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Advanced olive selections with enhanced quality for minor constituents

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SUMMARY: Squalene, phytosterols and tocopherols are minor constituents of paramount importance for the olive fruit and oil quality. The objective of this research was to conduct a two-year evaluation of these compounds in the fruits of seven advanced breeding selections. They were mainly selected for early bearing and high oil content from progenies of crosses between the cultivars 'Arbequina' and 'Picual'. An analysis of variance showed high genotypic effects, non-significant year effects, and genotype x year interactions of low magnitude. The selections showed great variability for the traits, surpassing in some cases the parental values. One selection with total tocopherol content of 263 mg·kg^{-1} fruit flesh, compared to a maximum of 148 mg·kg^{-1} in the parents, and another one with Δ^5-avenasterol concentration of 30.7% of total sterols, compared to a maximum of 22.1% in the parents, were the most relevant phenotypes. These selections may play an important role for improving olive fruit and oil quality for specific market niches.

KEYWORDS: *Breeding; Fruit quality; Olive; Phytosterols; Squalene; Tocopherols*

RESUMEN: ***Selecciones avanzadas de olivo con calidad mejorada para compuestos menores***. Compuestos como el escualeno, los fitoesteroles y los tocoferoles tienen una enorme importancia para la calidad del fruto y del aceite de oliva. El objetivo de este trabajo fue la evaluación durante dos años de estos compuestos en los frutos de siete selecciones avanzadas de olivo, seleccionadas principalmente para entrada temprana en producción y alto contenido en aceite a partir de las descendencias de cruzamientos entre los cultivares 'Arbequina' y 'Picual'. El análisis de la varianza mostró, para la mayoría de los caracteres, un elevado efecto del genotipo, ausencia de efecto del factor año, e interacciones entre año y genotipo de baja magnitud. Las selecciones mostraron gran variabilidad para todos los caracteres, sobrepasando en algunos casos los valores de los parentales. Entre las selecciones con valores superiores a los parentales, destacaron una selección con un contenido en tocoferoles de 263 mg·kg^{-1} pulpa frente a un máximo de 148 mg·kg^{-1} en los parentales, y otra selección con una concentración de Δ^5-avenasterol del 30.7% del total de esteroles, frente a un máximo de 22.1% en los parentales. Estas selecciones pueden desempeñar un papel importante en la mejora de la calidad del fruto y el aceite de oliva para determinados segmentos de mercado.

PALABRAS CLAVE*: Calidad de fruto; Escualeno; Fitoesteroles; Mejora; Olivo; Tocoferoles*

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1. INTRODUCTION

The olive is one of the most ancient domesticated tree crops (Besnard *et al*., 2013). Olive oil consumption is considered a hallmark of the traditional Mediterranean diet, which has been associated with reduced mortality caused by cardiovascular diseases (Psaltopoulou *et al*., 2004). The beneficial nutritional properties of olive oil were traditionally associated with its high content in monounsaturated oleic acid, in contrast to higher poly-unsaturation levels in seed oils until comparative studies with high-oleic seed oils suggested an additional role of minor constituents (Pérez-Jiménez *et al*., 1995). Minor components in olive oil associated with health-promoting effects include polar phenolic compounds, squalene, tocopherols, and sterols (Boskou, 2009).

The necessity of breeding efforts in olive has not been as evident as in other fruit species (Byrne, 2012). In fact, systematic breeding programs have been scarce until very recently, producing only a limited number of cultivars so far (Bellini *et al*., 2008). The main objectives in olive breeding are early bearing, high productivity, resistance to biotic and abiotic stresses, suitability to different growing systems, mechanical harvesting aptitude, and oil content and quality (León *et al*., 2008; Fabbri *et al*., 2009). At the first stages of olive breeding, the focus is mainly placed on vigor and fruit traits. Fruit and oil quality traits are generally considered only in the last selection steps. Several authors have reported improved cultivars/advanced selections with enhanced oleic acid, tocopherol, and/or total phenolic contents (Manaï *et al*., 2007; Baccouri *et al*., 2007; Ripa *et al*., 2008; León *et al*., 2011; De la Rosa *et al*., 2013). Practically no breeding research has been conducted on other oil quality traits such as squalene and phytosterols.

Squalene is one of the most biologically active constituents of olive oil due to its powerful antioxidant and anti-carcenogenic properties (Sotiroudis and Kyrtopoulos, 2008). Chemically, squalene is a terpenoid hydrocarbon synthesized as a biochemical intermediate at the initial steps of the phytosterol biosynthesis. Virgin olive oil is the richest vegetable source of squalene (Boskou, 2009). Phytosterols are compounds that play an important nutritional role by reducing cholesterol absorption due to their similar chemical structure and biological function as cholesterol (Piironen *et al*., 2000). From a technological perspective, phytosterols such as Δ^5-avenasterol that contain an ethylidene group in their structure confer higher antioxidant activity at frying temperatures than other sterols (Rossell, 2001). The presence of this sterol in olive oil has been associated with retarded polymerization in heated triacylglycerols (Boskou, 2011). Tocopherols are compounds with free radical scavenging activity, both *in vivo* (vitamin E) as well as *in vitro*. α-tocopherol, which is the tocopherol homologue with the highest vitamin E activity (Eitenmiller and Lee, 2004), is the predominant tocopherol form in olive oil (Boskou, 2009). Total tocopherol content in olive oil is much lower than in seed oils, including sunflower oil that also contains primarily α-tocopherol (Gunstone and Harwood, 2007).

One of the main constraints for the selection of oil quality traits is the scarce information about their genetic control and heritability (Fabbri *et al*., 2009). Most of the studies have focused on the fatty acid profile, with several studies reporting a great genotypic effect for the concentration of the major fatty acids and subsequent feasibility of selection for this trait (Ayton *et al*., 2001; León *et al*., 2008; Ripa *et al*., 2008; Rjiba *et al*., 2010; De la Rosa *et al*., 2013). For total phenolic content, the effect of the genotype has been found to be non-significant (Ripa *et al*., 2008) or of very low magnitude (El Riachy *et al*., 2011). Conversely, the genotypic effect was reported to be slightly higher than the year effect for tocopherol content, with low genotype × year interaction (Beltrán *et al*., 2010). Temperature and rainfall are the main environmental factors which influence olive oil quality (Aparicio and Luna, 2002). Beltrán *et al*. (2005, 2010) reported higher tocopherol content associated with lower rainfall, though a similar effect has not been observed for different irrigation levels (Gómez-Rico *et al*., 2007). Irrigation has been found to significantly influence the squalene content of olive oil (Martinelli *et al*., 2012). The altitude at which the trees are grown and the degree of maturity of the fruits are also two important factors affecting olive oil quality (Aparicio and Luna, 2002).

Seven olive breeding seedlings coming from crosses between the cultivars 'Arbequina' and 'Picual' were initially selected for their early cropping and oil content (León *et al*., 2004). The selections have been previously evaluated for fruit traits and fatty acid composition (De la Rosa *et al*., 2013). The objective of this research was to evaluate the variability for squalene content and tocopherol and phytosterol contents and profiles in those selections.

2. MATERIALS AND METHODS

The Spanish cultivars 'Picual' and 'Arbequina' and seven breeding selections derived from crosses between them, namely UC-I-22-90, UC-I-2-35, UC-I-32-78, UC-I-36-41, UC-I-36-43, UC-I-37-69, and UC-I-42-48 were used. A detailed pedigree of the selections was previously reported (De la Rosa *et al*., 2013). A field trial was established in an open field in Cabra (Córdoba, Spain) in June 2007 at 6×5 m spacing using plants propagated by semi-hardwood stem cuttings. A random sample of around 500 g of fruits was collected from three trees per genotype (replications) in mid-November of 2010 and 2011. Average maximum, mean and minimum temperature and rainfall for both years are shown in Table 1. The ripening

Table 1. Average annual minimum temperature (Tmin), maximum temperature (Tmax), mean temperature (Tmean), and total rainfall in the experimental field in 2010 and 2011.

Year	Tmin (°C)	Tmax (°C)	Tmean (°C)	Rainfall (mm)
2010	11.0	21.4	15.9	1058
2011	12.2	23.3	17.3	491

index at the time of harvest, averaged over the two years, was 3.5 for 'Picual' and 2.8 for 'Arbequina'. The ripening index for the selections ranged from 2.0 (UC-I-36-43) to 3.8 (UC-I-37-69). Considering all the genotypes, the ripening index was higher in 2010 (3.45) than in 2011 (2.48). More detailed information on the ripening index of individual selections was reported by De la Rosa *et al*. (2013).

A subsample of around 30 g was stored at −80 °C shortly after harvest and lyophilized. The stones were then removed and the flesh was milled in a laboratory ball mill. The ground samples were then stored at −20 °C and analyzed in duplicate for squalene content, phytosterol content and profile, and tocopherol content and profile following procedures previously reported (Velasco *et al*., 2014).

Data were analyzed by the General Linear Model procedure of IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA) using the following model:

$$Yij=\mu+Gi+Yj+GYij+\varepsilon ij$$

Where: Yij is the observation of genotype i in year j; μ is the general mean; Gi and Yj are effects of genotype i and year j, respectively; GYij is genotype × year interaction of genotype i with year j, and εijr is the residual error of genotype i in year j. Genotypes and years were considered as random variables. Pearson's correlation coefficients among oil quality traits were computed using IBM SPSS Statistics 20.0.

3. RESULTS

The analysis of variance revealed a significant effect of genotype for all the traits, whereas the year effect was only significant for tocopherol content and campesterol and γ-tocopherol concentrations (Table 2). The case of tocopherol content was particularly relevant, as the effect of year was much higher than the genotypic effect. The average tocopherol contents were 123 mg·kg^{-1} in 2010 and 180 mg·kg^{-1} in 2011. The genotype × year effect was significant for most of the traits, except for squalene content and concentrations of campesterol and stigmasterol in the phytosterol fraction. When significant, the genotype × year effect was in all cases of low magnitude compared to the main effects.

The cultivars 'Arbequina' and 'Picual' showed very contrasting levels for most of the traits, particularly for squalene contents (1800 and 5207 mg·kg^{-1} dry flesh weight, respectively), phytosterol contents (1485 and 1109 mg·kg^{-1} dry flesh weight, respectively), phytosterol profile (e.g. Δ^5-avenasterol concentration of 22.1 and 10.2% of total phytosterols, respectively), and tocopherol profile (e.g. α-tocopherol concentration of 99.1 and 92.2% of total tocopherols, respectively) (Table 3). The breeding selections derived from them showed great variability for all the traits, covering in most cases the ranges of variation between the parents and presenting for some of the traits lower or higher levels than the parents, e.g. for phytosterol content, concentrations of stigmasterol, β-sitosterol and Δ^5-avenasterol, and total tocopherol content (Table 3). The selection UC-I-36-41 showed a significantly lower phytosterol content than both parents, whereas no selection with significantly higher phytosterol content was identified (Table 3). For phytosterol profile, the selection UC-I-2-35 had a lower β-sitosterol concentration (62.6% of total sterols) and a higher Δ^5-avenasterol concentration (30.7%) than both parents, whereas UC-I-36-41 showed a higher stigmasterol concentration (Table 3). The selection UC-I-37-69 exhibited a higher tocopherol content (263.2 mg·kg^{-1} fruit flesh) than both parents (125.0 mg·kg^{-1} in 'Picual' and 147.9 mg·kg^{-1} in 'Arbequina'). Phenotypes with higher levels than the parents for the mentioned traits were observed for both years. This is shown in Figure 1 for squalene, phytosterol and tocopherol contents, and the concentration of Δ^5-avenasterol. No significant correlations among traits were observed in the average values of

Table 2. Analysis of variance (mean squares) for squalene content (mg·kg^{-1} dry fruit flesh), tocopherol content (mg·kg^{-1} dry fruit flesh), concentration of α- and γ-tocopherol (% of total tocopherols), phytosterol content (mg·kg^{-1} dry fruit flesh), and concentrations of campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol (% of total sterols) in the fruits of seven advanced olive selections and their progenitors 'Arbequina' and 'Picual', grown in Cabra (Córdoba) in 2010 and 2011.

	Df[a]	Squalene	Phytosterol	Campesterol	Stigmasterol	β-sitosterol	Δ^5-avenasterol	Tocopherol	α-T	γ-T
Genotype[b]	8	11119134**	437332**	5.5**	1.3**	243.3**	244.1**	11514**	24.2**	22.9**
Year	1	653915^{ns}	18460^{ns}	3.7**	0.6^{ns}	3.0^{ns}	13.4^{ns}	43814**	0.1^{ns}	9.6*
GxY	8	219379^{ns}	33439**	0.3^{ns}	0.1^{ns}	33.7**	26.8**	1338*	1.3**	1.6**
Error	36	220096	9649	1.0	0.2	5.3	5.0	544	0.1	0.1

[a]df=Degree of freedom.
[b]**=significant at $P\leq.01$; *=significant at $P\leq0.05$; ns=not significant at $P\leq0.05$.

TABLE 3. Two-year average values for squalene content (mg·kg^{-1} dry fruit flesh), tocopherol content (mg· kg^{-1} dry fruit flesh), concentration of α- and γ-tocopherol (% of total tocopherols), phytosterol content (mg·kg^{-1} dry fruit flesh), and concentrations of campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol (% of total sterols) in the fruits of olive cultivars 'Arbequina' and 'Picual' and seven advanced selections derived from them, grown in Cabra (Córdoba) in 2010 and 2011.

	Squalene	Phytosterol	Campesterol	Stigmasterol	β-sitosterol	Δ^5-avenasterol	Tocopherol	α-T	γ-T
Arbequina	1800	1485	3.2	0.7	72.8	22.1	147.9	99.1	0.1
Picual	5207	1109	3.0	1.2	83.7	10.2	125.0	92.2	6.6
Selections	2408–5711	759–1570	1.9–4.9	0.8–2.1	62.6–80.4	10.4–30.7	127.4–263.2	95.8–98.2	0.6–3.0
UC-I-22-90	4895	1563	4.1	1.2	75.8	16.4	129.3	96.8	2.1
UC-I-2-35	4196	1068	3.7	0.8	**62.6**[a]	**30.7**[a]	132.1	97.6	1.2
UC-I-32-78	3311	1356	1.9	0.9	79.6	15.4	146.5	95.8	3.0
UC-I-36-41	2408	**759**[a]	4.0	**2.1**[a]	69.0	22.4	165.6	98.2	0.6
UC-I-36-43	3109	1570	2.9	1.3	76.7	17.8	129.4	97.9	0.7
UC-I-37-69	2672	1107	4.9	1.7	80.4	10.4	**263.2**[a]	97.2	1.9
UC-I-42-48	5711	1288	2.1	0.9	75.8	19.4	127.4	97.4	1.5
$LSD_{0.05}$	777	163	1.7	0.7	3.8	3.7	39	0.6	0.5

[a]Values significantly lower or higher than those of the parents.

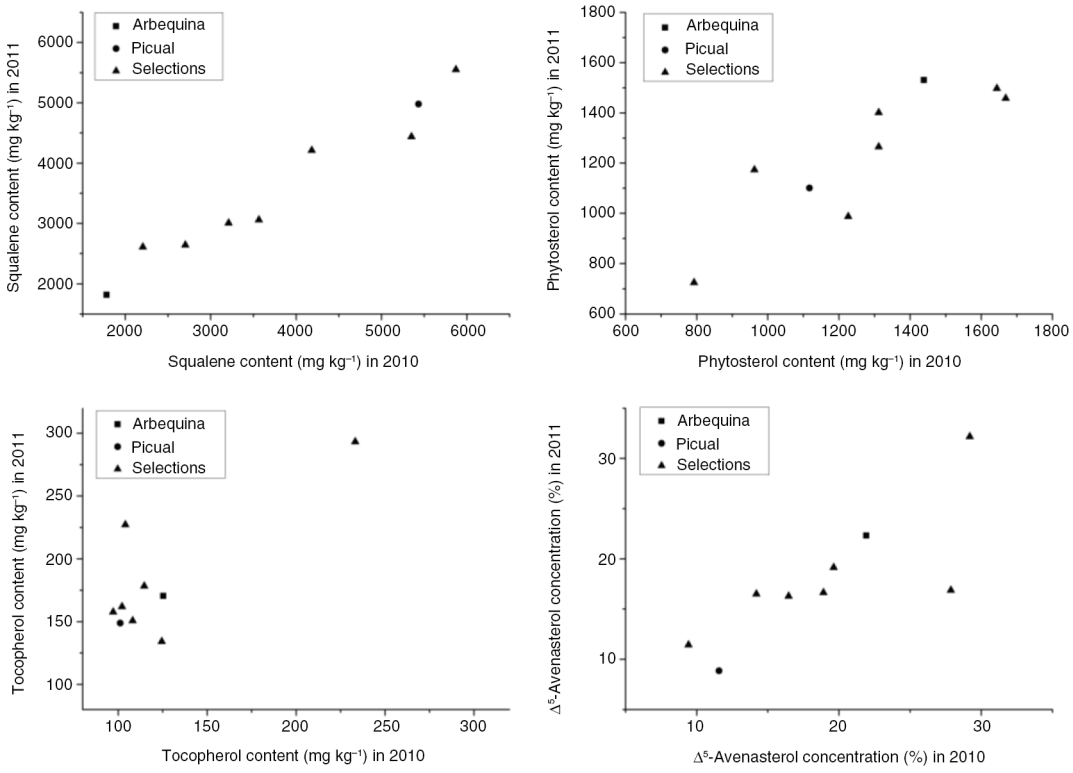

FIGURE 1. Values for squalene, phytosterol and tocopherol contents (mg·kg^{-1} dry weight) and Δ^5-avenasterol concentration (% of total sterols) in the fruit flesh of olive cultivars 'Arbequina', 'Picual', and seven advanced selections derived from them, grown in Cabra (Córdoba, Spain) in 2010 and 2011.

the seven selections, except for the expected negative correlation between the major components of phytosterol and tocopherol profiles, i.e r=−0.96 between the concentrations of β-sitosterol and Δ^5-avenasterol, and r=−0.99 between the concentrations of α- and γ-tocopherol. Two-year average values for the parents and selections are shown in Table 3. It is important to emphasize that no significant correlations were detected between phytosterol contents and the levels of their common precursor, squalene.

4. DISCUSSION

The analysis of variance revealed high genotype effects, non-significant or low-magnitude year effects, and genotype x year interactions of low magnitude. The main exception was total tocopherol content, for which a strong year effect was detected. Previous studies have associated total tocopherol content in olives with annual rainfall, with high annual rainfall reducing the tocopherol content (Beltrán *et al*., 2005; 2010). A similar effect has been observed in the present research; tocopherol contents in the genotypes included in the study averaged 123.3 mg·kg^{-1} in 2010, which was a very wet year with an annual rainfall of 1058 mm, whereas tocopherol content averaged 180.3 mg·kg^{-1} in 2011, a much dryer year with an annual rainfall of 491 mm (Table 1).

The cultivars 'Picual' and 'Arbequina' have contrasting oil qualities, particularly for fatty acid profile, squalene content, phytosterol content and profile, and total phenolic content (Allouche *et al*., 2007). Both cultivars differed largely for squalene content and phytosterol content and profile in the present study. One of the most relevant results of this research was that the progenies from crosses between both cultivars showed segregation for some traits that surpassed the values found in the parents, e.g. for total phytosterol and tocopherol contents, and for the concentrations of stigmasterol, β-sitosterol and Δ^5-avenasterol. Broad segregation in olive progenies has been reported previously for reproductive traits (Ben Sadok *et al*., 2013) as well as for oil quality traits such as fatty acid profile, tocopherol content and profile, polyphenols, and carotenoids (León *et al*., 2011). The most interesting variation identified in the present research consisted of increased levels of Δ^5-avenasterol (30.7% of total sterols) in the selection UC-I-2-35 and tocopherols (263.2 mg·kg^{-1} fruit flesh) in the selection UC-I-37-69 (Table 3).

The typical range of variation of Δ^5-avenasterol in olive oil is between 5 and 20% of total sterols (Boskou *et al*., 2006). Values up to 36% have been reported exceptionally in some Greek oils (Boskou *et al*., 2006), probably reflecting particular environmental and/or processing conditions. Sterol composition in olive oil is affected by cultivar, crop year, geographic factors, degree of fruit ripeness, storage time of fruits prior to oil extraction, and processing (Boskou *et al*., 2006). The cultivars with the highest Δ^5-avenasterol content are Koroneiki (23.9%) (Vekiari *et al*., 2010), Arbequina (23.2%) (Gracia *et al*., 2009), and Leccino (21.4%) (Gül and Seker, 2006). Accordingly, the high Δ^5-avenasterol levels in the selection UC-I-2-35, observed in both years of the experiment (Figure 1), are exceptional within olive oil cultivars. Additional research should determine their effect on the oxidative stability of the olive oil.

Increasing tocopherol content is an important breeding objective for improving the nutritional quality of olive oil. The tocopherol content in virgin olive oil typically ranges from 100 to 250 mg·kg^{-1} oil (Boskou, 2009), although higher values of up to 510 mg·kg^{-1} oil have been reported for some cultivars in single environments (Psomiadou *et al*., 2000; Beltrán *et al*., 2010). Tocopherol content is influenced by the same factors mentioned above for Δ^5-avenasterol, i.e. cultivar, crop year, geographic factors, degree of fruit ripeness, storage time of fruits prior to oil extraction, and processing (Boskou *et al*., 2006). Nitrogen fertilization has also been found to influence tocopherol content in olive oil, with high fertilization levels decreasing tocopherol contents (Fernández-Escobar *et al*., 2006). The maximum average tocopherol content identified in the present research, 263.2 mg·kg^{-1} fruit flesh in selection UC-I-37-69, was exceptionally high compared to the values found in both parents (148 mg·kg^{-1} in 'Arbequina' and 125 mg·kg^{-1} in 'Picual').

Virgin olive oil is the richest vegetable source of squalene, with typical levels between 200 and 7500 mg·kg^{-1} (Boskou, 2009). The squalene content in olive oil mainly depends on the cultivar and the extraction and refining technologies (Wiesman, 2009; Nergiz and Çelikkale, 2011). The squalene content of the extracted oil has been reported to decrease during maturation (Sakohui *et al*., 2011; Ben Mansour *et al*., 2015a). However, Fernández-Cuesta *et al*. (2013) found that, at the fruit pulp level, squalene content was scarcely related to maturation. Squalene content has also been reported to be affected by the geographic area (Ben Mansour *et al*., 2015b) and agronomic practices such as irrigation (Martinelli *et al*., 2012). In the present research, one of the selections (UC-I-42-48) showed high squalene content. Although the squalene content of this selection was not significantly higher than the best parent, 'Picual', the values in the fruits of the selection were higher than in the parent in both years, i.e. 5871 mg·kg^{-1} in UC-I-42-48 compared to 5434 mg·kg^{-1} in 'Picual' in 2010, and 5552 mg·kg^{-1} in UC-I-42-48 compared to 4980 mg·kg^{-1} in 'Picual' in 2011 (Figure 1).

The genotypes included in this study are advanced selections of a breeding program in which the main initial selection criteria have been early bearing and high oil content. They have also been characterized for fatty acid profile and fruit traits (De la Rosa *et al*., 2013). The most promising selections in that research, UC-I-42-48 and UC-I-2-35, show other additional interesting oil quality attributes. For example, UC-I-42-48 showed very high squalene content, whereas UC-I-2-35 showed very high Δ^5-avenasterol content. Interestingly, the latter selection is also characterized by high oleic acid content (De la Rosa *et al*., 2013), which is one of the main factors contributing to oil thermo-stability (Allouche *et al*., 2007). Further research is required to investigate the contribution of Δ^5-avenasterol to the thermo-stability of the oil as

well as alleged synergistic effects between high oleic acid and high Δ^5-avenasterol contents.

5. CONCLUSIONS

The present research identified great genetic variability for squalene and tocopherol contents and phytosterol content and profile in advanced olive selections, including phenotypes with higher levels than the parents for tocopherol content and Δ^5-avenasterol concentration. These selections may play an important role in improving olive fruit and oil quality for specific market niches.

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