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The embryo and the endosperm contribute equally to argan seed oil yield but confer distinct lipid features to argan oil

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ABSTRACT

In the perspective of studying lipid biosynthesis in the argan seed, the anatomy, ploidy level and lipid composition of mature seed tissues were investigated using an experimental design including two locations in Algeria and four years of study. Using flow cytometry, we determined that mature argan seeds consist of two well-developed tissues, the embryo and the endosperm. The lipid content of the embryo was higher than that of the endosperm, but the dry weight of the endosperm was higher. Consequently, both tissues contribute equally to seed oil yield. Considerable differences in fatty acid composition were observed between the two tissues. In particular, the endosperm 18:2 percentage was twofold higher than that of the embryo. The tocopherol content of the endosperm was also markedly higher than that of the embryo. In contrast, the endosperm and the embryo had similar sterol and triterpene alcohol contents and compositions.

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1. Introduction

Argan oil is extracted from the seeds of *Argania spinosa* (L.) Skeels, the only species of the tropical family *Sapotaceae* found in North Africa. It is endemic to Morocco, mostly in the western region where argan forest covers about 800,000 ha (El Mousadik & Petit, 1996), but smaller populations are also observed in Algeria in the district of Tindouf (about 3000 ha; Kaabèche, Benkheira, & de Foucault, 2010). *A. spinosa* can survive extreme arid conditions and plays an essential ecological role in its natural range. Because in addition to oil, the argan tree traditionally provides multiple goods and services such as wood for fibre and fuel, leaves and fruits as forage for local cattle, it is also of key social importance (Charrouf & Guillaume, 1999). However, this multipurpose tree is now endangered because of increasing aridity and, to a lesser extent, the extraction of wood for fuel (Le Polain de Waroux & Lambin, 2012).

There has been an exceptional increase in the number of studies dedicated to argan oil in the two last decades (for a review, see Charrouf & Guillaume, 2008; El Monfalouti, Guillaume, Denhez, & Charrouf, 2010; Guillaume & Charrouf, 2011). Thanks to several findings regarding its nutritional properties and culinary value, argan oil recently emerged from obscurity and has become the most expensive edible oil in the world (Lybbert, Aboudrare, Chaloud, Magnan, & Nash, 2011). Even more spectacular is the increasing interest in argan oil and extracts for cosmetic applications as illustrated by the numerous cosmetic patents filed in the USA and Europe (Lybbert, 2007). Its main applications in dermo-cosmetology rely on its moisturising and anti-aging properties (Guillaume & Charrouf, 2011). Potential therapeutic benefits of argan oil are also currently under study (El Monfalouti et al., 2010). Because of its very high price, there is increasing demand for efficient methods to detect argan oil adulteration (Faez et al., 2013; Hilali, Charrouf, Soulhi, Hachimi, & Guillaume, 2007; Salghi, Armbruster, & Schwack, 2014). The major fatty acids (FA) of argan oil are oleic (18:1; 43–53%), linoleic (18:2; 25–37%) and palmitic (16:0; 11–16%) acids (Gharby et al., 2012; Hilali, Charrouf, Soulhi, Hachimi, & Guillaume, 2005; Yaghmur, Aserin, Mizrahi, Nerd, & Garti, 2001; Yousfi et al., 2009). Argan oil also

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contains significant amounts of tocopherols (400–900 mg/kg oil), mostly gamma isoform, sterols and triterpene alcohols (TA) (Charrouf, 1984; Hilali et al., 2005; Khallouki et al., 2003; Marfil et al., 2011; Taribak et al., 2013).

In contrast with the abundant literature dealing with the chemistry, technology and potential applications of argan oil, very little is known about argan seed biology. Argan fruit development and maturation requires 9–12 months (Bani-Aameur, Louali, & Dupuis, 1998) and oil accumulates in the seed from mid-development up to about 50% of the dry matter (% DM) (38–62% DM; Ait Aabd, Msanda, & El Mousadik, 2013; Chernane, Hafidi, El Hadrami, & Ajana, 2000; Nerd, Irijimovich, & Mizrahi, 1998). Whether the method used to extract the oil is traditional or semi-industrialised, the argan seed –also called 'kernel'– is always processed as a whole. However, our preliminary observations of the argan seed structure revealed two distinct tissues of equal volume, a feature previously unreported in the literature. While the inner tissue clearly corresponds to the embryo, with two well-developed cotyledons, the nature of the outer tissue, which likely consists in an embryo-nourishing structure, could not be resolved between endosperm, as in coffee, castor bean or oil palm seeds (Brown et al., 2012; Dussert et al., 2013; Joët et al., 2009), or perisperm, as in the sugar beet seed (Catusse, Strub, Job, Van Dorsselaer, & Job, 2008). The endosperm of angiosperm seeds originates from the fusion of the bi-nucleate central cell of the embryo sac with one of the two male gametes delivered by the pollen tube and is therefore typically triploid, whereas the perisperm derives from the nucellus, a maternal, diploid tissue. These distinct genetic features may greatly impact lipid biosynthesis during seed development. Determining the nature of the tissue that makes up the outer argan seed is therefore a prerequisite for further investigations of lipid metabolism in this species.

Since the two main tissues that comprise the argan seed are apparently of similar volume, their lipid composition needs to be determined separately to understand the respective contribution of each type of tissue to argan seed oil content and argan oil characteristics. For instance, in oil palm, the oil content and FA composition of the endosperm and the embryo differ markedly (Dussert et al., 2013). In the coffee seed, the perisperm and the endosperm also display contrasted FA composition (Joët et al., 2009). Thus, to pave the way for further studies on lipid biosynthesis in argan, we analysed the ploidy level of the two tissues using flow cytometry, and their respective mass, oil content, FA, tocopherol, carotenoid, sterol and TA composition were profiled using mature seeds collected at two locations.

2. Materials and methods

2.1. Plant material

Mature argan (*A. spinosa* Skeels) fruits were harvested in four consecutive years (2010–2013) at two locations (Supp. Fig. S1): Stidia (on the west coast of northern Algeria) and Tindouf (in the westernmost province of southern Algeria). At Stidia, fruits were collected on a single well-developed tree of unknown origin growing in an orchard, while at Tindouf fruits were harvested on nine trees belonging to a small natural argan population growing in this province of Algeria. This population is located at Touaref Bou-âam, which is 90 km north of Tindouf (Supp. Fig. S1). The same trees were used each year. About 200 fruits were harvested each year at Stidia. At Tindouf, 50 fruits were collected on each of the nine trees studied and pooled to form a representative sample of the population. Fresh seeds were treated immediately after extraction from fresh fruits and used for flow cytometry and histology. Fresh leaves were also harvested for flow cytometry measurements. For chemical analyses, the seeds were extracted from the fresh fruits, and the endosperm and embryo were then separated, flash-frozen in liquid nitrogen, freeze-dried and stored at −80 °C until analysis. The dry mass of both tissues was measured using 30 individual seeds.

2.2. Histology and flow cytometry

For polysaccharide and protein determination, samples of fresh tissue were fixed as described by Buffard-Morel, Verdeil, and Pannetier (1992), and then each section was double stained with periodic acid-Schiff (PAS) and naphthol blue-black (NBB). To visualise lipids, samples were fixed in 4% paraformaldehyde and phosphate-buffered saline (PBS) in the presence of 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for 16 h, then washed with PBS 1X, sectioned (30 μ) with a vibratom, stained with Nile red (4 μg/ml in PBS and 0.4% v/v dimethyl sulfoxide) for 15 min, and analysed by fluorescence microscopy (Leica DMRB equipped with a I3 filter: excitation at 450–490 nm, and emission at 515-nm). Flow cytometry was used to estimate DNA content in the two argan seed tissues and leaves. Nuclei were extracted by chopping seed tissues or leaves in a lysis buffer containing 4′,6-diamino-2-phenylindole dihydrochloride hydrate (DAPI) (Galbraith et al., 1983). The nucleus solution was then filtered through a 20 μm mesh nylon cloth (Partec, Germany) and nuclei were stained for 30 min at 4 °C. DAPI-stained samples were analysed using a Partec Cyflow®Ploidy Analyser (Germany), tuned to UV excitation at 365 nm and emission at 455 nm. Fluorescence of about 30,000 nuclei was measured for each sample (leaf, whole seed, embryo or endosperm).

2.3. Lipid extraction, fatty acid, tocopherol and carotenoid analysis

Prior to lipid extraction, freeze-dried seed tissues were reduced to a fine powder using an analytical grinder (IKA A15, Germany). Total lipids were extracted from 1 g samples of powder using a modified Folch method as described previously (Laffargue, De Kochko, & Dussert, 2007). For each lipid class and tissue × location × year combination, analyses were performed in triplicate from three different extractions using a completely random experimental design. Fatty acid methyl esters (FAME) were prepared according to the ISO-5509 standard, and GC analyses of FAME were performed as previously described (Laffargue et al., 2007). Tocopherols and carotenoids were measured by HPLC as previously described (Montufar et al., 2010). Only traces of carotenoids were detected in the two seed tissues and results are not further discussed.

2.4. Sterol and triterpene alcohol analysis

Sterols and triterpene alcohols were purified by successive saponification of total lipids and thin-layer chromatography (TLC) of the unsaponifiable lipids. Saponification was performed as described by Dussert, Laffargue, de Kochko, and Joët (2008) using 100 mg samples of lipids combined with 0.4 mg of 5-α-cholestanol (internal standard). The unsaponifiable fraction was spotted on pre-coated TLC plates (silica G 60, 20 × 20 cm, 0.25 mm thickness) from Merck (Darmstadt, Germany). After developing twice in n-hexane/diethyl ether/acetic acid (70:30:1, v/v), as described by Kornfeldt and Croon (1981), a 2′,7′-dichlorofluorescein solution was sprayed on the plates and the bands corresponding to 4-desmethyl- and 4,4′-dimethyl-sterols (TA) were identified by comparing their retention factor with that of 5-α-cholestanol and lupeol standards, respectively. The monomethylsterol band was almost undetectable by eye and was therefore not scraped off the plate. Both 4-desmethyl- and 4,4′-dimethyl-sterol fractions were analysed by GC as described in Dussert et al. (2008) after addition

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