STARCH GRAIN ANALYSIS AS A MICROSCOPIC DIAGNOSTIC FEATURE IN THE IDENTIFICATION OF PLANT MATERIAL[1]

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Cortella, A. R. *and* **M. L. Pochettino,** (*Laboratorio de Etnobotánica y Botánica Aplicada (LEBA), Facultad de Ciencias Naturales y Museo, U.N.L.P. Paseo del Bosque s/n., 1900 - La Plata, Argentina.*) STARCH GRAIN ANALYSIS AS A MICROSCOPIC DIAGNOSTIC FEATURE IN THE IDENTIFICATION OF PLANT MATERIAL. *Economic Botany 48(2): 171–181. 1994. The starch grain is a diagnostic feature of multiple applications according to the peculiarities and origin of the plant material to be determined. Its morphological characteristics are studied through the analysis of samples from different origin and different preservation conditions, as flours, commercial starches, drugs, spices and archaeological remains (especially carbonized material). The usefulness and importance of the methods and techniques applied in each case are also discussed.*

Analisis del grano de almidon como caracter microscopico de diagnostico en la identificacion de material vegetal. *El grano de almidón constituye un caracter de diagnóstico de variada utilidad en función de las particularidades y origen del material vegetal a determinar. Se realiza un estudio de sus características morfológicas mediante el análisis de muestras provenientes de distintos orígenes y en diferentes estados de conservación, desde harinas y féculas hasta drogas, especias y material arqueológico, en particular carbonizado. Se discuten asimismo la utilidad e importancia de los métodos y técnicas utilizadas en cada caso.*

Key words: starch grains; microscopic characters; scanning electron microscopy; archaeological identification.

It is very difficult to identify vegetal material when it is in a fragmentary condition, partially decomposed or carbonized, or when reproductive organs are not available. Samples of this sort may come from different sources such as markets, as in the case of spices or commercial starches, or archaeological remains obtained directly or through flotation techniques (Smith 1985). Often, diagnostic microscopic features must be found to identify these samples. In such undertakings specialized techniques and experience with plant anatomy is necessary as suggested by Hastorf and Popper (1988).

In samples of spices, drugs and some desiccated archaeological material (Ugent et al. 1981, 1982, 1984, 1986, 1987), the histological elements are nearly unaltered and can be identified; but in other samples, such as carbonized archaeological remains, the identification task is rather difficult because only a few histological elements can survive the carbonization process (Pochettino and Cortella 1989–1990).

The starch grain is considered an especially significant feature because of its ubiquity in plants, its physical characteristics, its varied morphology and its resistance to grinding, drying and sometimes even to the carbonization process. Because the size, shape and structure of starch grains in a given plant vary within well-defined limits, grains coming from different species often can be distinguished (Czaja 1978; Trease and Evans 1986).

The aims of this paper are: to evaluate the relative importance of the starch grain in the identification of a variety of vegetal samples according to the history and characteristics of the sample; to illustrate the morphological variability of the starch grain as a diagnostic character; and to compare the efficacy various methods used for its study.

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General Features of Starch Grain

The starch grain is quasi crystalline, insoluble in water and slightly hydrated so that a large amount of carbohydrate can be stored in a small volume. It varies in size and shape and generally shows layers centered around a point—the hilum—which may be in the center or distorted towards a pole. It is common to find fissures emanating from the hilum and which appear to be the result of grain dehydration (Cutter 1969; Esau 1972). Generally grains are associated with small amounts of noncarbohydrate material such as proteins, fatty acids and inorganic substances (Williams 1968).

Among green plants, starch is the first assimilation product and in many plants it is the last product formed before the resting period. During photosynthesis, starch is formed in the chloroplasts, then hydrolyzed and synthesized as storage starch in the amyloplasts. An amyloplast may contain one or more starch grains. Several grains of joint origin form a compound starch grain.

The storage starch is deposited in different organs such as stems or roots. Reserve starch may be deposited at the embryo (cotyledons), endosperm or perisperm (Winton and Winton 1932) and it is largely unaltered during dormancy.

The first systematic studies on the botany and morphology of starch were carried out by Nägeli [1858] and Meyer [1895] (both cited by Czaja, 1978) who demonstrated the extraordinary variability of starch grains. Later, Reichert (1913) studied hundreds of starch grains using polarized light. Several papers have reported differences at species level by means of morphological descriptions (Seideman 1964, 1966; Ulmann 1969). In many cases, differentiation is achieved through quantitative techniques (Wellendorf 1975a,b) including image analytic and multivariate analysis (Baum and Bailey 1987).

Transitory starch—which is not considered in the present paper—is an intermediate product found at growing points, non-ripe fruit, flower buds before anthesis, pollen (Baker and Baker 1979) and secretory tissues (Fahn 1979).

Materials and Methods

The materials used in our study vary in origin and conditions of preservation, they include vegetal organs sold at markets or found in archaeological sites—dehydrated or carbonized samples of seeds, stems, roots—commercial starches or flours, fragmented or pulverized material—spices or herbal products. In each case, a large number of samples were examined. They were sent to LEBA by official institutions and private laboratories or collected by members of the research team.

Studies were conducted using binocular stereoscopic microscope, light microscope (LM), phase contrast, petrographic microscope, scanning electron microscope (SEM). When it was necessary X-ray diffractometer was used.

With I_2-IK we can infer from the coloring achieved, the proportion of branched molecules forming a grain. Amylose stains dark blue and amylopectine, brownish red or purple (Gahan 1984). Congo red dye was employed to observe grain damage (Sterling 1968).

Preparations for LM observation were mounted with water : glycerin 1:1 (Trease and Evans 1986) or lactophenol (Wallis 1968). Sodium hypochlorite and hydrogen peroxide 50% and 20% and lactic acid were used as decolorizing agents, especially for the observation of carbonized archaeological material. For SEM observation, the material was dehydrated in increasing series of alcohols, cut in different directions and mounted on stubs, using a stereoscopic microscope. Preparations were metallized with Au-Pd, observed and micrographed with SEM Jeol JSM-T 100. When it was necessary to isolate the starch grain from the tissue, alkaline treatment with sodium hydroxide 0.15% was employed and then the material was washed and screened (Wosiacki 1985).

Results and Discussion

The employment of starch grains as diagnostic features is of variable usefulness, depending on the type, origin, characteristics and preservation conditions of the sample to be identified.

Drugs and Spices

This material is generally fragmented or powdered. Different and important histological elements, depending on the vegetal organ of origin, can easily be identified by LM observation. The presence or absence of starch grains in some samples constitutes definitive evidence that adulteration or substitution has taken place. Adulteration of spices with flours or commercial starches is easily and frequently detected. We observed commercial samples of red pepper and paprika (*Capsicum annuum* L.) mixed with abundant

starch grains from beans (*Phaseolus* sp.) associated with the artificial coloring agents. Adulteration is also frequent with other organs of the same plant. A study of different commercial samples of coca leaves (*Erythroxylon coca* Lam.) allowed us to identify abundant seed fragments in coca tea sold in bags, which can be detected because of the characteristic amylaceous parenchyma.

Some spices have characteristic starches of typical shape and size, as the nutmeg (*Myristica fragrans* Houttuyn), which has simple or compound starch grains from 2 to 10 units, of 3–22 μm diameter (Youngken 1959) and well-differentiated hilum (Trease and Evans 1986) (Fig. 1, 2). The starch grain is also an important feature to differentiate commercial cinnamon. Ceylon cinnamon (*Cinnamomum zeylanicum* Nees) has simple and compound starch grains in groups of 2–4; the isolated grains are of spheroid, plane-convex or polygonal shape; generally the diameter is less than 10 μm (Fig. 3). The China cinnamon [*Cinnamomum cassia* (Nees) Nees ex Blume] presents individual grains of up to 22 μm diameter or more (Winton and Winton 1932; Youngken 1959) (Fig. 4). In distinguishing samples of these two species, the starch grain, together with other histological elements, contributes to the identification and may be the defining character.

Flours, Commercial Starches and Amylaceous Plants

Consumption of starch all over the world is great, but there are comparatively only a few plants which can supply high volumes of starch.

In the case of vegetal organs with amylaceous storage tissues and products manufactured with them, such as commercial starches and flours, the starch grain is usually the only diagnostic feature available for identification. For LM examination preparations must be mounted with a somewhat viscous liquid. A rapid means for determining three dimensional grain shape (French 1984; Wallis 1968) is to generate a streaming movement in the mounting medium which cause the grains to roll. Using this technique, drawings and photomicrographs may be prepared for comparison with reference collections and published sources, and measurements may be made.

Light microscope resolution allows the observation of characteristics of the starch grain, such

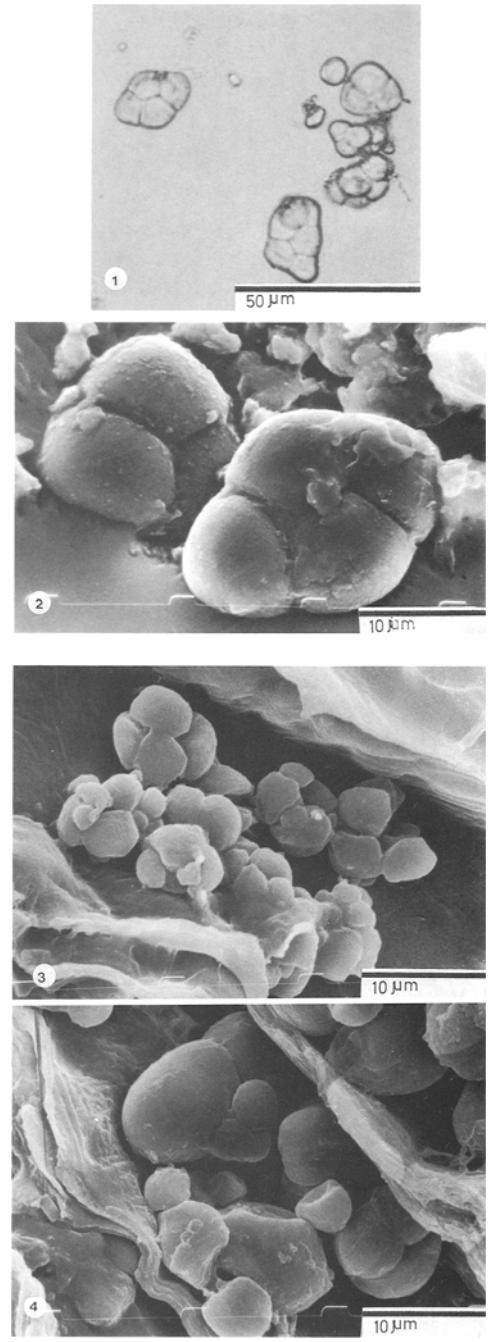

Fig. 1–4. **1)** *Myristica fragrans*: LM observation; **2)** Idem: SEM micrograph; **3)** *Cinnamomum zeylanicum* bark: SEM micrograph; **4)** *Cinnamomum cassia* bark: SEM micrograph.

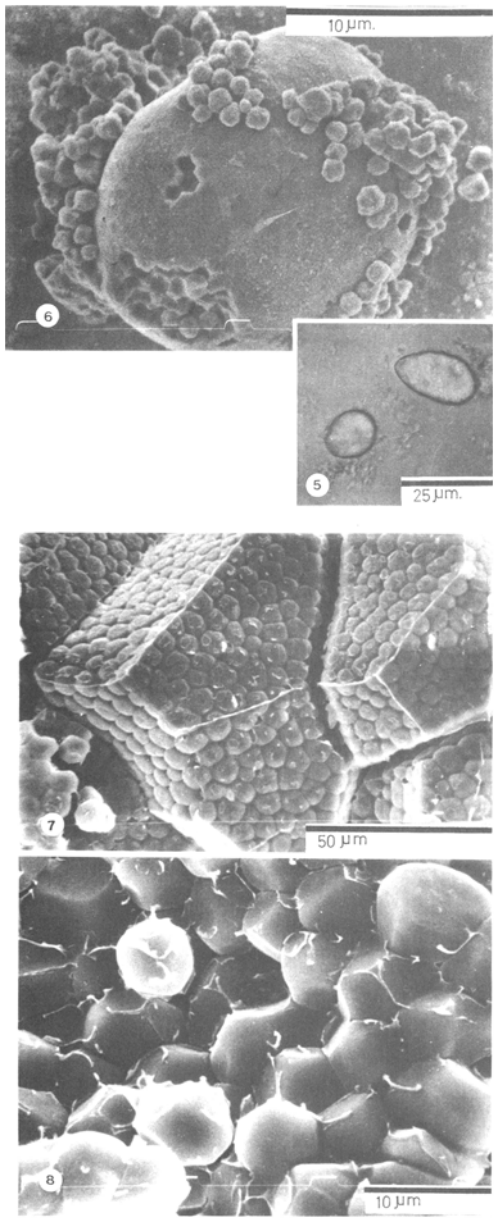

Fig. 5-8. **5)** *Chenopodium quinoa*: clustered grains from perisperm, LM photomicrograph; **6)** Idem: SEM micrograph. **7)** *Fagopyrum esculentum*: perisperm cells with single starch grains, SEM micrograph; **8)** Idem: detail of single grains.

as layers, fissures and hilum position. When complex compound grains with subunits of ca. 1 µm and indefinite number, such as quinoa (*Chenopodium quinoa* Willd.) and cañagua (*Ch. pallidicaule* Aell.), are being observed SEM must be used (Fig. 5, 6). The same is true in the case for single grains of small size, compactly disposed and filling the whole perispermatic cells, as those found in *Amaranthus* spp. and *Fagopyrum esculentum* Moench. In these cases, simple LM observation can lead to mistake a parenchyma cell full of small grains for a compound grain (Fig. 7, 8).

Potato starch presents different characteristics depending on the origin of the sample and its degree of preservation. Observation with LM shows the typical characteristics of the grain as described by Winton and Winton (1932) (Fig. 9). Observation by SEM shows the grain to be covered by a film which might be the remains of the amyloplast (Fig. 10). This film disappears when treated with acid or in material preserved in formalin-acetic acid-alcohol 70% (Fig. 11). The chuño de papa (Towle 1961) presents deteriorated grains, probably due to the potato dehydration, but which are easily detected through phase contrast (Fig. 12) and Congo red dyeing. Normal grains do not absorb stain components unless they are damaged or swollen. Starch grain morphology in wheat, barley and rye flours is similar, using either LM or SEM. Quantitative microscopic analysis is necessary for their identification. In order to determine if flour is pure or mixed, Wellendorf (1975b) applied the "starch index" described by Wallis (1922). Size distribution is bimodal: small and large grains are detected.

Using SEM, it is possible to observe the changes at the surface or inside the starch grain both from seeds during the germination process (Rodríguez et al. 1988) and from underground organs during sprouting (Fig. 13, 14, 15). Fungal amylase activity can also cause damage in the surface or internal structure of the grain, as described by Fuwa et al. (1978).

Some legume seeds stored for long periods at high temperature and humidity, develop the "hard-to-cook" (HTC) phenomenon. They require longer cooking periods and have less nutritive value. SEM observations showed that the starch grains of these HTC legume had surface damage. Paredes-López et al. (1988) consider that damage is caused by enzymatic activity during storage.

Corn starch is widely utilized as food and for industrial purposes. The endosperm represents 85% of the dry caryopsis weight. Its constitution determines the structure and feeding value of the

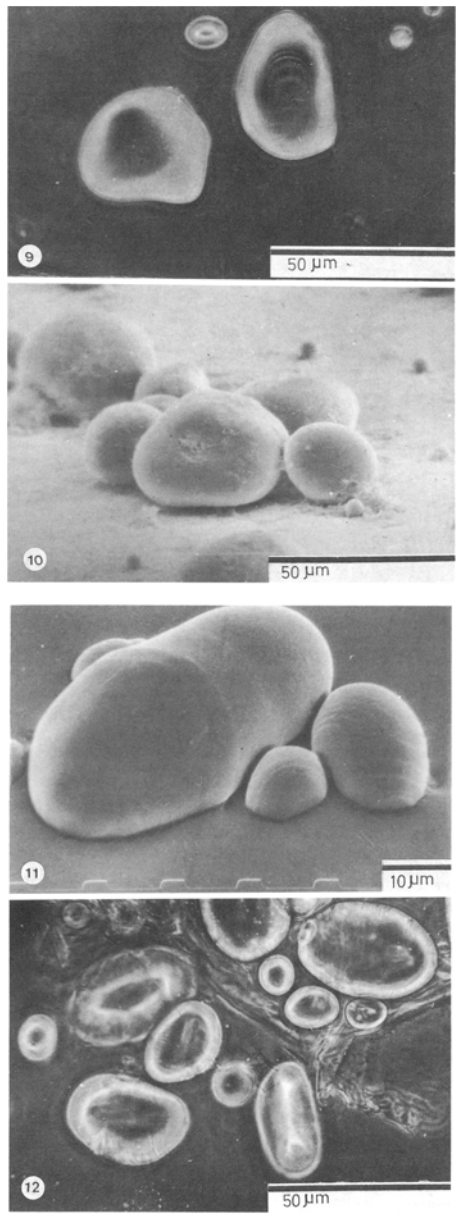

Fig. 9-12. **9)** *Solanum tuberosum*: LM and phase contrast observation of a fresh tuber; **10)** Idem: SEM observation; **11)** Idem: SEM observation of material preserved in formalyn-acetic acid-alcohol; **12)** Idem: LM and phase contrast observation of *chuño*.

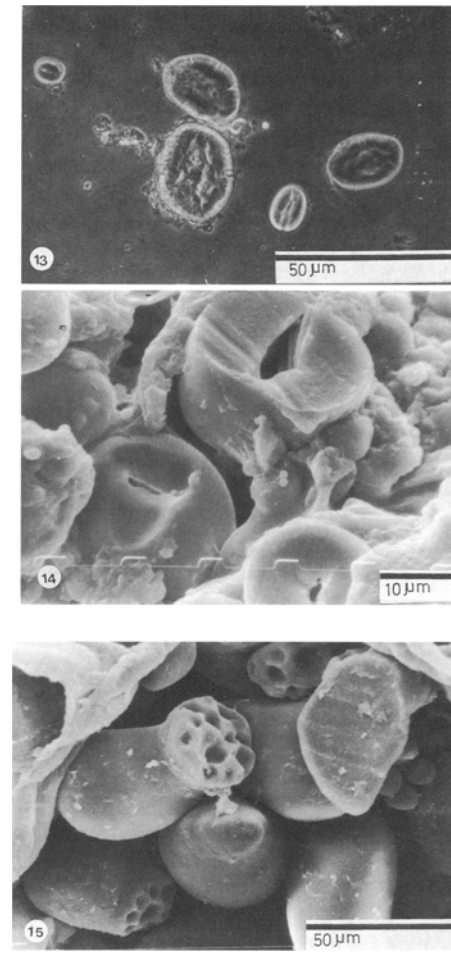

Fig. 13-15. **13)** *Pisum sativum*: LM and phase contrast observation of a germinated seed; **14)** *Phaseolus vulgaris*: SEM micrograph of a germinated seed; **15)** *Oxalis tuberosa*: SEM micrograph of a sprouting tuber.

different types of corn which have been classified according to the type of starch they have and its distribution in the endosperm. Corn with horny endosperm (hard and translucent), with floury endosperm (white) and with waxy endosperm (translucent) can easily be identified by sight or with the help of a stereoscopic microscope (León 1987).

The morphological characteristics of the starch grains in the three types of endosperm mentioned above have been elucidated by Winton and Winton (1932) and Trease and Evans (1986). Horny endosperm grains are angular, usually four or five-sided, compactly disposed and joined by zein and other proteins, forming a solid mass. Using LM, a central triangular hilum is observed, with a star-shaped fissure; the grain does not present layers and its size is about 10–30 µm. Floury endosperm grains are about 2–35 µm and spheric in shape. By means of SEM, morphological differences between floury endosperm and horny

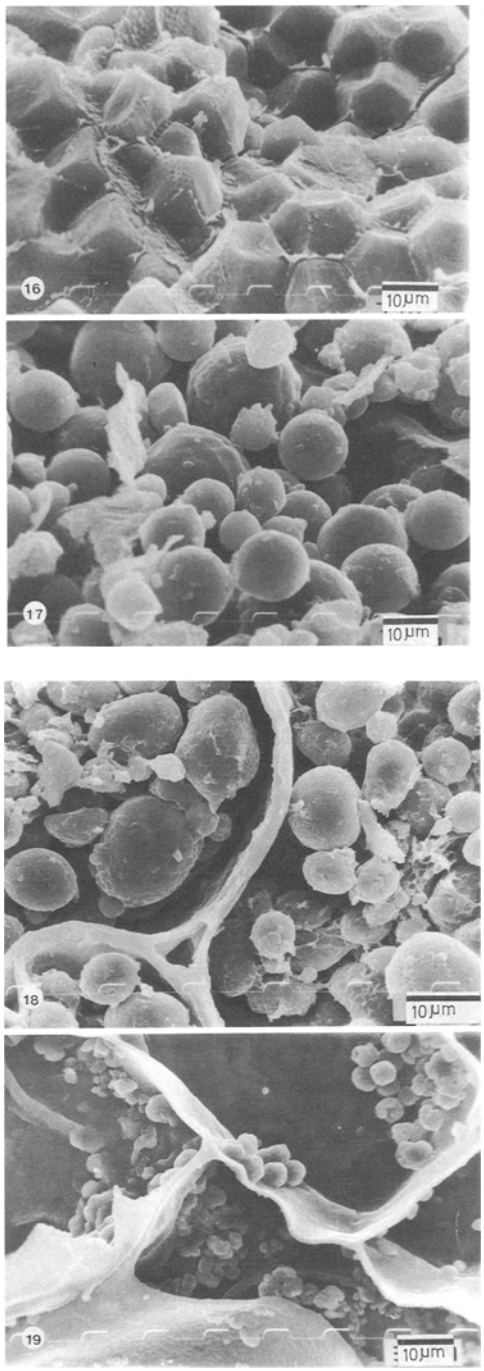

Fig. 16-19. **16)** *Zea mays*: horny endosperm, SEM micrograph; **17)** Idem: floury endosperm, SEM micrograph; **18)** *Arachis hypogaea*: SEM micrograph of cotyledons; **19)** *Chenopodium quinoa*: seed covers starch grains, SEM micrograph.

endosperm starches can be observed. Angular structure with compact grains showing a grooved or dimpled surface as the result of the crater-shaped impressions caused by zein bodies, was confirmed in horny endosperm (Fig. 16). This cannot be seen with LM. In the floury endosperm, spherical grains of variable size usually have smooth or more regular surfaces and are loose packed in the cell (Fig. 17).

Most Fabaceae seeds present a special type of starch grains known as bean-shaped or kidney-shaped (Wellendorf 1965). There are some exceptions, as the starch grains from *Arachis* cotyledons. They are masked by lipid drops and proteic bodies. After eliminating the fixed oils and proteic substances by means of solvents of different polarity, spherical and sub-spherical grains, generally with reticular surface, were observed (Fig. 18).

In the review of South American pseudocereals (Cortella and Pochettino 1990), distinct morphological differences in the starch grains present in the perisperm and in the seed covers were observed (Fig. 5, 19). This would confirm French's (1984) observation that isolated grains from different parts of the same plant can be completely different.

Archaeological Vegetal Material

The most difficult samples to analyze and understand are those obtained from archaeological sites. Desiccation and carbonization processes lead to the destruction of the histological elements. Carbonized samples are the most difficult to handle and identify, as few diagnostic characters, including starch, are preserved unaltered (Pochettino and Cortella 1989).

Starch grains are seldom found in archaeological remains, especially in carbonized material, because they are destroyed or partially hydrolyzed, and lose their polarization property. But some of them maintain their typical shape and can be dyed with I_2-IK. A larger quantity of grains can be found in desiccated samples but, although they are not misshaped by swelling due to heat and humidity, they are frequently invaded by fungi and sometimes have fissures indicating dehydration.

Resistent starch grains are difficult to see because they are often hidden inside the carbonized mass. In order to observe them, the sample must be decolorized with strong oxidizing agents and polarized light microscope (if the crystalline

structure is not modified) and phase contrast microscopy must be used (Fig. 20). This technique was used by Ugent et al. (1981, 1982, 1984, 1986) to observe starch in desiccated underground storage organs found in many Peruvian archaeological sites.

Indirect Evidence of Starch Presence

Light microscope observation of carbonized seeds of bean and corn show conspicuous structures with round perforations (Fig. 21). Utilizing SEM these structures were identified as the amylaceous parenchyma in which starch had disappeared leaving holes or "molds" on a compact stroma (Fig. 22, 23). To check this finding, material was compared with modern samples and it was seen that starch grains in parenchyma cells are submerged in a proteic stroma where they leave their traces or cavities. These traces are related to the size and shape of the grain (Fig. 23). In this way, cultivated and wild varieties of *Phaseolus vulgaris* could be differentiated and the presence of *Phaseolus vulgaris* var. *aborigineus* in archaeological contexts could be confirmed (Pochettino and Scatollin 1991).

In the case of other organs such as stems, roots and desiccated material, traces or marks of starch grains could be observed in the inside wall of the cell (Fig. 24, 25).

Discussion

The common light microscope, with 2000–2500 Å (0.2–0.25 μm) resolution allows the observation of the hilum, shape and size of the grains and swelling produced by heat and/or chemical agents. Comparing these features, different species can be distinguished (Seideman 1964, 1966; Wellendorf 1963). Light microscope observations make it hard to distinguish the surface from the inner structure.

Native starch grains are better observed with a polarization microscope. Grains are seen as distorted spherocrystals with the typical dark cross (Fig. 26). The apparent intensity of the double refraction of light rays depends on the grain thickness, the degree of crystallinity and the orientation of crystallites. It is widely accepted that the optical axis coincides with the direction of the starch chain as in other biopolymers (French 1984). Damaged regions are only partially or not birefringent in the polarized light. This has been observed in archaeological starch, either carbonized or desiccated.

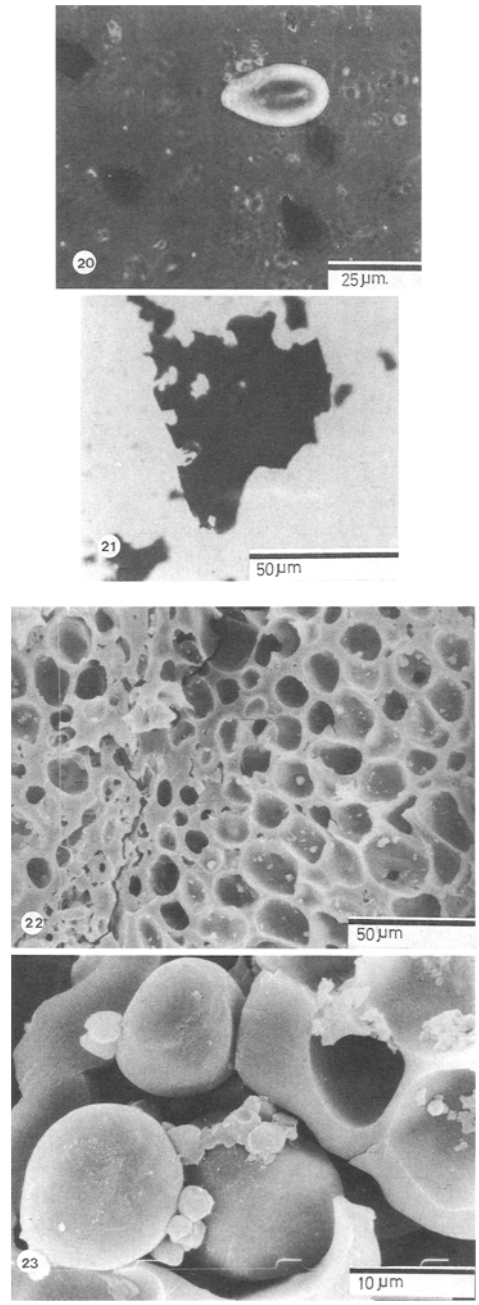

Fig. 20-23. **20)** *Phaseolus vulgaris* carbonized seed: LM and phase contrast photomicrograph; **21)** Idem: traces of starch grains, LM photomicrograph; **22)** Carbonized *Zea mays*: traces of grains, SEM micrograph; **23)** Idem: grains and traces, SEM micrograph.

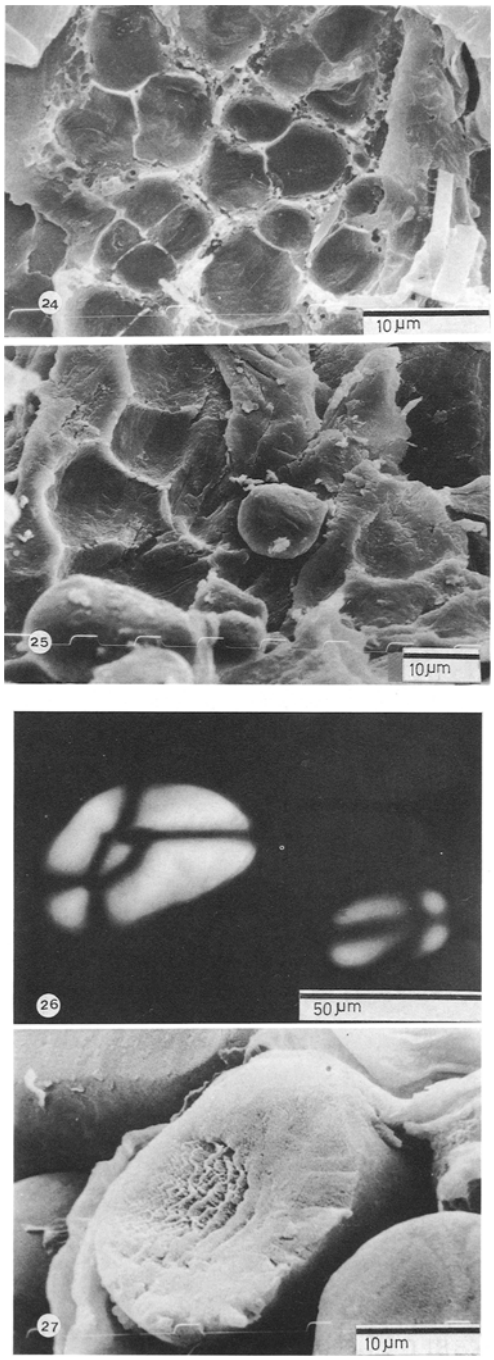

Fig. 24-27. **24)** *Cinnamomum cassia* bark: traces of starch grains, SEM micrograph; **25)** *Oxalis tuberosa* tuber: grains and traces, SEM micrograph; **26)** *Solanum tuberosum*: polarization microscope photomicrograph; **27)** *Oxalis tuberosa*: grain cut, SEM micrograph.

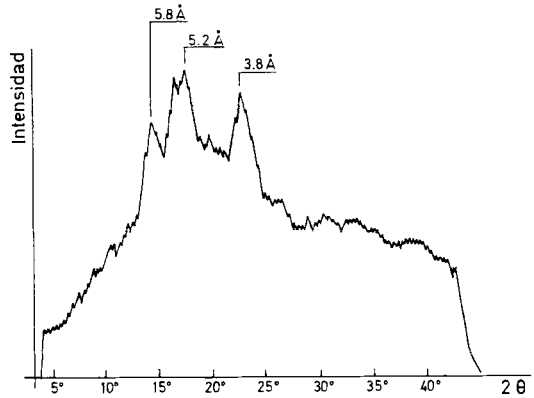

Fig. 28. X-ray diffractometer tracing from *Chenopodium pallidicaule* perisperm starch: A-pattern.

X-ray diffraction studies show different traces depending on the sample origin and this is one of the few ways to classify starch according to physical properties. Generally, cereal starch has a type A pattern, tubers have a type B pattern and roots and seeds have a type C pattern (Abd Allah et al. 1987) (Fig. 28). There are exceptions, and perhaps there will be more, since a wide variety of starches is being isolated and characterized by X-ray diffraction (Abd Allah et al. 1987; Eliasson et al. 1987; Zobel, 1988).

The scanning electron microscope can be employed to study the surface detailed structure of starch grain as well as its internal structures in different sections (Fig. 27). Use of SEM has two major advantages over use of LM: it has much greater depth of focus than the light microscope and the resolution is 70 Å or less, depending on the instrument used (Fitt and Snyder 1984).

Staining is a useful technique to identify different features of starch grains. Starch reacts with I_2-IK: long chain molecules stain deep blue, while short chain molecules stain red-brown, so the grain stains differentially according to the amylose–amylopectine proportion (Hixon and Brimhall 1968). Congo red is used as a quick method for detecting damage and fissures.

Conclusions

Starch grains are an important diagnostic feature in the identification of vegetal material because of their abundance, ubiquity, and varied morphological characteristics. From the taxonomic point of view, there are groups in which

TABLE 1. TECHNIQUES FOR THE STUDY OF STARCH GRAINS.

Main techniques used	Information obtained	Examples
Light microscopy. Resolution: 2000–2500 Å.	Morphology (qualitative and quantitative).	Commercial starches. Spices. Herbal products. Mixture of flours. Amylaceous parenchyma.
Stains		
I_2-IK	Short chain starch molecules (red brown) or long chain starch molecules (deep blue).	Waxy starch. Floury starch.
Congo Red	Damaged starch granules.	*Chuño*. Germinating seeds.
Phase contrast microscopy	Identification of starch in material with very few or masked grains.	Carbonized archaeological remains.
	Damaged grains.	Hydrolized grains in flours or commercial starches.
Petrographic microscope	Localization of starch in tissues where grains are masked with other ergastic substances.	No amylaceous tissues: *Arachis* cotyledons; seed coats (*Chenopodium* spp.)
	Evidence of starch crystallinity (birefringence) or damaged regions (no birefringence).	Commercial starches. Flours. Spices.
Scanning electron microscopy. Resolution: 70 Å	Surface detailed structure.	Hard-to-cook (HTC) seeds. Sprouting tubers.
	Identification of complex compound grains with indefinite number of subunits, or grains smaller than 1 μm.	Pseudocereals (*Chenopodium, Fagopyrum*).
X-Ray Diffraction	Evidence of starch crystallinity.	
	Information about sample origin.	Pseudocereals (A pattern).

starch grains are distinctive features while in other groups they are of relative value and other features must be used. Starch grains are utilized as diagnostic characters in samples such as flours, spices or herbal drugs. But in archaeological remains, starch has usually disappeared so that grain impressions left in the tissue constitute a valuable diagnostic element for comparison with fresh material.

Various instruments and techniques should be used both for the morphological description and for checking the grain physical condition from which the previous history of the sample can be inferred (Table 1).

Research work and techniques generally deal with morphology, features, and properties of secondary starch because of its abundance and economical interest. In the future, it will be productive to study starch grains as a diagnostic feature found in non-reserve tissue.

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Book Review

Biogeography. An Ecological and Evolutionary Approach. C. B. Cox and P. D. Moore. Blackwell Scientific Publications, London, 1993. x + 326 pp. 2nd edition. $34.95. (paperback). ISBN 0-632-02967-6.

In the preface of their 1973 edition of *Biogeography,* Cox and Moore outlined a multi-disciplinary approach to biogeographic study. This was well used to illustrate the complexities of biological diversity, and related considerations in biogeographic study. The current (1993) edition uses the same format, with topics progressing from basic, through those complex enough for use in advanced studies. This edition has information enough to have created two chapters more than the first. Much is in the form of examples, many of which have replaced the originals. Some that were sacrificed were well suited to the text; their replacements are equally applicable, but may be less well known. The new edition has increased its figures and tables by nearly 100% over the original, and more than three times as many citations appear in the 1993 edition. More importantly, of this increase in citations, nearly two thirds are additions since the last (1985) publication.

Much new material seems to be aimed at neutralizing historically contended issues, by suggesting that too little is known about complex phenomena to attribute them to one or a few established rules. This may create some anxiety among reductionists, but holistic considerations are valuable. Uncountable factors affect living systems, their adaptations, and interactions. Single factor explanations are valuable pedagogical tools, and are indispensable for hypothesis testing, but depending on these may limit understanding. We need this sort of reminder that natural systems are enormously complex, even though we test one hypothesis at a time.

In contrast, reference to vicariance and dispersal appears to be appended after most of the text was created. The introduction and definitions are adequate, but the most complete explanation of these concepts occurs after comparisons of distributions. Throughout the text there are places in which consideration of these theories may be appropriate. For example, the text emphasizes colonization of continents by mammals and Angiosperms; earlier text, however, indicates evolutionary development of these groups before continental breakup. Dispersal of taxa may be a valid explanation here, but vicariance seems to be an option. The question here may be: Is it better to offer an explanation after indicating differences in distribution, or to introduce the explanation before the examples? Either format is acceptable; the authors have chosen to present these alternatives as possible explanations after the comparisons have been made. Whether these concepts should be introduced earlier can be determined by an instructor; without such guidance this later introduction should cause no problem for the serious reader.

The text is usually clear and concise. In a few places it is difficult to follow and occasional editorial oversights exist. For example, the initial paragraphs describing tectonic activity are difficult, but are followed by systematic, detailed, and well-explained treatments of plate movement, climate, and biotic changes. The explanations are complemented by helpful graphics, but these are presented in different styles. This creates a minor obstacle in following the illustrations. Graphics with the same format would be welcomed here.

The explanation of the K/T extinction considers both gradual and punctuated extinctions. This attention to the possibility of both phenomena may seem like fence-sitting, but is a reasonable consideration based on limited available information. This again illustrates the complexity of global ecological events over evolutionary time. Indeed, there is little to indicate that both could not have occurred.

In summary, Cox and Moore present Biogeography without apology for the complications involved in its study. The underlying complexity, however, is hardly noticed as detailed explanations based on available information make up much of the text. This edition is well suited for introductory studies, and will be a valuable reference for those with advanced interests in biogeographic study.

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