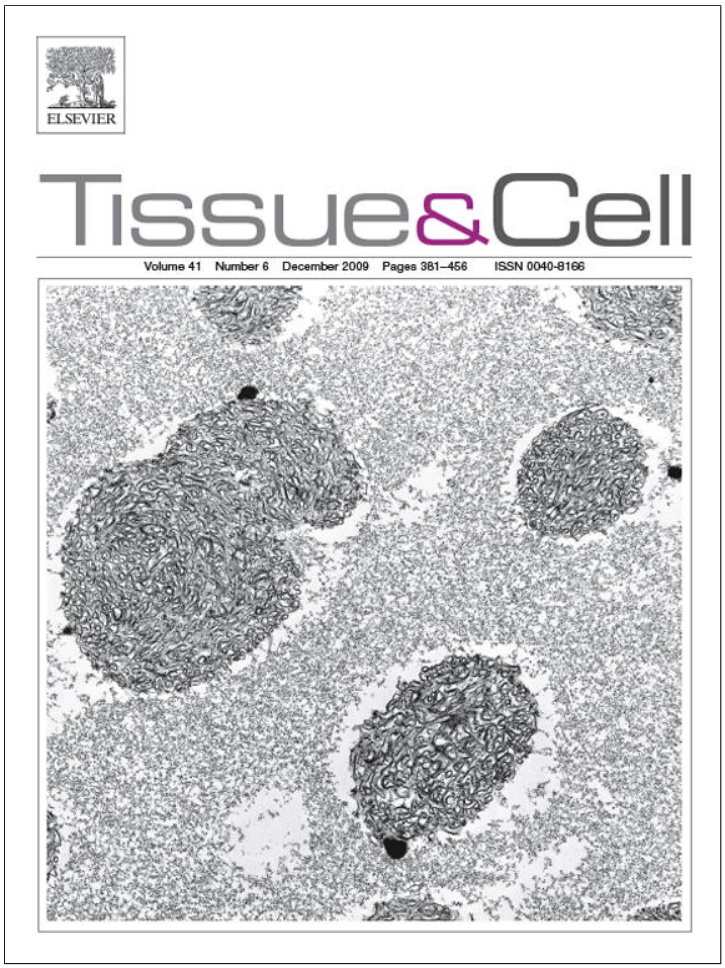

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Short communication

Ultrastructural study of archaeological *Vitis vinifera* L. seeds using rapid-freeze fixation and substitution

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ABSTRACT

The ultrastructure of *Vitis vinifera* seeds from different archaeological sites was studied. Preservation status differed between sites. Preliminary investigations of grape seeds from Poggio Bacherina (Chianciano Terme, Siena) and Miranduolo (Chiusdino, Siena) showed collapsed or charred tegument, making this material suitable for morphometric studies only. Rapid-freeze fixation and substitution of grape seeds from Shahr-I Sokhta in Iran and via De' Castellani in Florence revealed well preserved tegument suitable for chemical and cytochemical analysis. Energy dispersive X-ray microanalysis was used to determine chemical composition. Cytochemical analysis based on fluorescent staining with DAPI suggested the presence of cytoplasm residues.

1. Introduction

Relatively little is known about the economic and cultural importance of *Vitis vinifera*, its influence on communities and the origins of grape cultivation. Morphometric measurements are used in archaeobotany to distinguish cultivated and wild grape seeds (Mangafa and Kotsakis, 1996), but despite their widespread use, the consistency of morphometric studies is still debated. Morphological study of fresh material using classical chemical fixation shows that grape seeds have a cuticle, an epidermis and two teguments enclosing the albumen and embryo (Ribereau and Peynaud, 1971; Mullins et al., 1992), while a recent study of ultrastructure of Cabernet seeds showed three teguments (external, intermediate and inner) (Cadot et al., 2006).

To obtain finely detailed morphology of plant cells, plant tissue may be subjected to rapid freezing and substitution (Lancelle et al., 1987; Lancelle and Hepler, 1989). This technique preserves cytoplasm better than chemical fixation, which is known to damage fine structure (Ross et al., 2000).

In archaeobotanical specimens, the presence of plant DNA has been investigated using the fluorescent cytochemical stain 4′,6-diamidino-2-phenylindole (DAPI) (Kawamuro et al., 1995; Suyama et al., 1996; Milanesi et al., 2006). Chemical signals related to proteins and other biochemical molecules in cells subject to charcoalification have been detected by X-ray microanalysis (Wang, 2006).

The aim of the present study was to investigate the ultrastructure of grape seed fragments from archaeological sites in Tuscany (Poggio Bacherina, Miranduolo castle and Florence) and Iran (Shahr-I Sokhta), and to compare it with that of fresh seeds of *Vitis vinifera* as control. Morphological studies were carried out by rapid-freeze fixation and substitution to determine the state of conservation of tegument tissues. The possible presence of cytoplasm residues in the inner tegument was investigated by chemical and cytochemical analysis.

2. Materials and methods

2.1. Sampling selection

Archaeological grape seeds (*Vitis vinifera*) obtained from excavation sites at Poggio Bacherina (Fig. 1A), Miranduolo (Fig. 1B), Shahr-I Sokhta (fragments only; Fig. 1C) and Florence (Fig. 1D) were identified morphometrically and documented with light micrographs. Destructive analysis of archaeological material was compared with that of fresh seeds cultivated in the botanical gardens of Siena University (control). Deformed seeds from Poggio Bacherina described in Paolucci (1993) were dated to the second century BC, seeds

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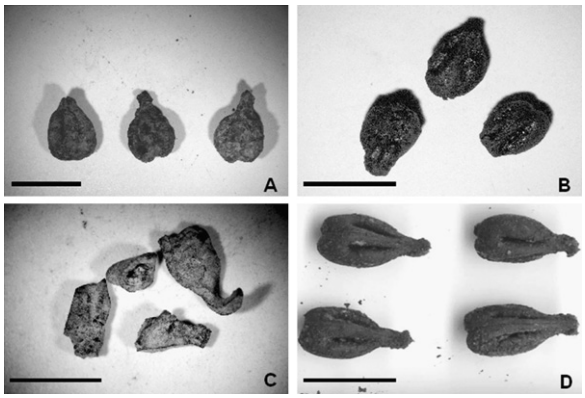


Fig. 1. Light micrograph of seed fragments from Poggio Bacherina (A), bar = 5 cm; Miranduolo castle (B), bar = 6 cm; Shahr-I Sokhta (C), bar = 2 cm; Florence (D), bar = 7 cm.

from Miranduolo were dated between the late 10th and early 11th century (Nardini and Valenti, 2003), seed fragments from Shahr-I Sokhta described in Tosi (1978) were dated to 23rd century BC, and seeds from Florence (Cantini et al., 2007) were dated to the 13th century. Samples were stored separately in sterile Falcon capsules.

2.2. Rapid freezing

Fresh control and archaeological seeds were divided into fragments measuring approximately 1 mm × 1 mm × 0.5 mm using a microscope pointer. The material was prepared for electron microscopy as described by Lancelle et al. (1987). The fragments were fast frozen in liquid propane at −160 °C and transferred to liquid nitrogen at −196 °C.

2.3. Scanning electronmicroscopy and X-ray microanalysis

Some fragments in liquid nitrogen were gradually brought to room temperature, glued to standard vacuum-clean stubs and coated with graphite (Edwards, carbon scancoat, S150A). Seed fragments for ultrastructural analysis were observed by scanning electron microscope (Philips XL20). The instrument was equipped with an X-ray microanalysis probe, which we used at 20 kV acceleration voltage to determine the chemical elements in seed tegument cells. Concentrations had an error of about 1%. Mean concentrations and standard deviations of each element were calculated from random determinations at five different spots on the samples. The X-ray beam was 1 μm wide and penetrated to a depth of 2 μm.

2.4. Resin embedding and polymerization

The remaining fragments in liquid nitrogen were progressively substituted in acetone at −80 °C and then gradually embedded in LR White resin (Lancelle and Hepler, 1989). To avoid heating the samples, polymerization was performed at 4 °C for 24 h with a UV lamp. Some of the samples were then observed by transmission electron microscope and the others were treated with DAPI cytochemical stain for fluorescence microscopy.

2.5. Transmission electronmicroscopy

Serial sections 0.8 μm thick were cut with an LKB III ultramicrotome fitted with a diamond blade. They were mounted on gold grids, contrasted for 3 min with 3% glutaraldehyde and 1% osmium tetroxide, stained with 2% uranyl acetate and lead citrate and observed with a Philips Morgagni 268D.

2.6. DAPI fluorescence microscopy

DAPI staining of archaeological materials has already been described in Kawamuro et al. (1995) and Suyama et al. (1996). Serial sections about 2 μm thick were cut with an LKB III ultramicrotome fitted with a glass blade. They were mounted on light microscope slides and stained. The slides were observed with a Zeiss Axiophot 400 fluorescence microscope.

3. Results

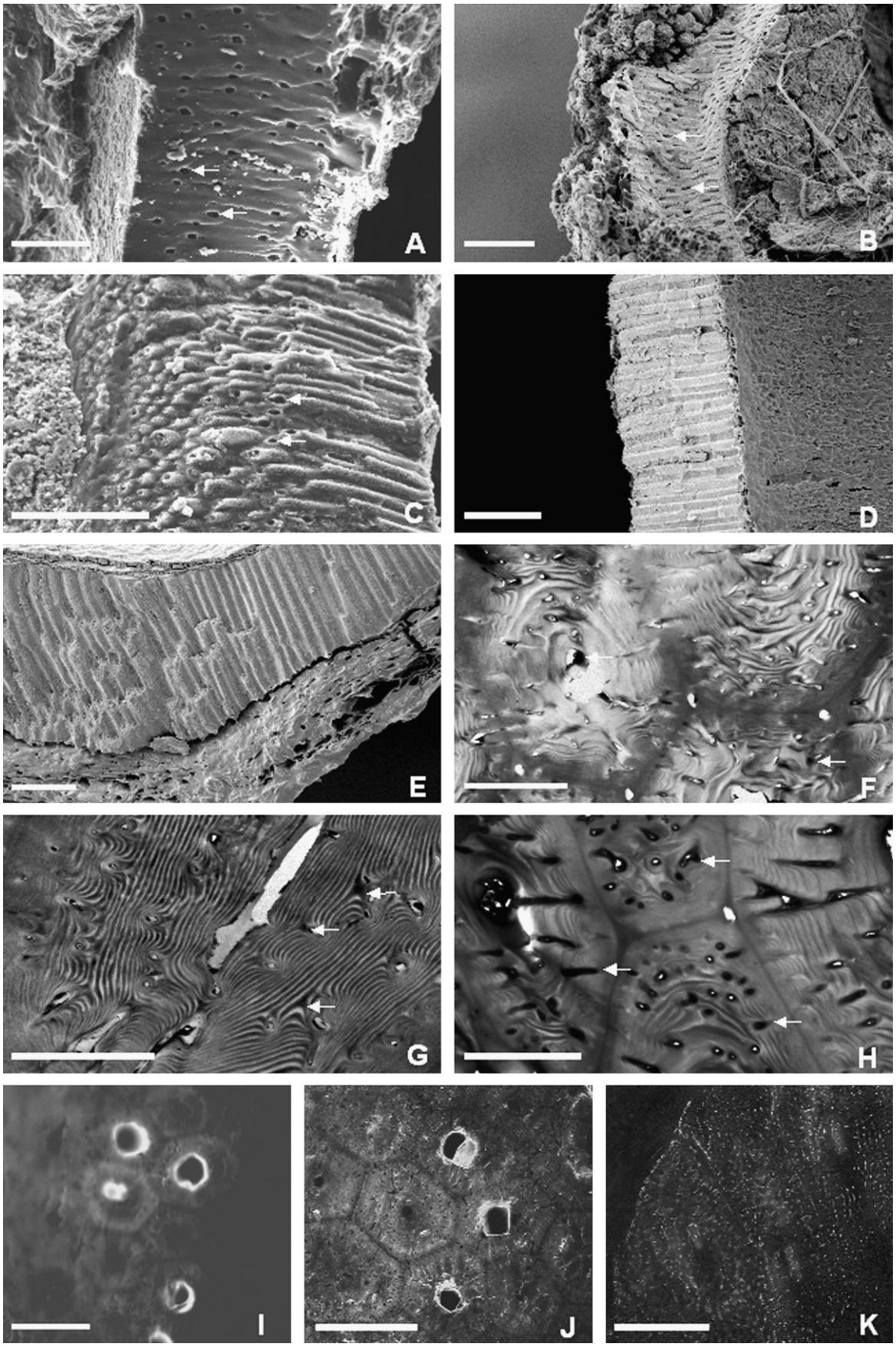
3.1. Light and scanning electronmicroscopy

Light micrographs of grape seeds recovered from sediments of the Etruscan archaeological site at Poggio Bacherina, showed deformed tegument tissue (Fig. 1A). This is evident in the scanning electron micrographs: the tegument cells appear dark, compact and full of holes (Fig. 2A). Light micrographs of seeds from the hearth of Miranduolo castle showed externally very dark epidermis (Fig. 1B). Scanning micrographs showed tegument tissue fractured obliquely with folds, corrosion and cells containing empty spaces (Fig. 2B). Light micrographs of seed fragments from Shahr-I Sokhta (Fig. 1C), showed tissues dispersed in clear crystallized sediment, recognizable by the typical shape and thickness of teguments. Scanning micrographs showed tissue with elongated tegument cells in relief. The tissue was not subject to sediment pressure and was well preserved, presumably due to saline conditions causing osmotic dehydration. The rapid-freeze process opened cell structure, revealing small holes (Fig. 2C). Light micrographs of shriveled pyriform seeds from Florence showed deep fissures flanking the central longitudinal ridge on the ventral side (Fig. 1D). When seeds were fractured, the interior of the endosperm was absent and the embryo sac was empty. Scanning micrographs after fast freezing showed tegument tissue fractured parallel to cell structure, detachment of walls and opening of membranes containing dense material (Fig. 2D). The cells were in close contact, typical of submerged material recovered from canals. Scanning micrographs of fresh teguments (control) showed cuticle, epidermis and elongated cells having a diameter of 20 μm and a length of about 150 μm (Fig. 2E).

3.2. Transmission electronmicroscopy and DAPI cytochemical staining

Seeds from Poggio Bacherina and Miranduolo were suitable for morphometric studies and microanalysis but not for morphological study.

Fig. 2. (A) Ultrastructure of tegument cells of grape seeds from Poggio Bacherina observed by scanning electronmicroscopy. High magnifications showing dark, compact tegument cells with many holes (arrows). (B) Ultrastructure of tegument cells of grape seeds from Miranduolo observed by scanning electronmicroscopy. High magnification of obliquely fractured tegument tissue, showing folds, corrosion and empty cells with holes (arrows). (C) Ultrastructure of tegument cells of grape seeds from Shahr-I Sokhta observed by scanning electronmicroscopy.High magnification showed tissue with elongated well preserved tegument cells in relief, not subject to sediment pressure. (D) High magnification of tegument cells of Florence specimens observed by scanning electronmicroscopy, showing well preserved cells and dense cytoplasmic material (arrow). (E) High magnifications of tegument of fresh control seeds observed by scanning electronmicroscopy, showing well preserved cells and dense cytoplasmic material (arrow). Bar = 100 μm. (F–H) High magnification of specimens from Shahr-I Sokhta (F), Florence (G) and fresh controls (H) by transmission microscopy after rapid-freeze fixation and substitution, showing tegument cells containing cytoplasmic material (black bodies). Bar = 10 μm. (I–K) Cytochemical analysis showing DAPI-stained and visible fluorescence of nucleic acids in Shahr-I Sokhta (I), Florence (J) and control tissue samples (K). Bar = 20 μm.



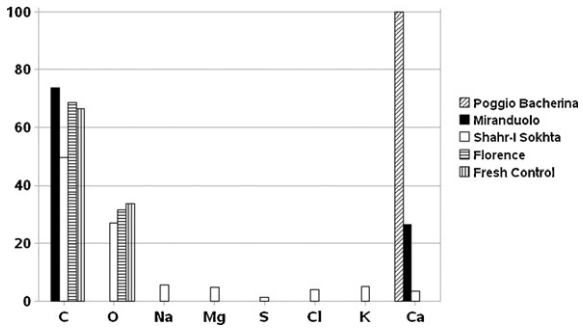


Fig. 3. X-ray element analysis of all specimens.

The ultrastructure of sections of freeze-substituted specimens of Shahr-I Sokhta (Fig. 2F), Florence (Fig. 2G) and fresh control material (Fig. 2H) showed cells with thin lignin cell walls in close contact with each other. In cross-section, specimens showed intercellular holes containing electrondense material which presumably acted as reserves of secondary metabolites. DAPI staining of Shahr-I Sokhta specimens showed peaks of fluorescence inside but only around the periphery of holes (Fig. 2I). In Florence specimens, peaks were more intense (Fig. 2J). DAPI staining of fresh control seeds showed diffuse fluorescence (Fig. 2K).

3.3. X-ray microanalysis

The results of X-ray microanalysis (percentage composition of elements) of teguments from all sites are shown in Fig. 3. In Poggio Bacherina specimens, only Ca (100%) was found in tegument tissue due to permineralization by external inorganic elements. Charred seeds from Miranduolo contained C (73.56%) and Ca (26.44%). Shahr-I Sokhta specimens contained Na (5.42%), Mg (4.61%), S (1.2%), K (4.85%) and Ca (3.48%), as well as concentrations of C (49.56%) and O (27%) giving a C:O ratio of 1.83; they also confirmed exposure to saline conditions (Na 5.42% and Cl 3.89%). Florence samples contained C (68.65%) and O (31.35%), with a C:O ratio of 2.1. Fresh control samples only contained C (66.35%) and O (33.65%), with a C:O ratio of 1.97. Shahr-I Sokhta and Florence samples had C:O ratios similar to fresh samples.

4. Discussion

Palaeobotanical material occurring in archaeological sites is almost always lost. Specimens of the type analyzed here can be regarded as precious. Very small fragments of archaeological grape seed specimens can provide information about the ultrastructure, chemistry and cytochemistry of *Vitis vinifera* tegument. Subfossil conservation may occur when fast burying creates a closed system, which reduces exogenous aerobic degradation. Liquid mineral substrates contribute to conservation, 'entombing' (Eglinton and Logan, 1991) or cementing subfossil cytoplasm and preventing its oxidation.

In Poggio Bacherina, Etruscan seeds were dispersed in aerobic environments where they underwent chemical permineralization, by which elements of the original tegument were substituted by other non-organic compounds. It was not possible to reconstruct original element compositions and their spatial distributions in tegument cells. Before entombing, seeds from Miranduolo castle were subject to anthropogenic processes that converted oxygen into volatile CO2, typical of hearth material. In such situations, tissue can also be preserved for a long time under aerobic conditions. Light and electron microscopy showed charred or collapsed tegument, making these specimens suitable only for morphometric studies (Nardini and Valenti, 2003). In the Iranian specimens, infiltration of chemical elements of sediment into tegument cells confirmed that samples were not subject to sediment pressure and were well preserved due to osmotic dehydration. Salinity contributes to osmotic dehydration (Hirayama et al., 1995), and transfer of water from the inner parts of the cells is accompanied by formation of anaerobic systems promoting preservation. In the Florence specimens, the matrix of urban sediments composed of microbes formed barriers that maintained substrate humidity, isolating and reducing exogenous degeneration (Manen et al., 2003).

Rapid-freeze fixation of fresh material, followed by embedding and polymerization in LR White acrylic resin is a method used to detect cytoskeletal structures, such as microfilaments, proteins and nucleic acids, damaged by conventional chemical fixation and embedding in epoxy resin (Ross et al., 2000). 4′,6-Diamidino-2-phenylindole (DAPI) binds DNA binder in plant cells (Larsen et al., 1989); it contains a fluorochrome excited by radiation having a wavelength of 358 nm, and emits in the visible spectrum. Emitted light is detected by fluorescence microscopy (Debapriya and Kumar Pal, 2008). Positive staining of subfossil material indicating cytoplasmic residues has been reported in pollen more than 150,000 years old (Suyama et al., 1996) and in even older pollen recovered from sediment (Gugerli et al., 2005). However, according to Lindahl (1993), cytoplasm cannot be expected to last for so long, except under extremely cold conditions such as permafrost or ice cores (Willerslev et al., 2007). In our Iranian and Florentine subfossil specimens, microanalysis confirmed the presence of elements associated with cytoplasm (Wang, 2006). A review of ranges of O:C ratios in tissues in the literature did not produce uniform results (Nguyen et al., 2004; Baldock and Smernick, 2002), but in our subfossil specimens the C:O ratio was close to that of fresh seeds, excluding charcoalification. Transmission electromicroscopy sections showed holes in tegument tissue containing electrondense material that presumably acted as a reserve of secondary metabolites. Rapid-freeze fixation samples showed well conserved areas rich in electrondense material, and positive DAPI staining confirmed the presence of DNA residues in subfossil plant cytoplasm (Poinar et al., 1996). Our preliminary data seem to indicate that salt-dehydrated and waterlogged subfossil seed teguments may be a good source of archaeobotanical cytoplasmic residues for historical research and comparison with modern cultivars.

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