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Application of x-ray microanalysis, diffraction and cytochemical techniques in the study of the structure and chemical composition of inclusions in *Olea europaea* leaves

J.D. Alche and M.I. Rodriguez-Garcia Estacion Experimental del Zaidin. Profesor Albareda 1. E-18008. Granada. Spain.

ABSTRACT: Two types of inclusions have been found in mesophyll cells of leaves of Olea europaea. The first type is located in the vacuole, and the application of X-Ray microanalysis, X-Ray diffraction and cytochemical techniques shown that these inclusions are composed of calcium oxalate. The second type of inclusion is intranuclear and its proteic nature is demonstrated by means of light microscopy stains. These crystal structures are probably well ordered in three dimensions.

1 INTRODUCTION

Calcium oxalate crystals are the most common form of calcium salts found in plants, and their presence has been widely documented in many different species (see review of Franceschi & Horner 1980). However, in some instances the composition of certain inclusions has been assumed to be calcium oxalate without proper investigation. The presence of calcium in these crystals can be shown by means of cytochemical techniques (Von Kossa 1901, Yasue 1969) as well as by more accurate methods such as X-Ray microanalysis, X-Ray diffraction, Raman analysis or infrared spectroscopy. These latter methods generally require that the crystals be isolated and in a fairly pure form. The function that they play in the plant is not clearly knew although it may be connected with the establishment of an adequate ionic balance in the plant.

The presence of intranuclear inclusions has been demonstrated in different species of Angiosperms (Fabbri & Menicanti 1970, Perrin 1970, Weintraub et al. 1971, Unzelman & Healey 1972, Vintejoux 1984, Alche & Rodriguez-Garcia 1988). The variation in their morphology and structure, as well as their constant presence in several families of Angiosperms make them useful for taxonomic purposes (Speta 1979, Bigazzi 1984). However details of their structure, chemical composition and localization in different tissues are still unclear, as is their possible functional significance in the plant.

2 MATERIAL AND METHODS

a) Conventional light and transmission electron microscopy (LM and TEM)

Small squares of about 1 mm. were sliced from the Olea europaea leaves nearest the olives. These squares were fixed in 3% glutaraldehyde in 25 mM cacodylate buffer, pH 7.2 for 2 hours washed three times with buffer, and them placed in 2% osmium tetroxide for 2 hours. They were then rinsed and dehydrated through an ethanol series and embedded in EPON (TEM with an intermediate step in propylene oxide) or LRWhite (LM). Sections for TEM were contrasted with uranyl acetate and lead citrate.

b) Cytochemycal techniques

The Von Kossa technique (1901) to detect the presence of calcium and the Cloramine T-Schiff technique to detect proteins were carried out on thin sections at LM level. Potassium pyroantimoniate (4 %) was added to the osmium tetroxide solution during post-fixation to detect the presence of divalent cations at TEM level, and the Tandler & Pellegrino de Iraldi procedure (in press) was carried out "in block" prior to embedding at both LM and TEM levels to show the presence of material with silver binding characteristics.

c) Optical diffraction

Several high-magnification TEM micrographs of intranuclear inclusions were examined using an optical diffractometer in order to determine the degree of quality of the crystals and their periodicity. This is inmediately clear from the number of diffraction points or spots in the back focal plane of a positive lens placed in front of or behind the micrograph.

d) X-Ray microanalysis

Sections were examined with a Ph420 transmission electron microscope equipped with an EDAX energy dispersive X-Ray spectrometer which is capable of analysing elements with an atomic number equal to or more than that of sodium ($Z \leq 11$). Elemental chemical analysis of vacuolar inclusions was performed at 120 KV.

3 RESULTS

Both the palisade and spongy mesophyll cells of Olea europaea leaves show the presence of two types of inclusions which are clearly different in their morphology and cellular localization (Fig 1).

The first type of inclusion body is located in the large central vacuole of these cells. It is often lost during sectionning (Fig 1) or as a result of beam damage. It is more or less elongated and rectangular in shape (Fig 2) and it shows high opacity to electrons. The number of these inclusions is higher in cells around the veins than in other areas of mesophyll, and they may frequently be observed in the veins themselves. These crystals react positively to the Von Kossa technique (Fig 3) and show precipitation of potassium piroantimoniate (Fig 4) in and around them, as well as the presence of argentafinic material (silver precipitate Fig5). X-Ray microanalysis spectra of the crystals (Fig 6) shows a peak identified as calcium, but it should be emphasized that the presence of elements with atomic number less than eleven can not be detected. Data obtained from X-Ray diffraction analysis of these crystals (Fig 7) indicates that they are composed of calcium oxalate when compared with theoretical data of seventeen ASTM standard calcium compounds (Table 1). ASTM: American Society for Testing and Materials

Table 1: ASTM data of several of the 17 calcium-compounds used, compaired with those obtained from vacuolar crystals in Olea europaea leaves. d:distance between reticulated plains of the crystal

	Experimental values of d	ASTM values of d: Calcite	Aragonite	100% Ca	Calcium oxalate ∝	Calcium oxalate β
Diagram 1	2.42	-	-	-	2.46	2.44
	2.66	-	2.70	-	2.63	2.64
	2.87	2.84	2.87	-	2.83	2.83
	2.91	3.03	2.87	-	2.92	2.92
Diagram 2	1.81	1.87	1.88	-	1.87	1.89
	2.24	-	-	-	2.25	2.22
	2.42	-	2.41	-	2.37	2.44
Diagram 3	2.55	-	-	-	2.50	2.49
	3.01	3.03	-	-	3.03	-
Diagram 4	2.24	-	-	-	2.25	2.22
	2.64	-	-	-	2.63	2.64
	2.94	-	-	-	2 03	2.95
	3.71	-	-	-	3.66	3.67
	5.92 ± 0.6	-	-	-	5.84	5.83
Diagram 5	1.92	1.93	-	-	1.94	1.94
	1.97	2.09	-	1.97	1.94	1.38
	2.09	2.09	2.11	-	2.08	
	2.40	-	2.41	-	2.37	2.39
	2.96	-	-	-	2.92	2.95
	3.71	-	-	-	3.66	3.67
	5.92	-	-	-	5.84 / 6.10	5.83 / 6.20

The second type of inclusion is intranuclear (Fig 1). It is more or less polyhedral in shape, similar in size to the nucleolus, and shows a clear periodic substructure of a set of parallel lines under great magnification (Fig 8). It's proteic nature is shown by a positive stain using the Cloramine T-Schiff technique (Fig 9). Neither a high amount of argentafinic material nor the presence of cations inside the crystalloids were demonstrated by means of the respective techniques (potassium pyroantimoniate and Tandler & Pellegrino de Iraldi procedure) (Figs 10 and 11) , although pyroantimoniate precipitate appears frequently around the inclusions (Fig 10). Preliminary observations on electronic micrographs of these cristalloids with an optical diffractometer show at least two pairs of reflections in different directions, proving that the crystal is ordered in two directions at least (results not shown). The periodicity most clearly visible was calculated in 150-160 Å.

4 DISCUSSION

The combination of cytochemical methods with X-Ray microanalysis and diffraction techniques allows a progressive approach to understanding the chemical composition of inclusion bodies located in the vacuole. The precipitation of potassium pyroantimoniate on the vacuolar inclusions shows the presence of cations but does not determine specifically what kind of cations are present. Techniques which use silver salts (Von Kossa 1901, Yasue 1969, Tandler & Pellegrino de Iraldi, in press) are based on a substitution of calcium by silver in the crystal, with the formation of a

new salt less soluble than the original, thus giving information about the presence or absence of calcium. The presence of calcium in vacuolar inclusions is also demonstrated by X-Ray microanalysis, which rejects the presence of others elements like P or S. The conclusion that these vacuolar inclusions are composed of calcium oxalate is only obtained after the analysis and coparison of X-Ray diffraction data. However, with the data obtained it is imposible to discern which of the two forms of calcium oxalate usually present in plants (the monohydrate whewellite form or the dihydrate weddellite form) is present in these crystals. In order to determine this, isolation and purification of the crystals seems to be necessary (Horner & Zindler-Frank 1982a, Clark et al. 1987).

The function of calcium oxalate in plants remain unclear (see review of Franceschi & Horner 1980, Horner & Zindler-Frank 1982b). However, various theories have been forward, namely the maintainance of an ionic equilibrium by the removal of excess oxalate, the storage of calcium against calcium-stress conditions or merely for structural support. Clearly, an investigation of the possible variation in these crystals in relation to metabolic changes in the plant is needed.

The Cloramine T-Schiff reaction demonstrates clearly the proteic nature of the intranuclear inclusions by means of an oxidization of free aminogroups to aldehyde groups, which are then made visible by reaction with a Schiff reagent, giving a red coloured end-product. Only a small amount of calcium and other cations inside the crystals is indicated by their practically negative response to the potassium pyroantimoniate test and the Tandler & Pellegrino de Iraldi techniques. However the peripheral presence of precipited pyroantimoniate around the crystalloids probably reflects a high degree of metabolic activity connected with synthesis, degradation or stabilysation processes occurring within them. The presence of symmetry in at least two directions in the crystal clearly indicates a very well ordered structure, probably along three dimensions. This means that its symmetries can be determined exactly, as can the unit cell volume and a good approximation of the molecular weight of the protein can be obtained (Hovmöller 1986).

It is possible that these intranuclear inclusions are used for protein storage, but the nucleus has not previously been considered a place for storing reserve substances in the cell. Thomas et al (1977) support the idea that the crystalloid body proteins are synthesized in the cytoplasm, and that they subsequently migrate towards the nucleus. A relationship has been observed in other species (Morassi & Bigazzi 1980, Bigazzi 1984) between these intranuclear inclusions and the nucleolus. However, only spatial proximity has been observed in Olea europaea, because of the large area occupied by the inclusions within the nucleus.

No relationship has been observed between both kinds of inclusions (vacuolar and intranuclear) in Olea europaea leaves.

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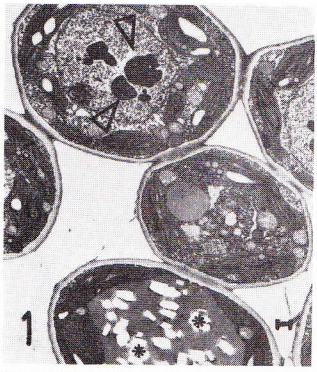
FIGURES

Fig 1:Olea europaea mesophyll cells showing both intranuclear (arrow) and vacuolar inclusions. Note that vacuolar inclusions (asterisc) came off the section during the sectioning process. Fig 2: High power view of a partially removed vacuolar inclusion. Fig 3: Light micrograph of spongy mesophyll cells stained with the Von Kossa technique. Fig 4: Potassium pyroantimoniate precipitation around and inside crystals in the vacuole. Fig 5: Tandler and Pellegrino de Iraldi technique. Silver precipitate appears strongly on crystals in the vacuole (arrows). Fig 6: X-Ray spectra of vacuolar crystal in Olea europaea leaves. On the abscisse is represented energy in KeV. The vertical scale represent counts per second. Peak of titanium is originated by grid material. Fig 7: X-Ray diffraction diagram of vacuolar crystals. Fig 8: High power view of a intranuclear inclusion showing periodic lattice. Fig 9: Ligh micrograph of palisade mesophyll cells: intranuclear inclusions show positive stain with the Cloramine T-Schiff technique. Fig 10: Potassium pyroantimoniate precipitation around intranuclear crystalloids. Fig 11: Tandler and Pellegrino de Iraldi technique. Silver precipitate appears strongly on the nucleus, particularly on nucleolus (NU) but not on intranuclear inclusions (IN).Bars reppresent 1 μm (10 μm in Figs 3 and 9).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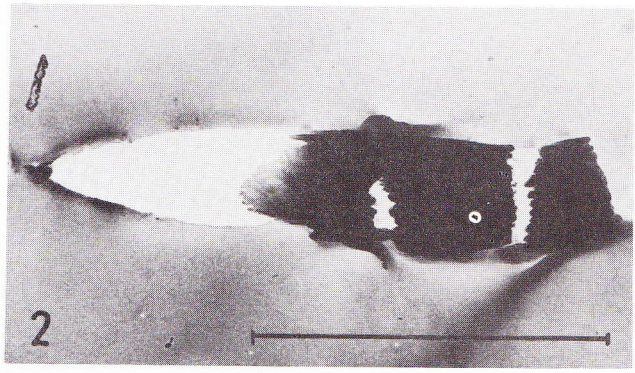
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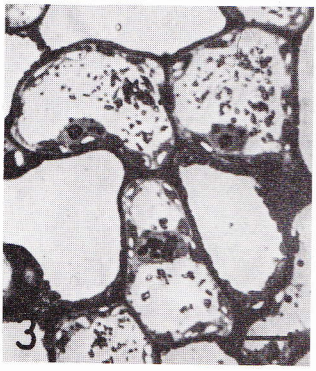
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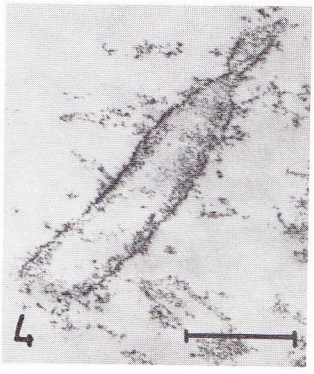
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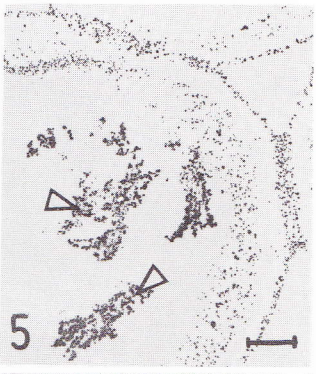
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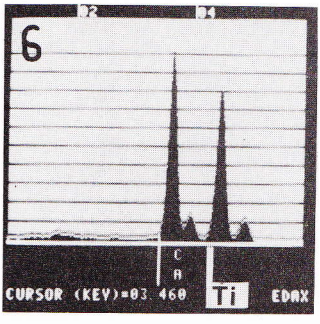
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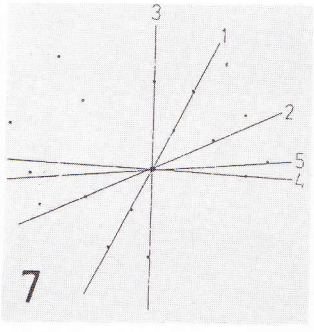
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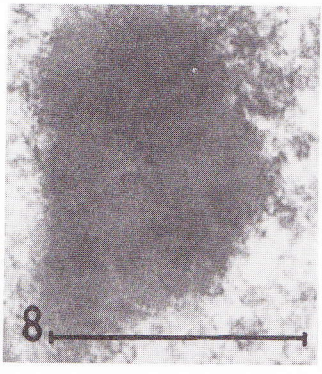
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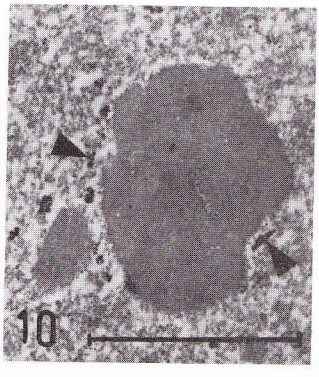
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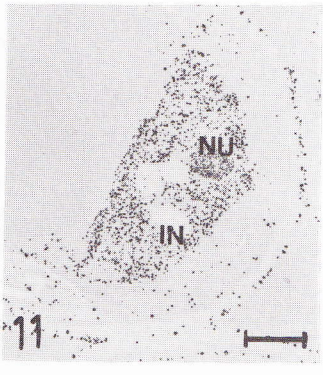
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