a quadratic tendency between both variables, which then affect in the same direction final tot-toc concentration (considering that  $\gamma$ -toc makes up 70% of tot-toc).

Seeding date influences the temperature to which soybean is exposed during critical development stages, including the seed-filling period, thus affecting tocopherol concentration and composition. Britz et al.<sup>62</sup> in Maryland reported significant and consistent effects of planting date on the  $\alpha$ -toc/tot-toc ratio and concluded that earlier planting dates (i.e., last week of May) exposed seed development to warmer conditions that may be responsible for greater  $\alpha$ -toc/tot-toc ratio compared to later seeding dates (i.e., end of June). A study conducted in six environments in Quebec during two years corroborated these findings. Seguin et al.<sup>69</sup> reported that earlier seeding (i.e., in mid-May) resulted in 45% greater  $\alpha$ -toc concentrations than seeding later in June;  $\delta$ -toc exhibited an opposite response being 24% higher with later seeding dates (i.e., mid to late June) than with earlier ones. In the case of  $\gamma$ - and tot-toc the response to seeding dates was highly inconsistent among environments; thus, Seguin et al.<sup>69</sup> concluded that specific tocopherols may be differently affected by environmental conditions, not only by air temperature but also by soil temperature, day length, and solar radiation levels. This in turn might affect the activities of key enzymes implicated in tocopherol biosynthesis and/or the expression of genes encoding for those enzymes. Some environmental factors are highly correlated in the field, for instance, solar radiation and temperature, hindering field data interpretation; thus, more studies in controlled environments are needed to resolve which specific environmental factors are responsible for the differential response observed for tocopherols to changing seeding dates.

At a given latitude the day length and temperature are highly correlated, making it difficult to attribute seed chemical variability to either factor on the basis of field data. It has been demonstrated that the apparent photoperiod effect might in reality be a temperature effect. Howell and Collins<sup>70</sup> concluded that temperature was more important than photoperiod and that most of the variation in fatty acid concentrations between locations and photoperiods was due to temperature. It is possible that for tocopherol concentrations, latitude may exert its effect mainly through temperature. Analyzing a large database involving 6 genotypes tested in 23 environments, Carrera et al.<sup>56</sup> observed that seeds from southern locations ( $>35^\circ$  S) sown at similar dates always exhibited approximately 58% less  $\alpha$ -toc and 84% more  $\delta$ -toc; also, an increase of  $\gamma$ - and tot-toc concentrations was detected from northern to southern Argentina. Such differences were

more significant at later sowing dates. Tocopherol variability was highly correlated with the average daily mean air temperature faced by the crop during the seed-filling period, accounting for 87 and 92% of the variation of  $\alpha$ - and  $\delta$ -toc concentrations, respectively. As a cultivar is grown farther south in Argentina, cool temperatures and photoperiod delay the beginning of the seed-filling period later into the year. Southern Argentinean locations result in exposure of soybean seed filling to lower temperatures than northern ones. Even at early seeding dates (i.e., October), photoperiodic control as latitude increases delayed crop cycle duration enough to result in most of the seed-filling period occurring beyond the second half of January, when temperatures start to decrease. This is consistent with previous results from Seguin et al.,<sup>69</sup> who observed large and consistent differences in tocopherol proportions along a north (cool temperatures)–south (warm temperatures) gradient in eastern Canada. Indeed, across experiments and years they reported  $\alpha$ -toc concentrations in cooler northern environments to be less than half of those observed in the warmer southern sites. The inverse was observed for  $\delta$ -toc concentrations, which were 67% greater at northern than at southern sites.

**Soil Moisture and Irrigation.** Soil moisture status during the seed-filling period is one of the most important factors affecting soybean seed composition. A greenhouse study reported that drought increased  $\alpha$ -toc concentrations by 42%, whereas  $\delta$ - and  $\gamma$ -toc were both decreased (by 20 and 5%, respectively) and the tot-toc concentration remained relatively constant when compared to irrigated plants.<sup>61</sup> Later these authors<sup>62</sup> corroborated their results in field trials, where they observed that the  $\alpha$ -toc/tot-toc ratio was highest for several genotypes, in environments characterized by severe soil moisture stress (precipitation 37–44% less than the 30-year average), the increase being as much as 3.5-fold above values observed in environments with average precipitations. Conflicting results emerge from a study conducted in 28 environments in Brazil,<sup>17</sup> whose authors analyzed tocopherol concentrations among locations grouped by precipitations. They failed to establish a relationship between precipitation and tocopherol concentrations. This could be due to confounding effects as a result of grouping locations without considering temperature, which differed by at least 3 °C between locations with similar precipitations, introducing possibly additional sources of variations in tocopherol response to precipitation. At locations with the same average temperatures but contrasting precipitations (approximately 4-fold variation) during the seed-filling period, a large increase in  $\alpha$ -toc concentration (245%) was observed along with concomitant reductions of  $\gamma$ -,  $\delta$ -, and tot-toc as precipitation decreased,<sup>17</sup> which is in agreement with previous studies of Britz and Kremer<sup>61</sup> and Britz et al.<sup>62</sup>

It is known that in drought-stricken environments (inductor of stomatal closure), warmer temperatures are experienced by transpiration-limited canopies (with reduced cooling capacity) compared to the prevailing air temperature. It is thus possible to hypothesize that water stress could exert its effect on tocopherol biosynthesis in part through an increase in temperature, which as aforementioned could affect activities of enzymes involved in tocopherol metabolism and/or the expression of genes encoding these enzymes. Because in field situations, it is common that drought conditions are accompanied by higher temperatures, as reported by Rose,<sup>68</sup> special attention should be given to the analysis of the impact of

water stress on seed composition under field conditions because it is highly probable that the response is due to a combination of environmental factors.<sup>71</sup> The combination of high temperature with drought modifies photosynthetic processes,<sup>72</sup> producing alterations that considerably exceed the simple additive effects of each stress alone.<sup>73</sup> Indeed, this combination of stresses has several unique aspects such as the superposition of high respiration levels with low levels of photosynthesis, stomatal closure, and high leaf temperatures,<sup>71</sup> affecting negatively oil constituents due to the dependence of seed oil synthesis on photoassimilate production.<sup>74</sup> Although the effect of drought, alone or in combination with heat stress, on yield production has been intensively researched, knowledge of its impact on soybean tocopherol metabolism is currently lacking in the literature.

**Soil Fertility and Fertilization.** Fertilization with nutrients can affect many physiological processes,<sup>75</sup> determining not only seed yield but also seed quality. The effect of P and K fertilization on soybean tocopherol concentrations was studied in a multilocation experiment by Seguin et al.,<sup>69</sup> who found negligible effects in fields with average to high initial soil P and K levels. To our knowledge the effect of other nutrients on soybean tocopherol concentration and composition has not yet been studied. Egesel et al.<sup>76</sup> reported that N fertilization increased the concentration of all individual tocopherols, as well as tot-toc concentration in rapeseed, with increases of 3, 20, 5, and 13% for  $\alpha$ -,  $\gamma$ -,  $\delta$ -, and tot-toc with N fertilization of 130 kg ha<sup>-1</sup> compared to an unfertilized control. At application rates >130 kg N ha<sup>-1</sup>, there was no further response, suggesting that minimal N levels might be needed to maximize tocopherol concentrations. Further research is needed not only to corroborate these findings but also to understand the biochemical basis and/or the physiological mechanisms for the possible roles of N, P, and K on tocopherol concentration and composition. Still, it is unknown whether macronutrient fertilization during crop development exerts modifications on tocopherol composition through a direct effect on the activities of the enzymes involved in their biosynthesis or if it is an indirect effect through other plant characters such as leaf area, seed number, and/or seed weight.

**Solar Radiation.** Very little is known about the effect of intercepted solar radiation on tocopherol metabolism in soybean, despite the well-known dependence of oil seed storage synthesis on photoassimilates, which is directly determined by solar radiation intercepted by plants.<sup>77,78</sup> Nolasco et al.<sup>79</sup> have shown that higher intercepted solar radiation per plant during the seed-filling period decreased the tot-toc concentration in oil in sunflower. Later, Izquierdo et al.,<sup>80</sup> through manipulative experiments, observed that a shading of 80% imposed on soybean during seed filling increased  $\beta$ -,  $\gamma$ -, and tot-toc concentrations by 80, 46, and 27%, respectively, when compared to an unshaded control, no effect being observed on  $\alpha$ - and  $\delta$ -toc concentrations. Because shading treatments resulted also in less oil content per seed than the control and this variable was negatively correlated with tot-toc concentration, these authors attributed much of the variation (58%) in tot-toc concentration to the effect of shade on seed oil content in soybean. The response was explained through a dilution-like effect, because an increase of solar radiation increased both oil and tocopherol content per seed, but the oil synthesis was increased more than tocopherol, thus explaining the observed decrease in oil tocopherol concentration as previously reported for sunflower by Nolasco et al.<sup>79</sup>

Under simulated canopy shade during seed development, Britz and Cavins<sup>81</sup> reported an increase of polyunsaturated fatty acids (i.e., linoleic and linolenic acid) in soybean oil. Recently, Bianculli et al.<sup>82</sup> corroborated these findings. Holden et al.<sup>83</sup> observed that the specific activity of  $\omega$ -6 desaturase (that catalyzes the conversion of oleic to linoleic acid) was high in endoplasmic reticulum membranes from seeds that matured under reduced light quantity. The positive correlation between linolenic acid and  $\delta$ -,  $\gamma$ -, and tot-toc in the oil of mature soybeans is well documented<sup>56,57,84</sup> In addition, Almonor et al.<sup>59</sup> reported that tocopherol concentration was significantly greater in genotypes with homozygous dominant alleles for  $\omega$ -6 desaturase compared to those with the homozygous recessive alleles within the same temperature treatment. It is, however, currently unknown if this association reflects only a correlation or if seed tocopherol concentrations are somehow affected directly by oil composition (i.e., fatty acids) rather than oil content per seed as previously suggested by Izquierdo et al.<sup>80</sup> and Nolasco et al.<sup>79</sup> More research is required to fully understand this relationship.

**Other Factors.** In the context of global climate change, which is likely to entail changes in average CO<sub>2</sub> levels in the atmosphere, it has been shown for some health-beneficial chemical components such as isoflavones that increased CO<sub>2</sub> concentrations in the atmosphere will lead to an increase in isoflavone concentrations.<sup>85,86</sup> Despite its importance, no study was found to assess the impact of different atmospheric CO<sub>2</sub> levels on soybean seed tocopherol concentration and composition.

The impacts of other crop management practices on soybean tocopherol concentrations such as seeding rate and row spacing were studied in Canada by Seguin et al.<sup>69</sup> These authors found  $\alpha$ -toc to be the most responsive form to these factors; for example, seeding at a rate of 40 seeds m<sup>-2</sup> resulted in 4% higher concentrations than seeding at a rate of 50 or 60 seeds m<sup>-2</sup>, in three of five environments, and row spacing of 36 cm or wider resulted in 6% higher concentrations compared to narrower row spacing (18 cm) in two of five environments.  $\delta$ -Toc was affected only by row spacing in an inverse way to  $\alpha$ -toc, increasing 4% at narrower row spacing (18 cm) than wider ones. Concentrations of  $\gamma$ - and tot-toc were not affected by either seeding rate or row spacing. Noticeably, these factors exhibited a much lower impact (maximum effect being <10%) on tocopherol concentrations compared to seeding date and location, which the authors attributed to the fact that the different treatments produced only subtle differences in microclimatic conditions faced by the crops. More data are needed to clarify the effects of agronomic practices on the synthesis and accumulation of tocopherols.

In summary, soybean seeds contain relatively high concentrations of tocopherols, which have potential uses by the nutraceutical and functional food industries. Reliable evidence highlights the essential role of tocopherols in human nutrition and health, as well as the diverse biological functions related to physiological processes of these compounds in plants. Genotypic effects, environmental effects, and their interactions all contribute to determining soybean tocopherol concentration and composition. The large variability in tocopherol concentrations attributable to genetic factors, together with their comparatively high heritability, suggests that selection for high-tocopherol and in particular for high- $\alpha$ -toc soybeans could be possible. The relatively simple biosynthesis pathway represents an opportunity for both metabolic engineering and marker-

assisted selection, both technologies having shown encouraging possibilities for breeders. The successful development and adoption of new soybean genotypes aimed for the functional food or nutraceutical markets requires acceptable agronomic performance; however, seed yield and seed weight might negatively correlate with tocopherols. Mounting evidence points to the large environmental effects on tocopherol concentration and composition, the most important factors (Table 2) being air temperature, soil moisture, soil fertility,

**Table 2. Soybean Seed  $\alpha$ -,  $\gamma$ -, and  $\delta$ -Tocopherol and Total Tocopherol Response to Environmental and Agronomic Factors**

variable	tocopherol <sup>a</sup>				references
	$\alpha$	$\gamma$	$\delta$	total	
air temperature	+	+/-	-	+/-	Carrera et al.; <sup>56</sup> Almonor et al.; <sup>59</sup> Chennupati et al. <sup>60</sup> Britz and Kremer; <sup>61</sup> Britz et al. <sup>62</sup>
seeding date	+	+/-	-	+/-	Britz et al.; <sup>62</sup> Seguin et al. <sup>69</sup>
latitude	+	-	-	-	Carrera et al.; <sup>56</sup> Seguin et al. <sup>69</sup>
drought	+	-	-	-	Britz and Kremer; <sup>61</sup> Britz et al. <sup>62</sup>
precipitation	-	+	+	+	Carrão-Panizzi and Erhan <sup>17</sup>
P fertilization	-	0	0	0	Seguin et al. <sup>69</sup>
K fertilization	0	0	0	0	Seguin et al. <sup>69</sup>
shading	0	+	0	+	Izquierdo et al. <sup>80</sup>
seeding rate	+	0	0	0	Seguin et al. <sup>69</sup>
row spacing	+	0	-	0	Seguin et al. <sup>69</sup>

<sup>a</sup>+, positive response; -, negative response; 0, lack of response; +/-, inconsistent response.

solar radiation, and some specific field management decisions (i.e., seeding date, row spacing, irrigation, fertilization). Elucidating the molecular and biochemical mechanisms underlying soybean tocopherol accumulation in response to these factors is essential to gain a better understanding of field conditions that will lead to the production of soybeans with stable target tocopherol concentrations that meet the needs of the functional food and nutraceutical industries.

## AUTHOR INFORMATION

### Corresponding Author

\*(C.S.C.) Phone: +54-351-4973636. Fax: +54-351-4974330. E-mail: [carrera.coty@gmail.com](mailto:carrera.coty@gmail.com).

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