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Abstract:	This study investigated the impact of controlled germination on colour, carotenoids and tocopherols of Andean lupin (Lupinus mutabilis Sweet) seeds. Two Andean lupin ecotypes, Cholo fuerte and Altagracia, were germinated in the dark for two, four and six days; colour coordinates (L*, a*, b*) were determined by colorimeter, carotenoids and tocopherols by HPLC. The germination significantly modified all the characteristics examined. The luminosity decreased with increasing germination time, while only minor changes were recorded for a* and b*. The sum of all carotenoids increased up to 12-fold during germination, with lutein always being the most abundant compound. The predominant tocol was γ-tocopherol; although total tocol content was almost unchanged, α-tocopherol increased from 0.7 to 74.8 mg/kg DM, improving by 4.3- fold the vitamin E activity, while γ-tocopherol decreased progressively. Our results demonstrate that germination promises to be a cost-effective approach to improve the nutritional properties of lupin seeds.
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Changes in colour, tocopherols and carotenoids during the germination of lupin seeds

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Dear Editor,

We are submitting our article “Changes in colour, tocopherols and carotenoids during the germination of lupin seeds” as a Short Communication for publication consideration in Journal of Food Composition and Analysis.

In the article we present the results of our research on the effects of controlled germination on colour, carotenoids and tocols of lupin seeds. To this end, seeds of two Andean lupin (*Lupinus mutabilis*) ecotypes were germinated in the dark for two, four and six days. Before (zero days or control) and during germination we assessed the colour coordinates with a colorimeter as well as the carotenoids and tocols composition and content by HPLC.

The germination significantly modified the characteristics examined. The luminosity decreased, the carotenoids increased and the tocols composition was deeply altered, improving the biological activity, because α-tocopherol increased while γ-tocopherol decreased. Therefore, germination in the dark promises to be a cost-effective approach to improve the nutritional properties of Andean lupin seeds.

We hope you will find our research interesting and suitable for publication in Journal of Food Composition and Analysis.

Looking forward to further word in due time, I remain sincerely yours

Andrea Brandolini

Highlights

- Seeds of two Andean lupins were germinated in the dark for two, four and six days
- The content of carotenoids (mainly lutein) augmented during germination
- The α-tocopherol increased while the γ-tocopherol decreased during the germination
- The increase in carotenoid and α-tocopherol improved lupins biological activity
- Germination is cost-effective in enhancing the nutritional value of Andean lupins

ABSTRACT

This study investigated the impact of controlled germination on colour, carotenoids and tocopherols of Andean lupin (*Lupinus mutabilis* Sweet) seeds. Two Andean lupin ecotypes, Cholo fuerte and Altagracia, were germinated in the dark for two, four and six days; colour coordinates (*L**, *a**, *b**) were determined by colorimeter, carotenoids and tocopherols by HPLC. The germination significantly modified all the characteristics examined. The luminosity decreased with increasing germination time, while only minor changes were recorded for *a** and *b**. The sum of all carotenoids increased up to 12-fold during germination, with lutein always being the most abundant compound. The predominant tocol was γ-tocopherol; although total tocol content was almost unchanged, α-tocopherol increased from 0.7 to 74.8 mg/kg DM, improving by 4.3- fold the vitamin E activity, while γ-tocopherol decreased progressively. Our results demonstrate that germination promises to be a cost-effective approach to improve the nutritional properties of lupin seeds.

Key words: vitamin A, vitamin E, lutein, lupin, sprouting.

1. Introduction

Lupins are appreciated for the composition of their seeds, rich in proteins (32-55 g/100 g DM), lipids (6-25 g/100 g DM), and minerals (2.4-5.2 g ash/100 g DM); their carbohydrates content (26-48 g/100 g DM) (Bähr et al., 2014; Carvajal-Larenas et al., 2016) is inferior to other *Faboideae*. Unfortunately, lupins are also rich in bitter and/or toxic alkaloids, that must be removed before consumption (Estivi et al., 2022a). *Lupinus mutabilis* Sweet, the Andean lupin, is traditionally cropped in cool mountain areas (2000-3800 m asl) of South America (Suomela et al., 2022). Its

diffusion to temperate regions is still limited because of lower yields in comparison to other Mediterranean species, possibly due to poor adaptation of the tested genotypes and dearth of improved varieties. The protein content is comparable to the other cropped lupin species, but the Andean lupin displays a lipids content (13.0-24.6 g/100 g DM; Briceño Berru et al., 2021; Carvajal-Larenas et al., 2016) higher than those of *L. albus*, *L. angustifolius* and *L. luteus*.

Lupin flour is used to enrich many products, such as cakes, snacks, burgers, biscuits, baby food and plant-based products, as well as a soy alternative in vegan milk and meat (Cremer, 1983; Ruales et al., 1988; Güemes-Vera et al., 2008). In fact, lupin isolates and protein concentrates have sensory, physical and functional properties (absorption of water and fats, stabilizing capacity, emulsifying and foaming activities, and gelling capacity) very similar to those of soy (Doxastakis, 2000; Gueguen & Cerletti, 1994; Moure et al., 2006).

Seed germination plays a fundamental role in the catabolism of storage compounds and anabolism of new molecules with positive nutritional properties, including those with antioxidant properties such as carotenoids and tocols. Germination is an inexpensive process to improve lupin flour quality by increasing antioxidant capacity, vitamin C and E activities (Fernandez-Orozco et al., 2006; Frias et al., 2005), reducing antinutritional factors like phytic acid (Dagnia et al., 1992; Mohammed et al., 2017) and oligosaccharides (Chilomer et al., 2013; Kaczmarska et al., 2017), and even by enhancing its flavour (Kaczmarska et al., 2018). Several studies report significant improvements in carotenoid and tocol contents in cereals (e.g. Aborus et al., 2018; Yang et al., 2001; Ziegler et al., 2016) as well as in different legumes, such as chickpeas (Khattak et al., 2008), soy and green mung bean (Mastropasqua et al., 2020), soy (Gu et al., 2017), mung bean (Alkaltham et al., 2020), green lentils, kidney beans and black beans (Riddoch et al., 1998), and 50 different species of *Fabaceae* (Fernández-Marín et al., 2017). Actually, Gan et al. (2017) defined

47 germination as a “green food engineering method”, because tocopherol content and antioxidant
48 capacity increase in seeds of diverse plant species, and even a short germination leads to a
49 significant improvement in nutritional quality, contributing to reduce the risk of malnutrition and
50 certain chronic diseases.
51 Therefore, aim of this research was to study the effect of germination in the dark up to six days
52 on colour and content of tocopherols and carotenoids of seeds from two ecotypes of Andean lupin,
53 Cholo fuerte and Altagracia.
54
55 **2. Materials and methods**
56 *2.1 Materials*
57 Two *Lupinus mutabilis* ecotypes from different regions of Peru (Altagracia, from La Libertad,
58 and Cholo fuerte, from Ancash), collected in November 2018, were kindly provided by the
59 Programa de Leguminosas y Oleaginosas of the Universidad Nacional Agraria la Molina, Lima,
60 Peru.
61
62 *2.2 Germination*
63 The seeds were disinfected with 0.1% sodium hypochlorite for 2 min, rinsed three times with
64 distilled water and immersed in water at room temperature (19-21 °C) for 12 h. After draining, the
65 seeds (200 g) were transferred to sterile stainless steel trays (26.5 x 35.5 cm), incubated in the dark
66 for 2, 4 and 6 days at 15 ±1 °C in an A-3920 Achieva germinator (Seedburo Equipment Company,
67 Des Plains, IL, USA), and sprayed with water six times a day for 8 s. The germinated seeds were
68 dried for 12 h at 50 °C, ground with a knife mixer (Braun, Kronberg im Taunus, Germany) for 30

seconds and passed through a 1.0 mm sieve. The flours were then placed in heat-sealed vacuum bags and stored at -20 °C.

The trials and the analyses were performed in triplicate.

2.3 Colour

The colour analysis, performed using a Chroma Meter CR-II tristimulus colorimeter (Minolta Italia S.p.A., Milan, Italy), using the standard-white reflector plate and illuminant C, led to the determination of the coordinate values of *L** (luminosity), *a** (red-green) and *b** (yellow-blue).

2.4 Carotenoids and tocopherols

The moisture was determined gravimetrically according to method 44–15.02 (AACC International). Carotenoids and tocopherols were extracted by thermal saponification and quantified by normal-phase HPLC as outlined by Brandolini et al. (2022). For carotenoid and tocopherol peak quantification, calibration curves were built using standards of lutein (Fluka, St. Louis, MO, USA), β-carotene (Sigma, St. Louis, MO, USA), zeaxanthin, β-cryptoxanthin (Extrasynthese, Genay, France), α-tocopherol (Fluka BioChemika, Buchs, Switzerland), β-tocopherol, γ-tocopherol, and δ-tocopherol (Supelco, Bellefonte, PA, USA). The tests were performed on three independent samples and the results are expressed as mg/kg DM.

2.5 Statistical analysis

To evaluate the effect of the different treatments and lupin ecotypes, the data were processed by two-way analysis of variance (ANOVA), considering germination time and genotype as factors. When significant differences ($p \leq 0.05$) were found, Fisher's least significant difference (LSD) was

computed at a 95% significance level. All analyses were performed using the statistical programme STATGRAPHICS® Centurion XVI (Statgraphics Technologies, Inc., The Plains, USA). Mean and standard error were calculated using the programme Excel 2016 (Microsoft®, Redmond, USA).

3. Results and discussion

3.1 Analysis of variance

The two-way ANOVA (Supplementary Table 1**)** evidenced that germination time had a highly significant effect on colour and carotenoid content; genotype and germination time-x-genotype interaction, even when significant, were far less relevant. On the other hand, germination time influenced all four tocopherols but was preponderant only for α-tocopherol, while the genotype was significant for β-, γ- and δ-tocopherol and was particularly relevant for γ-tocopherol; the time-x-genotype interaction was significant only for β-tocopherol content, albeit of minor importance.

3.2 Colour

The average values of the *L**, *a** and *b** coordinates of the Cholo fuerte and Altagracia seeds are presented in Table 1. At day 0 the Altagracia seeds were slightly less luminous than the Cholo fuerte ones, but the difference disappeared during germination. The *L** of both ecotypes increased significantly after two days of germination and slowly decreased afterward. The *a** component (red-green), instead, showed a progressive shift towards a green hue, while the *b** component (blue-yellow) after an initial slight decrease moved towards the yellow tinge. Hence, the increasingly darker and yellow-green colour of the flours (Fig. 1). Not many studies on lupin colour are present in literature. The *L. albus* flours studied by Mohamed & Rayas-Duerte (1995)

showed comparable values (*L**: 82.8, *a**: -2.0, *b**: 21.3), while those of *L. angustifolius* analysed by Rumiyati et al., (2015) were more luminous (*L**: 90.6, *a**: 1.3, *b**: 28.5). Briceño Berru et al. (2021) studied 33 different *L. mutabilis* ecotypes and recorded luminosities ranging from 67.2 in dark-hulled samples to to 87.5 in white-hulled samples, while *a** ranged between 0.3 and -3.4, and *b** varied between 22.0 and 33.3.

3.3 Carotenoids

The changes in carotenoid composition and content upon germination are illustrated in Fig. 2. For all compounds and for their total content an increasing trend of the values during sprouting is evident. As mentioned above (paragraph 3.1), the results of the two Andean lupins were not significantly different. Lutein was the most abundant carotenoid both before (1.5 mg/kg DM) and during germination (13.6 mg/kg DM after six days), followed by (α+β)-carotene and zeaxanthin; β-cryptoxanthin, absent in non-germinated seeds, increased up to 1 mg/kg DM after six days. The greatest percentual growth after six days occurred for (α+β)-carotene (3000%, i.e. from 0.2 to 6.2 mg/kg DM) and, of course, β-cryptoxanthin (not computable, going from not detectable to 1.0 mg/kg DM). Similar results were recorded during germination in the dark of soybean by Gu et al. (2017), who observed a hundred times increase of β-carotene content after four days; in *Vigna radiata* by Alkaltham et al. (2020), who recorded a 210% augment in total carotenoids after 21 h; and in chickpeas by Khattak et al. (2008), who spotted a 78% surge in total carotenoids after four days. The positive effect of germination on carotenoids content was evident also in cereals. For example, Hidalgo et al. (2019) recorded that germinated wheat and barley seeds reached concentrations (82.6–119.7 mg/kg DM) not far from highly-touted vegetables such as orange carrots (152 mg/kg fresh weight; Surles et al., 2004) and spinach (176.6–226.3 mg/kg fresh weight;

Kidmose et al., 2001); lutein was always the predominant carotenoid, but the (α+β)-carotene underwent sizeable increases.

According to Rodríguez-Villalón et al., (2009), in seeds germinated in the dark the methylerythritol 4-phosphate pathway provides the precursors for carotenoids synthesis. Furthermore, the biosynthesis of a carotenoids key precursor, geranylgeranyl diphosphate (DellaPenna & Pogson, 2006) is active during germination (Beck et al., 2013). When aetioplasts differentiate in the dark into chloroplasts, α-carotene, β-carotene, lutein and violaxanthin accumulate, while chlorophylls are synthesized only after exposure to the light.

3.4 Tocopherols

The tocopherol content of the two Andean lupin ecotypes is depicted in Fig. 3. In the non-sprouted seeds, Cholo fuerte showed a higher total tocopherol content than Altagracia (227 ± 25 and 167 ± 23 mg/kg DM, respectively), with a clear prevalence (94–98%) of γ-tocopherol, followed by the δ-, β- and α- homologues. These values, as well as γ- tocopherol prevalence, agree with the ranges indicated by Briceño-Berru et al. (2021) and Brandolini et al. (2022) in non-debittered Andean lupins, i.e. 172.1–249.8 and 228–231 mg/kg DM, respectively. During sprouting, changes in individual tocopherols were similar in both ecotypes. On average, α-tocopherol increased from almost undetectable (0.7 mg/kg DM) to 74.8 mg/kg DM, while γ-tocopherol decreased from 189.7 to 127.8 as the germination proceeded. The changes in β- and δ-tocopherol, even when significant, were almost negligible given their scarce amount. Overall, the total tocols quantity did not change substantially during germination, passing from 193.7 before sprouting to 205.0 mg/kg DM after six days. On the other hand, no tocotrienols were detected either before or after germination. Likewise, Frias et al. (2005) in *L. albus* observed a 29% γ-

tocopherol decrease (from 201 to 143 mg/kg DM) after six days of germination, a percentage comparable to ours (33%), while α- increased from 1.9 to 23 mg/kg DM. The same trend was spotted by Fernandez-Orozco et al. (2006) in the first six days of germination of *L. angustifolius*: γ-tocopherol went from 126.7 to 32.5 mg/kg DM, whereas α-tocopherol moved from 3 to 86 mg/kg DM. Similarly, working with *L. albus*, *Glycine max* and *Vigna radiata*, Gan et al. (2017) reported significant changes in α- and γ-tocopherol, accompanied by an increase in antioxidant capacity due to the improved α-tocopherol content.

An increase in α-tocopherol, coupled with a decrease in γ-tocopherol, is a phenomenon observed in several germinating legumes, such as peas (Fordham, Wells, & Chen, 1975), green mung beans (Marero et al., 1991), lentils (Frias et al., 2002), soybean (Lee & Chang, 1993; Plaza et al., 2003), but also in cereals (wheat; Yang et al., 2001) and pseudocereals (*Fagopyrum tataricum*; Zhou et al., 2015).

Our results clearly confirm that the decrease in γ-tocopherol (and, in minor measure, of δ-tocopherol) is compensated by an increase in the α-tocopherol homologue: therefore, it can be assumed that during germination a conversion between the two forms occurs. Jube and Borthakur (2006) report that γ-tocopherol is the precursor of α-tocopherol in the biosynthetic pathway. The conversion reaction is catalysed by the enzyme γ-tocopherol methyltransferase (γ-TMT, EC 2.1.1.95), which introduces a methylation in the -5 position using methionine as a donor of $-CH_3$: therefore, S-adenosyl-L-methionine + γ-tocopherol become S-adenosyl-L-homocysteine + α-tocopherol. The same enzyme catalyses the methylation of δ- tocopherol into β-tocopherol and the corresponding conversions among tocotrienols (Jube & Borthakur, 2006; enzyme.expasy.org). From a nutritional point of view, α-tocopherol has the greatest value because, compared to α-tocopherol (1.0), the vitamin activity of β-tocopherol is equivalent to 0.5, of γ-tocopherol to 0.1

and of δ-tocopherol to 0.03 (WHO/FAO, 2004); consequently, the conversion from γ-tocopherol to α-tocopherol leads to a 10-fold increase in biological activity. In our research the change from day 0 to day 6 was from 20.4 to 88.2 mg α-tocopherol equivalent/kg DM, corresponding to a 332% increase. Germination therefore represents a simple and effective way to improve the nutritional value of lupin flours. In soybean, Tavva et al. (2007), Dwiyanti et al., (2011), and Arun et al. (2014) studied the expression of the gene coding for γ-TMT and found that this enzyme is the factor limiting the conversion rate of γ-tocopherol to α-tocopherol.

In addition to its role as vitamin E, the α-tocopherol homologue has also the greatest antioxidant activity: α-tocopherol > β-tocopherol and γ-tocopherol > δ-tocopherol. Its lower phenolic hydrogen dissociation energy and reduction potential give α-tocopherol the greatest reaction rate with the peroxide radical, 1.5 and 3.4 times higher than those of the γ and δ homologues (Kim & Min, 2008).

Even with a negligible change in the total content (the variation was about +6%), the different behaviour and biological activities of the four tocopherols influence the nutritional properties of *L. mutabilis*. In addition, the lipophilic antioxidants are barely or non-affected by the debittering treatment (Córdova-Ramos et al., 2020); therefore, after germination the debittering of bitter varieties will result in minor or no losses of *ex-novo* synthesized carotenoids and modified tocopherols.

4. Conclusions

During germination, the flours became darker and shifted towards yellow-green; additionally, carotenoid content increased by 12-fold, and a significant transition from γ- to α-tocopherol was observed. These findings suggest that germination is a simple and cost-effective approach to

enhance vitamin A and E activities of the flour of Andean lupin. The increase in health awareness and the shift towards plant-based protein suggest that germinated lupin flour could become a valuable ingredient in the food industry. Further studies should investigate its use in the development of functional foods.

CRediT author statement

Estivi: Data curation, Investigation, Writing – original draft, Writing –review & editing. Pasqual-Chagman: Conceptualization, Writing – review & editing. Santa Cruz Olivos: Conceptualization, Investigation, Methodology, Writing – review & editing. Savasi: Methodology, Writing – review & editing. Brandolini: Data curation, Validation, Writing – original draft, Writing –review & editing. Hidalgo: Conceptualization, Supervision, Data curation, Investigation, Writing – review & editing.

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Data availability. Data will be made available on request.

Supplementary material. Supplementary Table 1 to this article can be found online at

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402

403 **Figures captions**

404 **Fig. 1**. Seeds and flours of the *L. mutabilis* ecotypes Cholo fuerte and Altagracia during the
405 germination.

406 **Figure 2**. The lutein, β-carotene, β-cryptoxanthin, zeaxanthin and total carotenoid content (mg/kg
407 DM) in *L. mutabilis* seeds of Cholo fuerte and Altagracia during the germination.

408 **Fig. 3**. The α-, β-, γ- and δ-tocopherol and total tocopherol content (mg/kg DM) in *L. mutabilis*
409 seeds of Cholo fuerte and Altagracia during the germination.

Figure 1

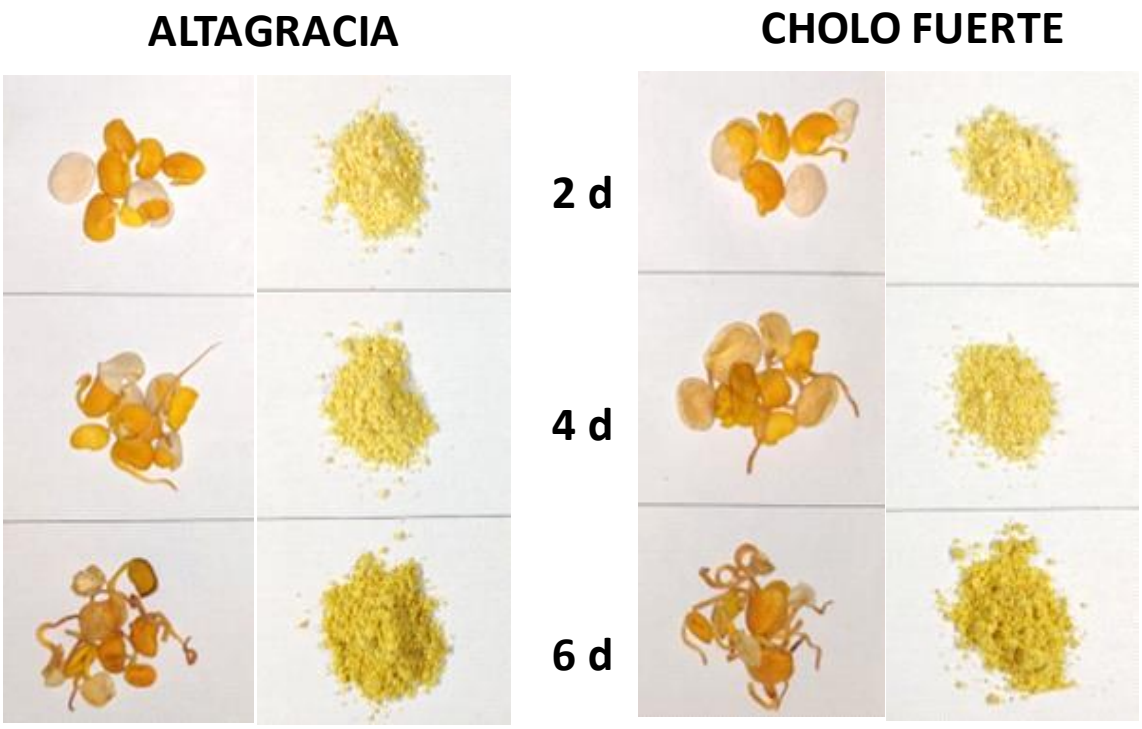

Figure 2

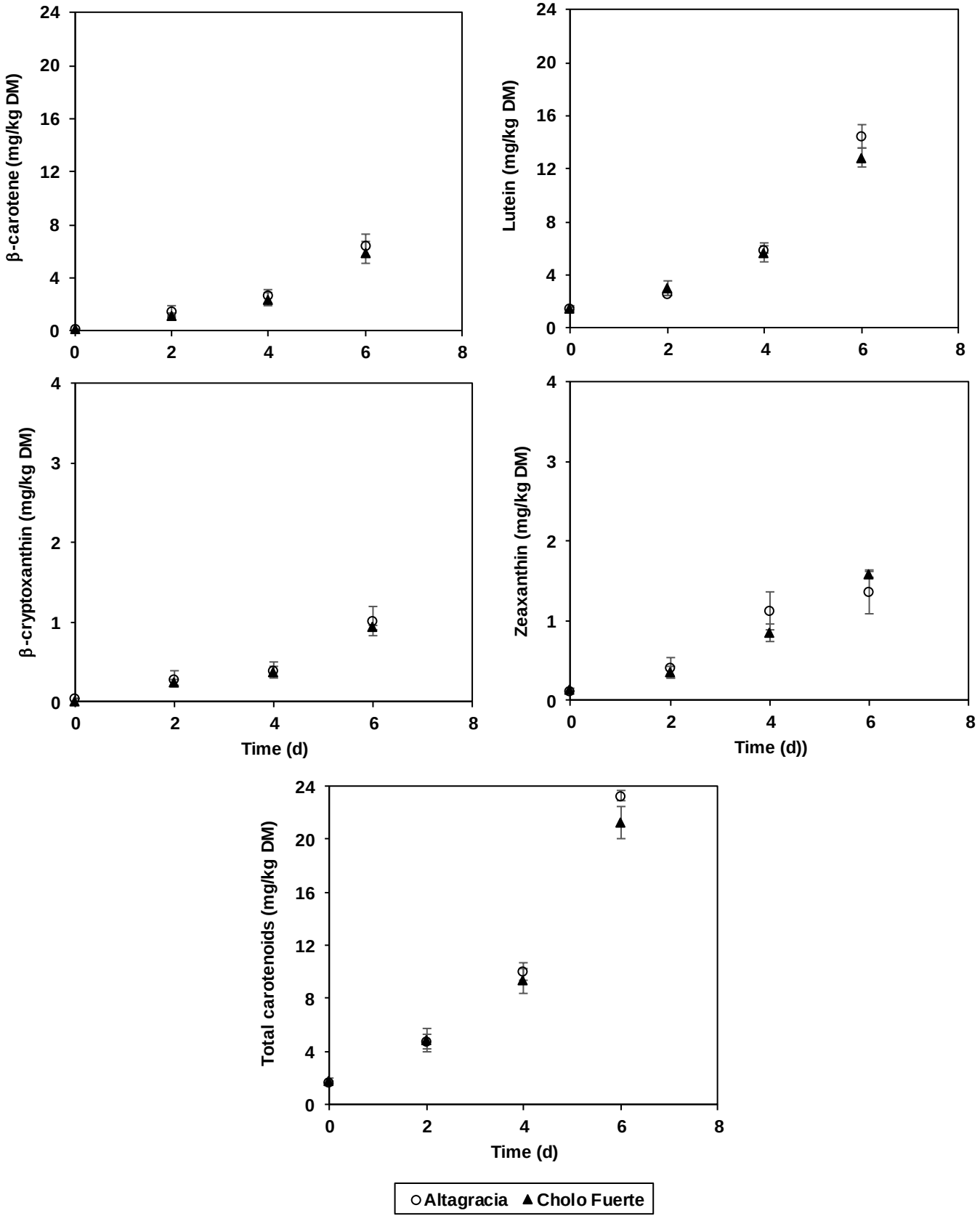

Figure 3

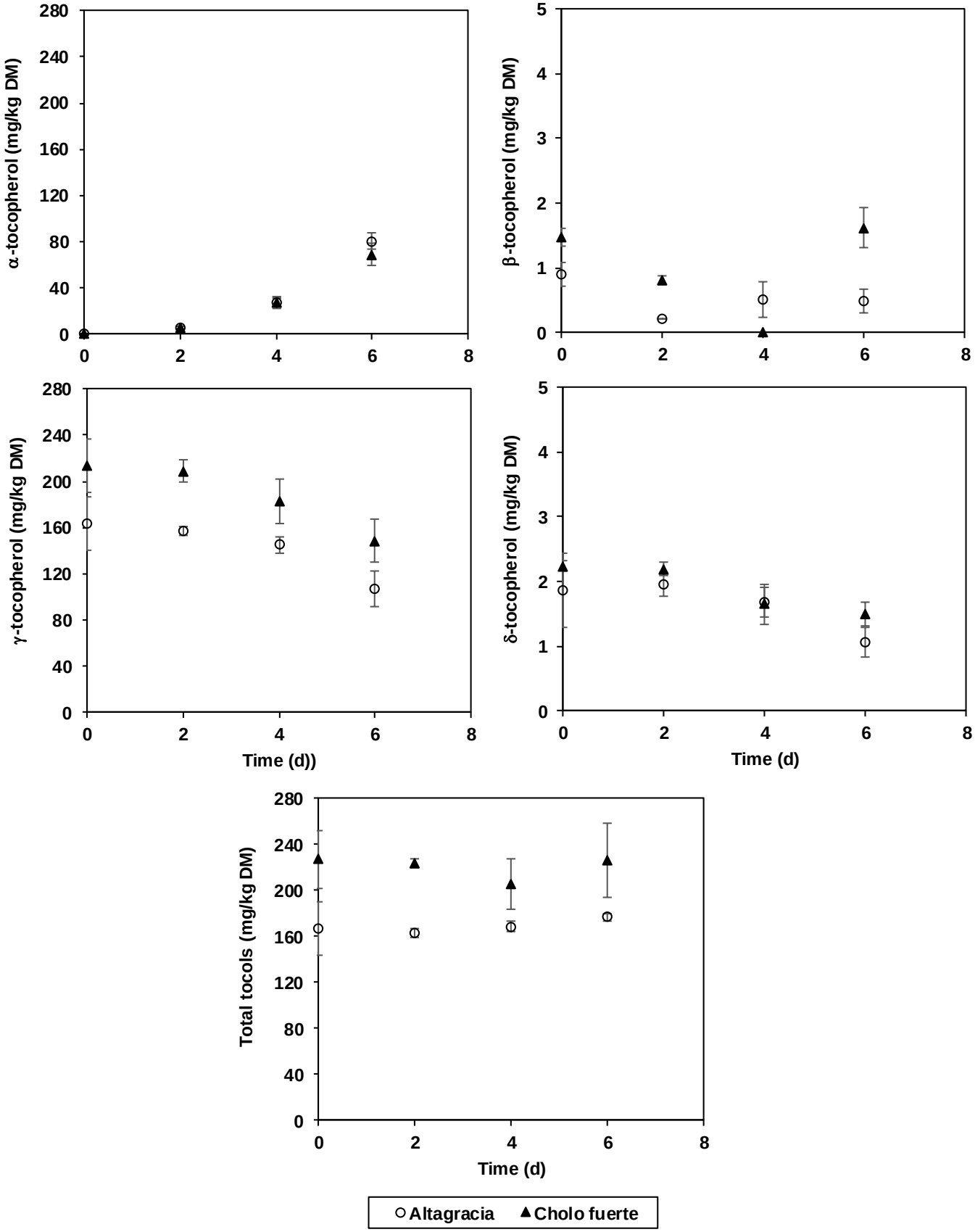

Table 1. Colour parameters results of the two *L. mutabilis* ecotypes. Different letters in the column indicate significant differences among samples at $p \leq 0.05$ according to LSD multiple range test.

	Days	*L**	*a**	*b**
Cholo fuerte	0	$80.9^{c} \pm 0.4$	$-2.4^{a} \pm 0.1$	$27.7^{b} \pm 0.2$
	2	$84.5^{a} \pm 0.4$	$-3.6^{b} \pm 0.1$	$26.5^{c} \pm 0.4$
	4	$83.3^{b} \pm 0.7$	$-4.8^{c} \pm 0.2$	$28.8^{a} \pm 0.6$
	6	$79.8^{d} \pm 0.7$	$-5.4^{d} \pm 0.4$	$32.3^{a} \pm 0.9$
Alta Gracia	0	$76.5^{c} \pm 0.7$	$-0.8^{a} \pm 0.3$	$28.9^{b} \pm 1.0$
	2	$84.9^{a} \pm 1.0$	$-4.1^{b} \pm 0.4$	$26.9^{c} \pm 1.1$
	4	$84.3^{a} \pm 0.7$	$-5.8^{c} \pm 0.1$	$29.4^{b} \pm 0.7$
	6	$79.4^{b} \pm 0.9$	$-6.0^{c} \pm 0.2$	$32.6^{a} \pm 1.1$

Supplementary Table 1. Two-ways ANOVA of colour, tocopherols, and carotenoids in Andean lupin seeds of ecotypes Cholo Fuerte and Altagracia during germination time.

	Time (T)	Ecotype (E)	T x E	Error
d.f.	3	1	3	23
Colour				
*L**	53.4***	4.59**	8.91***	0.46
*a**	21.4***	0.08	2.01***	0.05
*b**	35.1***	2.16	0.26	0.66
Carotenoids				
(α+β)-carotene	40.7***	0.36	0.11	0.26
β-cryptoxanthin	0.98***	0.01	0.001	0.01
Lutein	178.6***	0.61	1.23*	0.29
Zeaxanthin	2.22***	0.003	0.06	0.02
Total	490.9***	2.28	1.46	0.49
Tocopherols				
α-tocopherol	6848.6***	48.2	48.2	22.3
β-tocopherol	1.17***	1.17***	0.73***	0.04
γ-tocopherol	4631.5***	11877.1***	59.8	238.1
δ-tocopherol	0.85***	0.35*	0.07	0.05
Total	226.3	10786.6***	149.2	318.3
d.f.	3	1	3	15
Antioxidant capacity				
DPPHmethanol:H_2O	459.0***	0.72	7.24***	0.41
DPPHhexane	0.17*	0.17	0.03	0.04
ABTSmethanol:H_2O	11780.0***	863.6	103.7	323.2
ABTShexane	1.23*	1.64*	0.37	0.19

d.f: degrees of freedom; level of significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

Title: Changes in colour, tocopherols and carotenoids during the germination of lupin seeds

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Declaration of competing interest

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