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# Response of seed tocopherols in oilseed rape to nitrogen fertilizer sources and application rates[*#]

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**Abstract:** Tocopherols (Tocs) are vital scavengers of reactive oxygen species (ROS) and important seed oil quality indicators. Nitrogen (N) is one of the most important fertilizers in promoting biomass and grain yield in crop production. However, the effect of different sources and application rates of N on seed Toc contents in oilseed rape is poorly understood. In this study, pot trials were conducted to evaluate the effect of two sources of N fertilizer (urea and ammonium nitrate). Each source was applied to five oilseed rape genotypes (Zheshuang 72, Jiu-Er-1358, Zheshuang 758, Shiralee, and Pakola) at three different application rates (0.41 g/pot (N1), 0.81 g/pot (N2), and 1.20 g/pot (N3)). Results indicated that urea increased α-, γ-, and total Toc (T-Toc) more than did ammonium nitrate. N3 was proven as the most efficient application rate, which yielded high contents of γ-Toc and T-Toc. Highly significant correlations were observed between Toc isomers, T-Toc, and α-/γ-Toc ratio. These results clearly demonstrate that N sources and application rates significantly affect seed Toc contents in oilseed rape.

**Key words:** Oilseed rape (*Brassica napus* L.), Nitrogen rate, Urea, Ammonium nitrate, Tocopherol
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## 1 Introduction

Tocopherols (Tocs; vitamin E) are some of the most important lipid-soluble compounds and constitute a family of natural antioxidants (Burton and Ingold, 1986; Ingold *et al*., 1987; Bramley *et al*., 2000). They have four major isomers: α-, β-, γ-, and δ-Toc (Fig. 1) (Hussain *et al*., 2013b). α-Toc exhibits 100% biological activity of vitamin E. β-, γ-, and δ-Toc have only 30%, 15%, and 5% biological activity, respectively (Traber, 2006). Toc confers several human health benefits, including a decreased risk of cardiovascular diseases, improved immune function, and a slower progression of degenerative conditions, such as cataracts, arthritis, and age-related disorders of the nervous system (Traber and Sies, 1996; Pryor, 2000; Bell and Grochoski, 2008). Recent reports suggest that vitamin E (Toc) intake, either from the diet or from supplements, may reduce the risk of liver cancer (Zhang *et al*., 2012; Hussain *et al*., 2013a). Toc is synthesized mainly in plants. Rapeseed oil is an important source of Toc (DellaPenna, 2005; DellaPenna and Pogson, 2006). In rapeseed oil, γ-Toc is the most predominant isoform of Toc, followed by α-Toc. γ-Toc is highly potent in quenching reactive N species (Cooney *et al*., 1993; Christen *et al*., 1997). δ- and β-Toc are less significant because of their low or negligible concentrations (Goffman and Becker, 2002). On average, rapeseed oils contain about 64% γ-Toc, 35% α-Toc, and <1% δ-Toc (Goffman and Becker, 2001; 2002).

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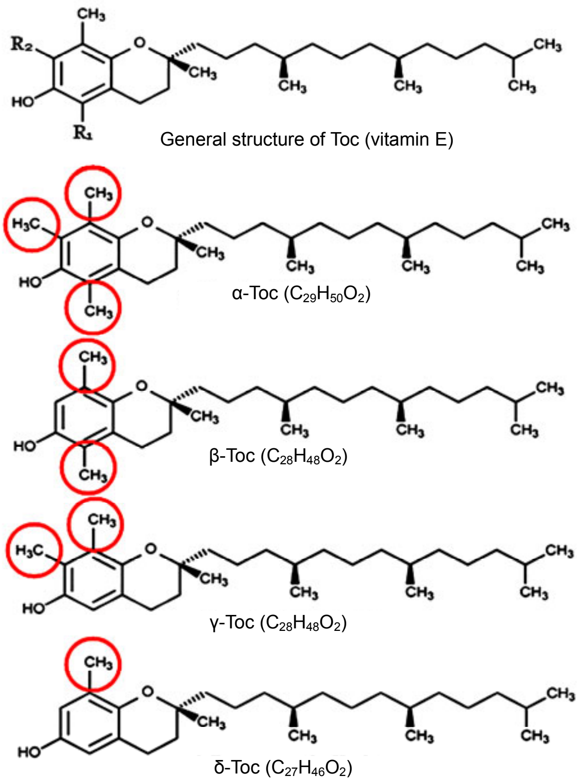

**Fig. 1 Chemical structures of tocopherol (Toc) and its isoforms (α-, β-, γ-, and δ-Toc)**
The number and position of methyl substituents (circled) in the aromatic ring are different. This figure is modified from Hussain *et al.* (2013b)

The Toc content in seeds of rape depends on the genotype and the interaction between the genotype and various environmental factors, of which fertilizer management is important (Marquard, 1985; 1990; Dolde *et al.*, 1999; Marwede *et al.*, 2004; 2005; Havlin *et al.*, 2005; Rathke *et al.*, 2005). N accounts for the largest amount of metabolic element that plants absorb from soil. N is also one of the most common limiting nutrients in the production of non-nitrogen-fixing crops. Therefore, N fertilizer is applied at higher rates than other fertilizers. In plants, Toc is found mostly in plastids, specifically in the chloroplast, a cell organelle containing chlorophyll and involved in photosynthesis (Gzyl-Malcher *et al.*, 2010). N is a major component of chlorophyll and amino acids. N is also found in other important biomolecules, such as adenosine triphosphate (ATP) and nucleic acids (Wagner, 2011). Toc and chlorophyll are localized in the same plant tissues and may be associated because N may enhance photosynthetic activity. Moreover, the function of Toc is highly associated with the maintenance of the integrity and normal photosynthetic functions of the cell membrane system (Havaux *et al.*, 2005; Collin *et al.*, 2008). The predominant forms of N in the soil are $NO_3^-$ and $NH_4^+$. Positively charged $NH_4^+$ is adsorbed by negatively charged soil particles and soil organic matter. Thus, $NH_4^+$ is protected from leaching. By contrast, negatively charged $NO_3^-$ is subject to leaching (Hofman and van Cleemput, 2004; Havlin *et al.*, 2005).

Rapeseed, in particular the canola type, has a high N demand. Efficient supply of N fertilizer is an important factor affecting its yield and seed quality (Grant and Bailey, 1993; Schjoerring *et al.*, 1995; Weisler *et al.*, 2001). Three main variables, including application rate, timing, and chemical form, are considered important in implementing an efficient N fertilizer management program. However, knowledge of the response of seed Toc in oilseed rape to N fertilizer sources and application rates is lacking. In this study, pot trials were conducted to evaluate the effects of two sources of N fertilizer (urea and ammonium nitrate). Three different rates of each source were applied to five oilseed rape genotypes from different ecological regions to determine the effects of these factors on the seed Toc contents.

## 2 Materials and methods

Pot trials were conducted in a greenhouse at the Huajiachi Campus of Zhejiang University (Hangzhou, China) during the growing season from 2010 to 2011. The aim was to evaluate the effects of the two sources of N fertilizer applied at three different rates on the yield components and seed Toc contents of five rapeseed (*Brassica napus* L.) genotypes: “Zheshuang 72”, “Jiu-Er-1358”, “Zheshuang 758”, “Shiralee”, and “Pakola”. The first three genotypes are the dominant cultivars in Zhejiang, China. The remaining two genotypes are widely distributed in Pakistan. Urea and ammonium nitrate (A-nitrate) were used as N sources. Three different doses of each source [0.41 g/pot (N1), 0.81 g/pot (N2), and 1.20 g/pot (N3)], equivalent to rates of 90, 180, and 270 kg/ha, were applied to each of the five genotypes. Thus, 30 treatment combinations (Table S1), each with four replicates, were tested and applied in a randomized complete block design. Each pot had the following dimensions: top diameter, 25 cm; height, 25 cm; and bottom diameter, 22 cm. Each pot was filled to the brim with 9.5 kg of soil. The soil was a silt-loam type containing 0.15% total N and 1.68% organic matter at pH 6.7. According to the experimental procedure, the amount of fertilizer was calculated on a per-pot basis and applied in two split doses: one-third was applied 5 d after seed emergence and the remainder at the flower initiation stage. All other agronomic practices and nutrients were applied according to the standard practices of oilseed rape production in Hangzhou, China. Sample preparation to extract seed Toc and analysis of Toc contents using gas chromatography-flame ionization detector (GC-FID) technique were done according to the method described by Hussain *et al.* (2013b). Statistical analyses of the data were done by analysis of variance (ANOVA) technique using the MSTATC Version 2.10 (MSDOS 1989; Michigan State University, East Lansing, MI). The least significant difference (LSD) test was used to compare the significant difference among means at $P\leq 0.05$ or $P\leq 0.01$.

Detailed materials and methods for Toc extraction and analysis are described in Data S1.

## 3 Results

### 3.1 Response of Toc to N fertilizer sources

Statistical analyses of the data (Table 1) indicated that N sources had significant effects on seed Toc contents. Significant variation ($P<0.01$) was observed among the contents of T-Toc and its isomers in response to different inorganic N sources, except for δ-Toc (Fig. 2). Urea yielded more seed α-, γ-, and T-Toc than the application of A-nitrate (Fig. 2). For the α-/γ-Toc ratio, the index value of A-nitrate (0.432) was higher than that of urea (0.428). Higher γ-Toc contributed to the lower α-/γ-Toc ratio in seeds of urea-treated plants. Therefore, in *B. napus*, urea fertilizer yielded higher seed Toc contents than did A-nitrate.

### 3.2 Response of Toc to N fertilizer application rates

Different N doses significantly ($P<0.01$; Table 1) affected γ-Toc (Fig. 3b) and T-Toc contents (Fig. 3d) as well as the α-/γ-Toc ratio (Fig. 3e). The contents of α- and δ-Toc isomers (Figs. 3a and 3c) did not show a significant response to different N rates, regardless of N sources. Considering the average mean values (irrespective of genotypes and N sources), higher γ-Toc (224.35 μg/g seed) and T-Toc (357.04 μg/g seed) contents were obtained with N3. By contrast, comparatively low γ-Toc (208.96 μg/g seed) and T-Toc (341.05 μg/g seed) contents were recorded with N2. For α-/γ-Toc ratio, N1 and N2 had higher index values (0.432 and 0.433, respectively) than N3 (0.406), as shown in Fig. 3e.

### 3.3 Variation in Toc contents of different genotypes of *B. napus*

Seed Toc content and composition varied among the genotypes (Table 1). Considering the mean values (irrespective of N sources and doses), Zheshuang 72 produced significantly ($P<0.01$) higher contents of α-, γ-, and T-Toc (91.67, 219.68, and 353.33 μg/g seed, respectively) (Figs. 4a, 4b, and 4d). Although Jiu-Er-1358 produced higher δ-Toc contents compared with other genotypes, it was statistically on par with Zheshuang 72 (Fig. 4c). The α-/γ-Toc ratio is an important index used to measure seed quality in terms of Toc content. Pakola had the highest α-/γ-Toc ratio (0.428), whereas Zheshuang 72 had the lowest (0.417), as shown in Fig. 4e.

**Table 1 Mean square values for the contents of tocopherol (Toc) isomers in seeds of *Brassica napus* L. following treatment with two different sources of nitrogen fertilizer applied at three different rates**

| Source of variation | DF | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | α-Toc | γ-Toc | δ-Toc | T-Toc | α-/γ-Toc ratio |
| Genotypes (G) | 4 | $7.84^{**}$ | $61.22^{**}$ | $10.29^{**}$ | $120.67^{**}$ | $0.0001^{**}$ |
| N-source (NS) | 1 | $13.59^{**}$ | $1195.49^{**}$ | $4.26^{ns}$ | $1310.83^{**}$ | $0.002^{**}$ |
| G×NS | 4 | $4.88^{*}$ | $39.81^{**}$ | $7.37^{**}$ | $72.98^{**}$ | $0.0001^{**}$ |
| N-rate (NR) | 2 | $2.34^{ns}$ | $2863.40^{**}$ | $3.43^{ns}$ | $2966.09^{**}$ | $0.009^{**}$ |
| G×NR | 8 | $10.59^{**}$ | $30.76^{**}$ | $0.88^{ns}$ | $59.17^{**}$ | $0.0002^{**}$ |
| NS×NR | 2 | $0.90^{ns}$ | $711.47^{**}$ | $5.08^{*}$ | $709.13^{**}$ | $0.002^{**}$ |
| G×NS×NR | 8 | $8.94^{**}$ | $54.14^{**}$ | $0.92^{ns}$ | $79.13^{**}$ | $0.0002^{**}$ |
| Error | 87 | 1.57 | 6.29 | 1.35 | 12.51 | 0.000 |

DF: degree of freedom. $^{ns}$ Not significant, $^{*}$ Significant at $P\leq 0.05$, $^{**}$ Significant at $P\leq 0.01$, analyzed by ANOVA

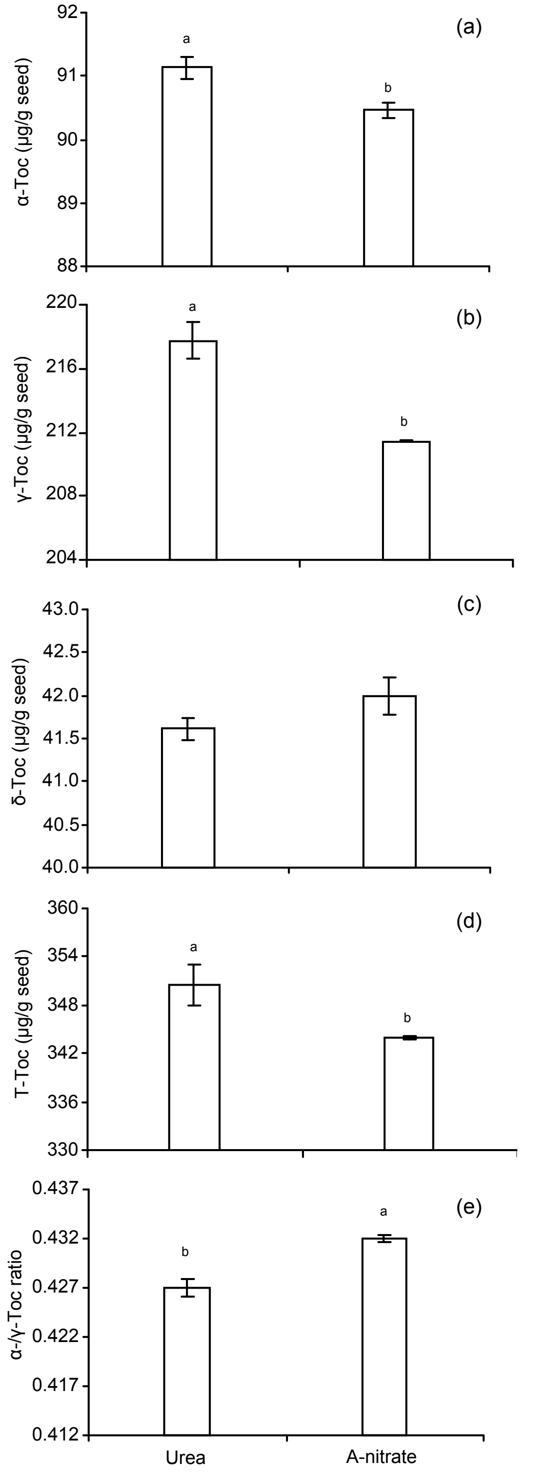

**Fig. 2 Effects of nitrogen fertilizer sources on tocopherol (Toc) contents of *Brassica napus* L. seed averaged over all the genotypes and nitrogen application rates**

(a) α-Toc; (b) γ-Toc; (c) δ-Toc; (d) T-Toc; (e) α-/γ-Toc ratio. Data are expressed as mean±SD (*n*=4). Different letters above the column show the significant differences among the two nitrogen sources

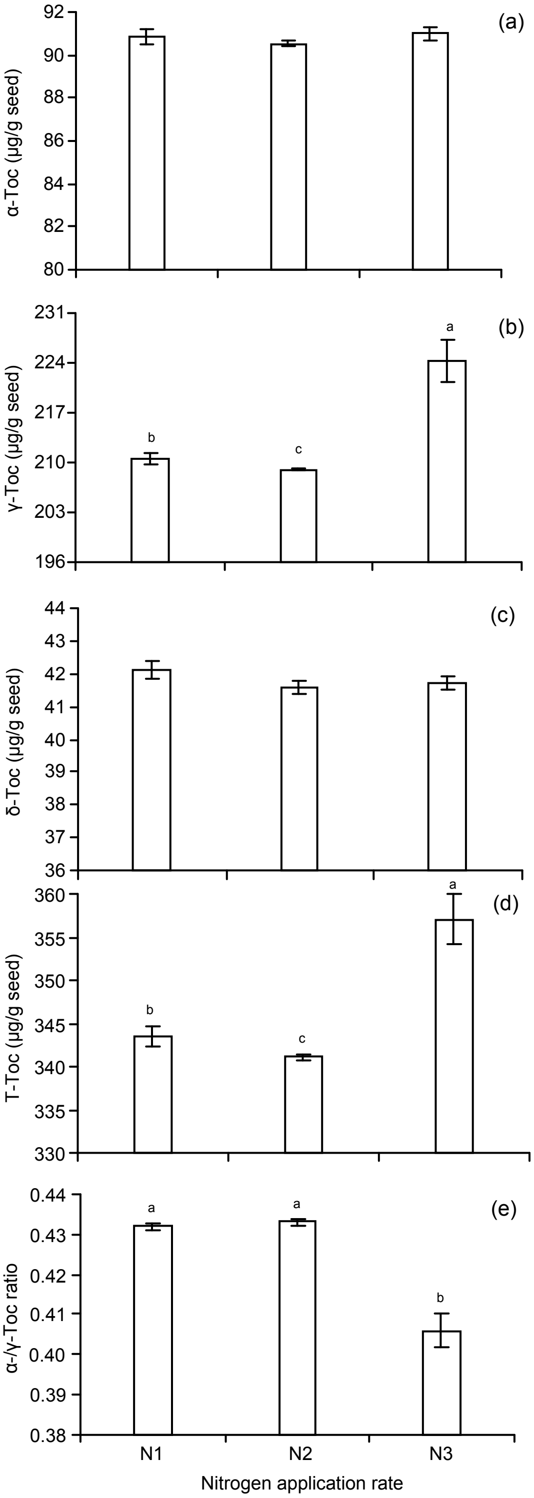

**Fig. 3 Effects of different nitrogen fertilizer application rates on tocopherol (Toc) contents of *Brassica napus* L. seed for all genotypes and nitrogen sources tested**

(a) α-Toc; (b) γ-Toc; (c) δ-Toc; (d) T-Toc; (e) α-/γ-Toc ratio. N1=90 kg/ha; N2=180 kg/ha; N3=270 kg/ha. Data are expressed as mean±SD (*n*=4). Different letters above the column show the significant differences among the three nitrogen application rates

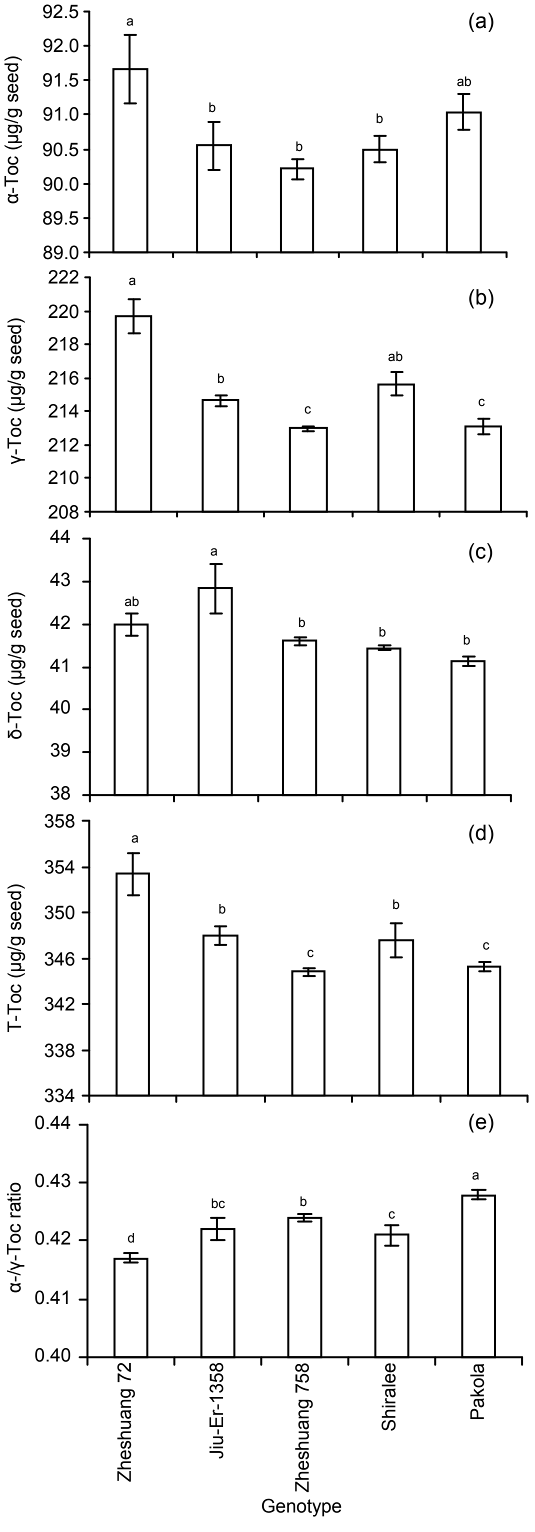

**Fig. 4 Comparison of the contents of total tocopherol (Toc) and its isoforms in the seed among *Brassica napus* L. genotypes**

(a) α-Toc; (b) γ-Toc; (c) δ-Toc; (d) T-Toc; (e) α-/γ-Toc ratio. Data are expressed as mean±SD ($n$=4). Different letters above the column show the significant differences among the genotypes

## 3.4 Response of Toc to interactions among factors

### 3.4.1 Response of Toc to the interaction between genotypes and N sources

A significant interaction was found between the genotypes and N sources for the α-, γ-, δ-, and T-Toc contents as well as the α-/γ-Toc ratio (Table 1). Urea-fertilized Zheshuang 72 plants yielded the highest α-Toc content (92.54 μg/g seed), whereas A-nitrate-fertilized Jiu-Er-1358 yielded the lowest (89.88 μg/g seed) (Fig. 5a). Zheshuang 72 and Shiralee interacted significantly with urea to yield higher γ-Toc contents of 221.46 and 219.830 μg/g seed, respectively, compared with other genotypes fertilized with either N source (Fig. 5b). Almost all of the A-nitrate-fertilized genotypes showed lower values of γ-Toc ranging from 210.21 μg/g seed (Zheshuang 758) to 212.37 μg/g seed (Jiu-Er-1358). A-nitrate-fertilized Jiu-Er-1358 plants produced higher δ-Toc content (43.95 μg/g seed), whereas urea-fertilized Pakola plants produced the lowest seed δ-Toc content (40.96 μg/g seed) (Fig. 5c). Urea-fertilized Zheshuang 72 plants yielded the highest seed T-Toc content (356.32 μg/g seed) (Fig. 5d), whereas A-nitrate-fertilized Zheshuang 758 plants produced the lowest T-Toc (341.99 μg/g seed). Zheshuang 72, Zheshuang 758, Shiralee, and Pakola fertilized with A-nitrate shared the highest α-/γ-Toc ratio (0.429). The lowest α-/γ-Toc ratio (0.413) was obtained from urea-fertilized Shiralee seeds (Fig. 5e).

### 3.4.2 Response of Toc to the interaction between genotypes and N application rates

ANOVA indicated significant ($P$<0.01) interaction between genotypes and N application rates for α-, γ-, and T-Toc contents, and for the α-/γ-Toc ratio (Table 1). N1-fertilized Zheshuang 72 plants produced the highest seed α-Toc content (93.69 μg/g seed) among all the combinations of genotypes and N rates. By contrast, the lowest α-Toc content (89.78 μg/g seed) was obtained from the seeds of N1-fertilized Zheshuang 758 (Fig. 6a). As the amount of N increased from N1 to N2, no significant differences were observed in the γ-Toc and T-Toc contents among the genotypes. However, as the N rate was further increased from N2 to N3, significant ($P$<0.01) differences among the genotypes were observed (Figs. 6b and 6c). The interaction between genotypes and N doses in terms of α-/γ-Toc ratio is shown in

Fig. 6d. N1-fertilized Zheshuang 72 plants yielded the highest α-/γ-Toc ratio (0.436), whereas the lowest α-/γ-Toc ratio (0.396) was obtained from N3-fertilized Shiralee plants.

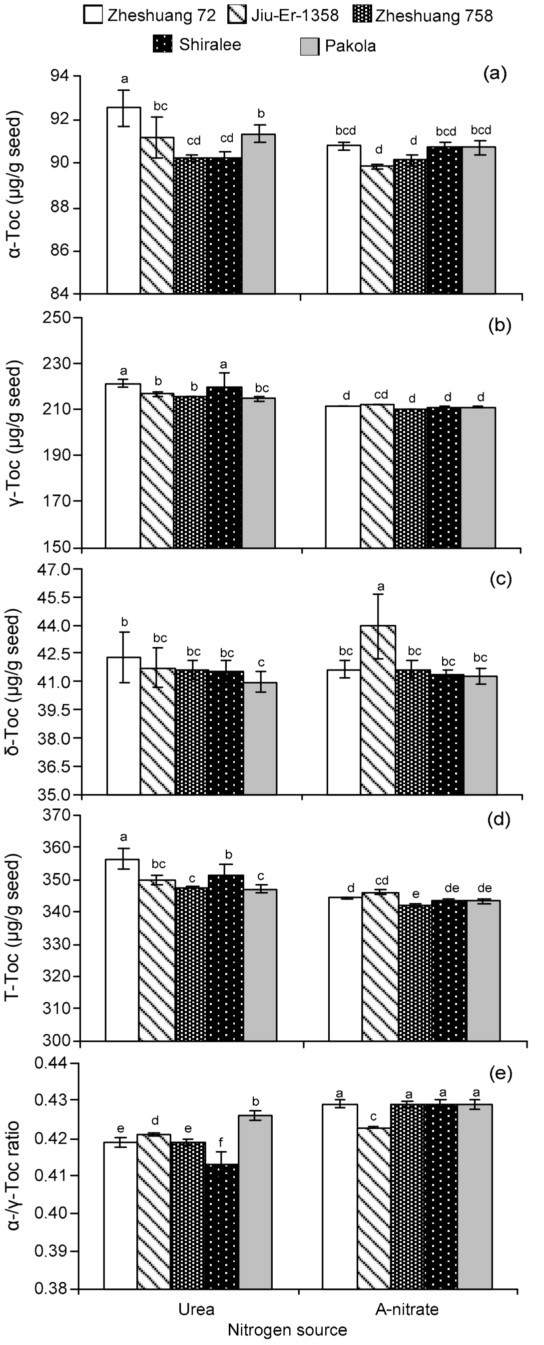

**Fig. 5 Effects of interaction between genotypes and nitrogen sources on the seed tocopherol (Toc) contents of *Brassica napus* L.**
(a) α-Toc; (b) γ-Toc; (c) δ-Toc; (d) T-Toc; (e) α-/γ-Toc ratio. Data are expressed as mean±SD (*n*=4). Different letters above the column show the significant differences among the genotypes at two nitrogen sources

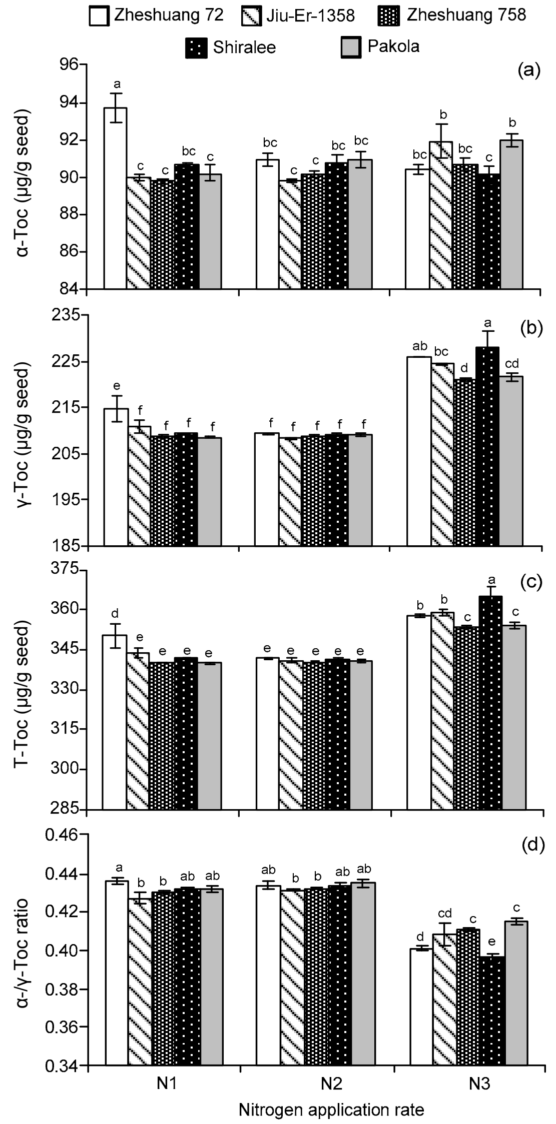

**Fig. 6 Effects of interactions between genotypes and nitrogen application rates on the tocopherol (Toc) contents of *Brassica napus* L. seeds**
(a) α-Toc; (b) γ-Toc; (c) T-Toc; (d) α-/γ-Toc ratio. N1= 90 kg/ha; N2=180 kg/ha; N3=270 kg/ha. Data are expressed as mean±SD (*n*=4). The different letters above the column show the significant differences among the genotypes at three different nitrogen application rates

### 3.4.3 Response of Toc to the interaction between N sources and N application rates

Significant (*P*<0.01) two-way interactions between sources and application rates of N were observed for γ-, δ-, and T-Toc contents, and for the α-/γ-Toc ratio of *B. napus* L. seeds (Table 1). Considering the average of the genotypes, the combination of urea and N3 yielded the highest γ-Toc content (232.24 μg/g seed), followed by the combinations of

A-nitrate and N3 (216.42 μg/g seed) and urea and N1 (212.22 μg/g seed) (Fig. 7a). Variation in the δ-Toc content of *B. napus* seeds was observed because of a significant ($P<0.05$) interaction between the sources and rates of N (Table 1). Considering the average of the five genotypes, urea-fertilized plots at N1 yielded the highest δ-Toc contents (42.35 μg/g seed) among all of the combinations of sources and application rates of N. By contrast, low δ-Toc contents (41.13 μg/g seed) were obtained after A-nitrate at N1 was applied (Fig. 7b). Urea applied at N3 yielded the highest T-Toc (364.93 μg/g seed, $P<0.01$) (Fig. 7c). The highest α-/γ-Toc ratio of 0.434 was obtained from urea-fertilized plants at N2, and the lowest (0.419) from urea-fertilized plants at N3 (Fig. 7d).

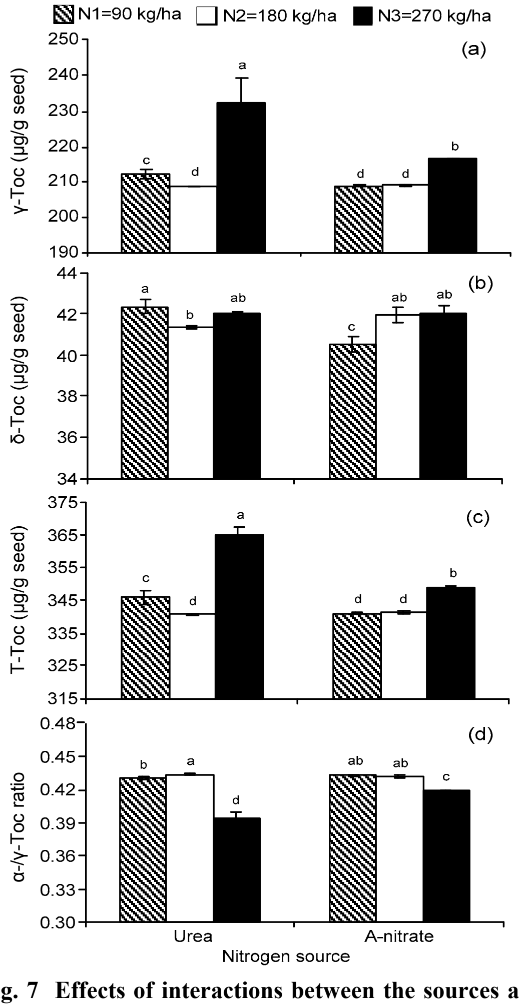

**Fig. 7 Effects of interactions between the sources and application rates of N on tocopherol (Toc) contents in *Brassica napus* L. seeds**

(a) γ-Toc; (b) δ-Toc; (c) T-Toc; (d) α-/γ-Toc ratio. Data are expressed as mean±SD ($n$=4). Different letters above the column show the significant differences among the nitrogen application rates at two nitrogen sources

#### 3.4.4 Correlations between Toc isomers, T-Toc, and the α-/γ-Toc ratio

To determine the effects of N sources, N application rates, and genotypes, correlations among Toc isomers, T-Toc, and the α-/γ-Toc ratio were analyzed using Pearson's method (Table 2). Toc isomer contents were highly, significantly ($P<0.001$), and positively correlated with T-Toc content. α-Toc content was also positively correlated with the α-/γ-Toc ratio. In contrast, γ-Toc and T-Toc contents were negatively correlated with the α-/γ-Toc ratio.

**Table 2 Pearson's correlation between tocopherol (Toc) isomers, total Toc, and α-/γ-Toc ratio**

| Toc isomer | α-Toc | γ-Toc | δ-Toc | T-Toc | α-/γ-Toc ratio |
|---|---|---|---|---|---|
| α-Toc | 1 | $0.396^{***}$ | $0.942^{***}$ | $0.778^{***}$ | $0.499^{***}$ |
| γ-Toc | | | $0.427^{***}$ | $0.879^{***}$ | $-0.586^{***}$ |
| δ-Toc | | | | $0.799^{***}$ | $0.403^{***}$ |
| T-Toc | | | | | $-0.147^{***}$ |

$^{***}$ Significant at $P\leq0.001$

## 4 Discussion

### 4.1 Response of Toc to N sources and application rates

Urea [$CO(NH_2)_2$] contains 45% to 46% N and is the major nitrogenous fertilizer in crop production. A-nitrate [$NH_4NO_3$] contains 34% N and is also applied as a nitrogenous fertilizer of crops. $NH_4NO_3$ contains ammonium and nitrate. This compound is less concentrated than urea. In principle, the form of N influences the yield in terms of crop physiology and fertilizer management by affecting the subsequent shoot growth, phytohormone balance, energy, and carbohydrate status of plants (Rathke *et al.*, 2006). In the present study, N sources significantly affected α-, γ-, and T-Toc contents. We found that urea as a source of N yielded more seed α-, γ-, and T-Toc compared with A-nitrate. The mechanisms of $NH_4^+$ and $NO_3^-$ uptake and assimilation include three important steps: (1) N uptake from the soil solution, (2) translocation to the site of assimilation, and (3) assimilation of inorganic N into organic N (Bloom, 1988). In addition, roots have a cation exchange capacity (CEC) because of the negative charges in the cell wall. This root CEC attracts positive ions (cations) such as ammonium ($NH_4^+$) but repels negative ions (anions) such as nitrate ($NO_3^-$). Urea fertilizers are rapidly transformed to the ammonium form in soils. $NH_4^+$ is assimilated immediately in the roots via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Bloom, 1988). Nitrate assimilation can occur in either the roots or the leaves by the nitrate reductase-nitrite reductase pathway. Such assimilation yields $NH_4^+$, which subsequently enters the GS-GOGAT pathway (Evans, 2001). However, the N of nitrate has a weak affinity to form surface complexes with soil minerals (Strahm and Harrison, 2006) because of its low degree of adsorption. Nitrate promotes organ elongation, whereas ammonium nutrition increases the yield components quantitatively in crop plants (Camberato and Bock, 1990a; 1990b; Gerendás and Sattelmacher, 1990). Thus, efficient conversion of urea into ammonium is more advantageous than A-nitrate in terms of regulating physiological processes in leaves to achieve higher production of Toc molecules in plastids. As a result, a more active source-sink (leaf-seed) relationship is associated with high rates of Toc assimilation in seeds.

The responses of γ-Toc, T-Toc, and Toc composition to different N application rates were significant. We observed a slight decrease in Toc contents in N2 compared with N1. For the growth and yield components (Table S2), N1- and N2-treated plants had almost the same plant height, but significantly higher numbers of branches and siliques were observed in N2-treated plants. No significant difference was observed between N1- and N2-treated plants in the number of seeds per silique. Seeds from N2-treated plants weighed less than those from N1-treated plants, indicating that the proportion of source to sink translocation during photo-assimilation was impaired in N2-fertilized plants. N2-fertilized plants also may not be supported by a balanced supply of exogenous N at later growth stages. Thus, tissue-assimilated N was utilized in vegetative and reproductive organ development simultaneously, resulting in lower Toc contents. In contrast, the assimilation of γ-Toc in *B. napus* seeds occurred mostly in plants fertilized with a higher dosage of N (such as N3), thereby yielding high T-Toc. These findings could be correlated with agronomic traits that contributed to the production of yield components in response to N3 (Table S2). To the best of our knowledge, only Egesel *et al.* (2008) have reported the effects of N fertilizer doses on seed Toc contents in oilseed rape. They examined the relationship between genotypic variation and three different nitrogen application rates, but the effect of N sources has not previously been investigated. Our findings are partially in agreement with those of Egesel *et al.* (2008) who reported that N-fertilized plots yielded higher levels of Toc, particularly γ-Toc, than non-fertilized plots, but they could not find any significant difference in T-Toc content between applications of 130 and 260 kg N/ha. In contrast, our results revealed a highly significant difference in T-Toc content between applications of 180 and 270 kg N/ha. High Toc contents were also observed in N3. Thus, a significant change in Toc contents following applications of >130 kg N/ha was not observed by Egesel *et al.* (2008) because N losses (by leaching) are higher under field conditions than in pot experimental conditions in a greenhouse. α-Toc did not show a significant response to N rates, and a higher α-/γ-Toc ratio was obtained with N2. A decrease in the index value was observed as γ-Toc production was further increased in N3. The availability of the last enzyme (γ-Toc methyltransferase) required in α-Toc synthesis could be the rate-limiting factor because γ-Toc is the immediate precursor of α-Toc (DellaPenna and Pogson, 2006).

## 4.2 Variation in Toc contents of the five genotypes

The genetic makeup of a crop is the main factor affecting the differences among various species of the same genus. Zheshuang 72, Zheshuang 758, Shiralee, and Pakola are double-low canola types, whereas Jiu-Er-1358 is a double-high type. Differences in agronomic traits were observed among all of the genotypes (Table S2). Variation in the contents of Toc and its isomers has been previously reported in oilseed crops (Dolde *et al.*, 1999; Rocheford *et al.*, 2002; Velasco *et al.*, 2002). In our study, a significant amount of genotypic variation was observed in seed T-Toc, Toc isomers, and Toc composition. Zheshuang 72 produced the highest T-Toc content. Agronomic traits such as a low number of branches and a moderate number of siliques per plant compared with other genotypes, resulted in efficient translocation of photoassimilates at the later stages of growth and development of the seeds. As a result, higher γ-Toc content in the seeds was recorded. This finding could explain why Zheshuang 72 seeds had higher T-Toc content, because γ-Toc is the predominant isomer in *B. napus* seeds. These findings are consistent with those of Tan (1989) and Demurin *et al.* (1996) who reported the predominance of γ-Toc in *B. napus* seeds. Toc composition is defined in terms of the α-/γ-Toc ratio, an important index used to measure seed quality. In the present study, an α-/γ-Toc ratio of 0.428 was obtained for Pakola. Studies have suggested that both α- and γ-Toc have important anti-oxidative and anti-inflammatory properties that may have preventative functions in chronic disease states associated with increased inflammation and oxidative stress (Brigelius-Flohe and Traber, 1999; Azzi and Stocker, 2000; Jiang *et al.*, 2000). Recently, Zhang *et al.* (2012) reported that Toc (vitamin E) intake, either from the diet or from supplements, may reduce the risk of liver cancer. Thus, the classification of large numbers of *B. napus* genotypes based on their Toc contents or α-/γ-Toc ratio should be further investigated to improve oilseed.

### 4.3 Interactions between the factors and Toc

N source and assimilation patterns can greatly influence intra-plant N status (Yoneyama and Kaneko, 1989; Yoneyama *et al.*, 1991; Evans *et al.*, 1996). $NO_3^-$, if used as a primary source, can induce significant intra-plant variation in N distribution, whereas $NH_4^+$ causes only slight variation. The contrasting patterns of intra-plant variation are probably caused by different assimilation patterns (Evans, 2001). Urea is rapidly transformed into the $NH_4^+$ form in the soils. $NH_4^+$ is assimilated immediately by the roots (Bloom, 1988). Therefore, organic N in shoots and roots is the product of a single assimilation event. By contrast, $NO_3^-$ assimilation can occur in both roots and shoots. For a majority of Toc and its isoforms, urea was proven as the most efficient source of N to achieve a positive interaction with genotypes or N application rates and obtain higher Toc values. The interaction effects of N application rates with genotypes or N sources suggested that N3 resulted in greater γ- and T-Toc contents in most combinations. Oilseed rape requires high amounts of N and the positive impact of N on the seed yield of winter oilseed rape has been described frequently (Bilsborrow *et al.*, 1993; Sieling and Christen, 1997; Sieling *et al.*, 1997; Behrens *et al.*, 2001; Rathke *et al.*, 2005; 2006). However, the efficiency of N can vary among genotypes. Differences among genotypes in the distribution of $NO_3^-$ reductase in a plant could also result in variation in discrimination during uptake. Differences between shoot and root N of plants grown with $NO_3^-$ were also observed, because nitrate reductase may be present in only roots, shoots or in both (Evans, 2001). Our results are consistent with those of Goffman *et al.* (1999) who reported significant correlations between the four Toc isoforms in Brassicaceae oils. A positive relation between γ-Toc and δ-Toc in soybean seeds has also been reported (Britz and Kremer, 2002). In contrast to our findings, γ-Toc is negatively correlated with δ-Toc and T-Toc in pumpkin seed oils (Stevenson *et al.*, 2007). Toc composition is often expressed in the form of the α-/γ-Toc ratio. α-Toc was positively correlated with Toc composition, whereas γ-Toc and T-Toc were negatively correlated with the α-/γ-Toc ratio, indicating the dominance of γ-Toc in oilseed rape. These results also supported our previous findings about the correlations between Toc isoforms and composition in 52 landraces and 15 breeding lines of *B. napus* (Li *et al.*, 2013).

Although our study provided insights into the effects of N sources and application rates on seed Toc in oilseed rape, our results pointed out a possible interaction between Toc and chlorophyll contents in plastids, and between Toc and N fertilizer sources and/or application rates. With the recent characterization of chlorophyll-derived phytol and phytyl phosphate kinase from *Arabidopsis* (Ischebeck *et al.*, 2006; Valentin *et al.*, 2006), it has been shown that the prenyl moiety of γ-Toc biosynthesis is derived from free phytol in seeds, indicating that phytol is recycled during chlorophyll breakdown (Peisker *et al.*, 1989; Rise *et al.*, 1989; Dörmann, 2007). Thus, the optimum plant growth caused by high N results initially in high photoassimilate storage in photosynthetic tissues and subsequently in efficient translocation to seeds. This observation may account for the increase in Toc content of seeds from N3-treated plants compared with those from plants treated with low rates of N in the current study. Increased N application rate was also found to be correlated with increased protein accumulation and concentration in canola seeds (Asare and Scarisbrick, 1995; Grant *et al.*, 2002; Lemke *et al.*, 2009). Furthermore, pot experiments used in nutrient studies, especially those involving N treatments, should be given preference compared with field research for evaluating genotypic performance. Crop recovery of applied N fertilizer is <50% during the year of application and about 65% over a period of five growing seasons (Krupnik *et al.*, 2004). In addition, results from studies in controlled environments can allow clearer interpretation of the treatment effects, such as N sources and application rates. Such studies can also minimize hidden errors, including leaching, immobilization, volatilization, and denitrification in field conditions (Tomar and Soper, 1987; Malhi *et al.*, 2007).

## 5 Conclusions

In summary, the data presented in this study demonstrated variation in seed Toc contents in oilseed rape based on N sources, N application rates, and genotypes. The following points can be drawn from this study: (1) Nitrogen, as the most limiting nutrient, should be further investigated for proper management. (2) Urea, as a source of N, was proven to be more efficient than A-nitrate in terms of higher Toc production per unit seed weight. (3) High rates of N should be preferred compared with low rates for oilseed rape because N3 exhibited the highest efficiency by producing more Toc contents per unit seed weight. (4) The potential concentrations of several metabolites, such as Toc, in oilseed crops are at a micro level. Therefore, a slight but significant difference because of factors such as N sources and application rates must be given importance and consideration. (5) Zheshuang 72 showed the highest genotypic variation in terms of seed Toc. Large germplasm pools of *B. napus* (including genotypes, breeding lines, and land races) should be evaluated to determine their metabolite profiles and correlative studies are needed to ensure better food and feed quality. In addition to Toc and fatty acids, other substances such as phytosterols, carotenoids, retinols, and terpenoids are important metabolites that should be studied comprehensively in *B. napus* to determine seed oil traits that are related to N fertilizer. (6) Comprehensive molecular studies are recommended to determine the functions of N in regulating metabolites that are used as seed quality indices.

## Compliance with ethics guidelines

Nazim HUSSAIN, Hui LI, Yu-xiao JIANG, Zahra JABEEN, Imran Haider SHAMSI, Essa ALI, and Li-xi JIANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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## List of electronic supplementary materials

Table S1 Treatment combinations of genotype×fertilizer sources×fertilizer application rates

Table S2 Response of agronomic traits in oilseed rape to nitrogen fertilizer sources and application rates

Data S1 Materials and methods

## 中文概要：

**本文题目：**氮肥种类与施量对油菜种子生育酚的影响

**Response of seed tocopherols in oilseed rape to nitrogen fertilizer sources and application rates**

**研究目的：**生育酚是菜籽重要的品质指标，氮肥是影响作物生物学与籽粒产量最常用的肥料。这项研究旨在搞清楚氮肥种类与施量对油菜种子生育酚含量与组分的确切影响，以及这种影响在基因型之间的差异。

**创新要点：**这篇论文研究了不同的氮肥种类（硝态氮与铵态氮）与低、中、高施用量对种子生育酚总量与组分的影响，并分析了其中的原因，为通过合理的氮肥施用方案配置，以达到最理想的菜籽生育酚含量或组分提供依据。

**研究方法：**采用盆钵实验控制氮肥施量与流失的精准方法，五种基因型、二种氮肥种类、三档施量水平，三重复控制误差；尝试用气相色谱法检测菜籽生育酚含量的新方法。

**重要结论：**尿素比硝酸氨更有利于菜籽总生育酚、阿尔法生育酚及伽马生育酚的有效形成；提高氮肥施量对于菜籽形成高含量的总生育酚与伽马生育酚非常有效，但对提高菜籽阿尔法生育酚含量的效果却不太明显。

**关键词组：**油菜；氮肥施量；尿素；硝酸氨；生育酚