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## On the Question of Pear Decline in Northern Greece

By

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With 10 Figures (4 Plates)

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#### Summary

ELEFTHERIOU E. P. & TAMOUTSELI D. C. 1985. On the question of pear decline in Northern Greece. — Phyton 25 (1): 123—133, with 10 figures (4 plates). — English with German summary.

Pear trees (*Pyrus communis*) exhibiting declinelike symptoms from an orchard in Northern Greece have been investigated by light, fluorescence and electron microscopy. The purpose was to demonstrate whether decline is caused by mycoplasmalike organisms (MLO) or from other factors. Observations indicate that no replacement phloem is formed, while necrosis of sieve tubes results from normal seasonal degeneration. Functioning phloem contains the usual cytoplasmic components in its highly specialized sieve tubes. No sieve-tube particles could structurally be identifiable as MLO, whige fluorescence microscopy gave negative results. It is concluded that MLO should be excluded from the decline etiology and the disease is probably caused by scion-rootstock incompatibility.

### Zusammenfassung

ELEFTHERIOU E. P. & TAMOUTSELI D. C. 1985. Zur Frage der Birnenverfall-Krankheit in Nordgriechenland. — Phyton 25 (1): 123—133, mit 10 Abbildungen auf 4 Tafeln. — Englisch mit deutscher Zusammenfassung.

Birnbäume (*Pyrus communis*) aus einem Garten in Nordgriechenland mit Symptomen ähnlich der Birnenverfall-Krankheit wurden licht-, fluoreszenz- und elektronenmikroskopisch daraufhin untersucht, ob diese Krankheit durch mycoplasma-ähnliche Organismen oder durch andere Faktoren verursacht ist. Die Beobachtungen ergaben, daß keine Neubildung von Siebröhren stattfindet, die Nekrose jedoch der jahreszeitlichen Degeneration

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entspricht. Arbeitsfähiges Phloem enthält die normalen cytoplasmatischen Komponenten, keine Strukturen in ihnen konnten als mycoplasma-ähnliche Organismen identifiziert werden. Es wird gefolgert, daß diese Organismen nicht als Ursache der pear-decline-Krankheit diskutiert werden sollten, sie ist möglicherweise durch eine Unverträglichkeit von Pfropfreis und Unterlage verursacht.

(Editor transl.)

#### Introduction

More than a decade has past since a condition of decline by several hundred pear trees in orchards of central and northern Greece was reported (Agrios 1972), though trees exhibiting various symptoms of decline have probably been present for many years. Recently there was an outbreak of the disease especially in the Imathia province, northern Greece, appeared in 1979—80 when the cultivar "Williams", propagated on quince rootstocks, was widely introduced in the area.

However, the question on the causal agent remains still unanswered, though the resulting damage to pear cultivators and pear industry has become serious for the country during the last four years. According to experts a percentage of more than  $50^{\circ}/_{\circ}$  of the recently planted "Williams" trees exhibit symptoms highly resembling the slow disease symptoms of the so called "pear decline".

Pear decline is a very destructive disease initially recognized as a new disease in some States of U. S. A. in about 1948 (see Blodgett & al. 1962). Later it was also observed in some European countries (Germany, Italy) and probably it occurs in other continents (see Agrics 1978). Pear decline causes either a slow, progressive weakening and final death of trees (slow decline) or a quick, sudden wilting and death of the trees (quick decline) (Woodbridge & al. 1957, Moller & al. 1978).

Pear decline, as well as several yellows-type diseases, were initially thought to be caused by viruses; however, the application of electron microscopy revealed that the disease is caused by mycoplasmalike organisms (MLO) present in the phloem sieve tubes (Hibino & Schneider 1970, Soma & Schneider 1971, Agrics 1978, Behnke & al. 1980, Schaper 1981). MLO are spherical to oblong particles of about 50 to 800 nm. The bodies are bounded by a single triple-layered "unit" membrane, they lack cell wall and contain cytoplasm, randomly distributed ribosomes and strands of nuclear material (see Agrics 1978, Behnke & al. 1980). The pathogens were found to be transmitted from plant to plant by the pear psylla (*Psylla pyricola* Först., Jensen & al. 1964) or by grafting (Blodgett & al. 1963, Schneider 1970). The disease is also associated with the rootstock (Blodgett & al. 1962). Expression of decline in various rootstocks is

influenced by both inherent susceptibility to the decline organisms and by natural vigour (Moller & al. 1978, Psarros 1981).

Besides the pear decline, similar decline disorders of pear have also been observed, whose relationship to pear decline have not been established. Such declining symptoms, not caused by MLO, are attributed to other factors like graft incompatibility (Agrics 1972).

Since the causal agent of declinelike disorders of pear trees in Greece has not been determined yet, no measure has been taken till now to face the disease or limit its expansion. Among experts in Greece there is a controversy about the etiology of the decline. Some claim they have good, although indirect, evidence that it is caused by MLO and they seek for a definite demonstration, while others, failing to transmit the disease by grafting scions from diseased to healthy trees, have a strong belief that the decline is the result of scion-rootstock incompatibility. In order to contribute to the solution of this question the present structural and ultrastructural investigation has been undertaken, aiming to demonstrate, if possible, the presence or absence of MLO from the sieve tubes of decline affected trees.

#### Materials and Methods

A four-year-old pear orchard located by the Nissi village in the province of Imathia, northern Greece, in which about 40% of the trees exhibited a condition of decline attracted our attention for investigation. The pear cultivars (*Pyrus communis L.*, cv. Williams) were grafted onto quince rootstocks (*Cydonia oblonga Mill.*, clone BA 29).

Three diseased trees have been selected for the present structural study. Samples have been taken on 23 June, 12 September and 11 November, 1983. Each time sampling included root cortex (roots 5—8 mm in diameter), stem cortex from current season's shoots, small veins from reddish leaf blades and segments of leaf petioles. Stem and root samples were cut with some wood so that to ensure the presence of intact phloem.

For fluorescence microscopy samples sized about  $2 \times 5$  mm were excised and immersed immediately in  $5^{\circ}/_{\circ}$  cold glutaraldehyde fixative in phosphate buffer 0.1 M, pH 7.0. The material was stored in a refrigerator in this solution until sectioning with a freezing microtome (Jung Fricocut Model 2700). The about 10  $\mu$ m thick sections were stained with a DNA indicator (4'-6-diamino-2-phenyl-indol [DAPI]: Serva, Heidelberg, West Germany) and they were examined by using a Leitz epifluorescence illuminator (Seemüller 1976, Schaper & Seemüller 1982).

For electron microscopy thin elongated strands were cut while immersed in fixative consisting of 5% glutaraldehyde and 4% para-

formaldehyde (Karnovsky 1965) in cacodylate buffer 0.05 M, pH 7.0. Transverse sections were cut at least an hour later. Fixation lasted 5 h in all at ambient temperature (about 20—22° C). The specimens were futher postfixed in 1% OsO4, dehydrated in ethanol series and propylene oxide and embedded in Spurr's epoxy resin (Spurr 1969). Semithin sections were stained with toluidine blue 0 and ultra-thin ones, all cut on a Reichert Om U-2 ultramicrotome, were examined and photographed with a Zeiss 9 S-2 electron microscope after double staining with uranyl acetate and lead citrate (Reynolds 1963).

#### Observations and Results

Declining pear trees were distributed in the orchard singly or in small groups. The symptoms of declining were: reduced overall size; little or no terminal growth; twigs lose green colour becoming brownish; sparse foliage; the few leaves were smaller in size and turned on reddish in late summer; no evident rolling, however, of lateral leaf margins was observed; most leaves drop off prematurely in autumn. The declining trees appeared the tendency for quick fruit production; the fruit, however, were few and small in size, rather rounded in shape and of poor quality. Cut and removal of a narrow strip of bark from the bud union of diseased trees revealed the presence of a distinct brown line on the trunk. Some of them died in autumn three years after planting, while others recovered.

Light microscope micrographs (Figs. 1—4), obtained from semithin sections cut prior to ultrathin sectioning, served for a preliminary examination of the phloem of stem, roots and leaves. Fig. 1 presents a general layout of tissues in leaf petiole. Xylem rays radiate from the inner border of the xylem to the pith outwards up to the border of phloem and cortex. At a centripetal direction, phloem consists of fiber-sclereids, non-functioning sieve tubes, and functioning sieve tubes adjacent to the cambium (Fig. 2). The cytological condition of the sieve tubes was determined by electron microscopy (e. g. Figs. 5 and 6). Small-sized vascular bundles were preferably examined in leaves (Figs. 3, 4).

The sieve tubes of the stem secondary phloem degenerate during autumn. The degeneration begins in the oldest part of the tissue and proceeds centripetally towards the cambial zone (at the direction of the arrow in Fig. 2). The number of sieve tubes degenerated depends upon the time of collection, being fewer in early and more in late autumn. Figs. 5 and 6 were obtained from stem samples collected on November. Fig. 5 shows a portion of phloem situated close to the cambium containing several sieve elements having a structure suggesting a functional conditon, some sieve elements at a collapsing process, and companion cells. As functional sieve tubes are considered those which are lined

by an intact plasmalemma and bear the normal cytoplasmic complements of mature sieve tubes (see Esau 1969: 386). The companion cells as well as the phloem parenchyma and ray cells have plastids bearing one or more sizeable starch grains. On the other hand the oldest part of secondary phloem consists of sieve tubes lacking any internal cell structure and having an entirely empty lumen (Fig. 6). They are readily distinguishable at the electron microscope and apparently they were not functioning at the time of collection. Some of them bear thick nacreous thickenings. However, non-functioning sieve tubes were encountered among functioning sieve tubes in the phloem close to the cambium (Fig. 5).

Several parenchyma cells scattered throughout the phloem contain crystals. The crystals are hard enough to cause damage to the glass knife during sectioning and they fail to be cut; the space occupied by a crystal appears as an entirely electron transparent area in the ultrathin sections (Fig. 5). Usually, the shape of the area denotes the outline of the crystal. All crystal-containing cells have a large clear area suggesting the development in the living cells of single prismatic or rhombohedral crystals.

Intimate examination of the sieve tubes of the functioning phloem reveals that most of them contain fine thread-like filaments, which are the characteristic protein of phloem, the so-called P-protein (Figs. 7—9). In some elements P-protein is abundant and dispersed throughout the cell lumen, while in others it is rather scant (Fig. 5).

Sieve-tube cell walls develop thick nacreous thickenings (Fig. 7). The inner face of the undamaged sieve tubes is lined by a continuous plasmalemma (Fig. 8). Several other structures are encountered close to or in contact with the plasmalemma, among which the most easily identifiable are endoplasmic reticulum (ER) cisternae (Figs. 7, 8) and mitochondria (Figs. 5, 8). Mitochondria are recognized by their double-membrane envelope, the rounded to oval shape and their constant size; inner cristae are not always evident. If sections are out of the middle region of the organelle their features are less distinguishable.

Besides the above components of the sieve elements, several other structures are also encountered. Variously-sized vesicles defined by single membranes, without or with low-contrasted contents are common (Figs. 8, 9). Multivesicular and multilamellar bodies normally are associated with the plasmalemma (Fig. 9). Sieve-element plastids contain starch grains delimited by the double-membrane envelope of the organelle. However, in disturbed sieve tubes the starch grains are released and/or disorganized into numerous small segments which disperse throughout the cell lumen (Fig. 10). The pores of sieve plates and lateral sieve areas in uninjured sieve tubes are usually occupied by electron

dense material including P-protein (Fig. 9), but in disturbed ones they are occluded by callose (Fig. 10).

Examination of phloem by fluorescence microscopy gave negative results. Not any specific fluorescence could be detected in any sieve tube after DAPI staining in any collection of the three diseased trees.

#### Discussion

Agrios (1972) was the first to report on the existence of a declining disease in pear orchards of Greece. The declining symptoms exhibited by many pear trees in Greek mainland were, according to Agrios, in most respects similar to those described for pear decline in North America, despite the differentiation in certain characteristics. Comparison of symptoms of the diseased trees in the orchard presently examined to those described by Agrios (1972), though similar in some aspects, differ in others. The latter refer mainly to the pre-seasonal reddening of leaves and terminal shoots, and the lack of an upward rolling of the leaves at their margins in ours, while Agrico describes the leaves of his "William" cultivar as turning "greenish-yellow to yellow-red" and "rolled upward". A demarcation line at the graft union of some declining trees was observed both by Agrios and us. However, the "William" trees examined by Agrios (1972) were grafted onto wild pear rootstocks, while ours on quince. The scion-rootstock combinations play very important role on the behaviour of pear decline diseases, and this mostly depends on the rootstock rather than the scion (Blodget & al. 1962, Moller & al. 1978).

EVERT (1960) described the phloem of pear bark and its seasonal changes. New phloem tissue forms each spring and functions through the summer. Then, sieve tubes, beginning with the older ones, gradually degenerate during autumn and early winter. Event proposed a diagram interpreting seasonal growth of stem secondary phloem. According to the diagram, we were expecting to find functional sieve tubes in June and September, while in November about half of the phloem should be functional and the rest nonfunctional. Nonfunctioning phloem denotes the phloem in which the sieve tubes have ceased to conduct, but the tissue is not entirely functionless (see Esau 1969: 231). Our results are in good agreement with these data. The first sieve tubes to cease conducting are those found away from the cambium, that is the initial elements formed in early spring. Degeneration further proceeds centripetally. Nonfunctioning sieve tubes do not obligatorily crush and/ or obliterate. According to Evert (1960) the degree of crushing of sieve elements appeared to depend on local tissue relations: the sieve elements were crushed only if they occurred next to the enlarging parenchyma cells of fiber-sclereids. Consequently, the degenerated sieve tubes we have observed are probably the result of a normal seasonal degeneration.

Sieve elements, being the highest specialized living cells of vascular plants, are of principle importance for the long distance transport for assimilates in all plant organs. Their significance is shown by their early, prompt, and continuous differentiation between source and sink (KOLLMANN & al. 1983). Any interruption of this transport system interferes with vital functions of the plant resulting in serious damage or even death. In experiments where phloem continuity is intentionally interrupted the gap created is bridged by short-time cell differentiation (Behnke & Schulz 1980, Kollmann & al. 1983). The normal seasonal degeneration in autumn is followed by next-spring phloem regeneration (Evert 1960). However, in severely diseased pear trees (see Blodgett & al. 1962, Agrios 1978), as well as apple trees (Schaper 1981, Schaper & SEEMÜLLER 1982), the sieve tubes of spring phloem become damaged, necrotic and somewhat collapsed, and a replacement phloem is formed. The replacement phloem is indicative of pre-seasonal degeneration or of a graft union phloem discontinuity. In all trees examined in the present study no replacement phloem could be found. The necrotic sieve tubes result probably from normal seasonal degeneration.

Crystal deposition in a variety of phloem parenchyma cells is a common phenomenon, while crystal accumulation characterizes non-conducting phloem (see Esau 1969: 245). In *Pyrus communis* the crystal-containing cells are encountered even among functioning sieve tubes (Fig. 5). These cells do not store starch and their protoplasts eventually become disorganized (see also Evert 1960).

The structural cytology of sieve tubes presently examined is in good agreement with the results of other investigations on pear trees (EVERT 1960, BEHNKE & al. 1980, SCHAPER 1981) and other higher plants (see Esau 1967, Evert 1977). All well preserved sieve elements of Pyrus communis are lined by intact plasmalemma and contain Pprotein, which was mostly encountered as dispersive filaments. Pprotein is a major characteristic component of the protoplasts of dicotyledonous sieve-tube members and occurs in several morphological forms (Evert 1977, Kollmann 1980, Behnke 1981). For a long period P-protein attracted the attention of phloem physiologists and cytologists in their search for the mechanism of translocation (for literature see e. g. Cronshaw 1975, Kollmann 1980). The sieve-element plastids of Pyrus communis contain starch grains and they are classified into the S-type sieve element plastids of Behnke's (1981) taxonomic system. Their appearance is similar to other dicotyledons (compare e. g. Behnke 1981, Gailhofer 1983).

Among the components of the sieve-tube elements of *Pyrus* communis mitochondria and ER cisternae, both parietally distributed

are the most consistent structures in functioning sieve tubes. Multivesicular and multilamellar bodies are also parietally encountered. Favourable images give clear evidence for continuity of the delimiting membrane and the plasmalemma. Such configurations have been termed as paramular bodies (Marchard & Robards 1968) and probably they are involved in the reduction of the plasmalemma surface (Kristen 1973).

Other components are the variously-sized vesicles confined by single membrane, but lacking any internal structure or electron density. Such structures, as well as mitochondria, ER profiles, starch grains, starch fragments, and paramular bodies are readily discernible from MLO observed in sieve tubes of decline-diseased pear trees. MLO have been identified either by fluorescent microscopy (SEEMÜLLER 1976, 1983, CAZELLES 1978, SCHAPER 1981, SCHAPER & SEEMÜLLER 1982) or by electron microscopy (Hibino & Schneider 1970, Soma & Schneider 1971, PARTHASARATHY 1973, WORLEY 1973, BEHNKE & al. 1980, SCHAPER 1981). They are confined by a unit membrane and contain internal structures such as ribosomes and DNA filaments (Agrios 1978, Behnke & al. 1980). The demonstration of MLO in sieve tubes has implicated these bodies as plant pathogens (Giannotti & al. 1968) and responsible for pear decline (Hibino & Schneider 1970, Agrios 1978, Moller & al. 1978). The cell complements observed in well preserved sieve tubes of all trees examined are in no way identifiable as MLO. Attempts, however, to demonstrate MLO in diseased trees by electron microscopy often meet with failure or reveal only a very low population of the organisms (see Seemüller 1983). Our electron microscopy investigation, however, was combined with light and fluorescence microscopy and the correlated results conform with the same conclusion: that presently and under the conditions and at the extent of our experiment, MLO should be excluded from the etiology of declinelike symptoms of the diseased trees.

## Concluding remarks

The absence of replacement phloem as it is revealed by light microscopy, the normal complement in organelles of mature functioning sieve tubes of secondary phloem examined by electron microscopy, the failure to identify any MLO in the sieve tubes, and the negative results of fluorescence microscopy, all suggest that the causal agent of decline of the pear trees in the orchard examined under the conditions and at the extent of our experiment is not attributed in all probability to any pathogens in phloem sieve tubes. Agrics (1972) has concluded that the declining symptoms of pear trees in orchards of central and northern Greece result from graft incompatibility. In the present study no evidence was obtained against that approach.

Recently, an ultrastructural investigation carried out on callus cells of pear grafted on callus cells of quince clearly indicated a graft incompatibility between pear and quince *in vitro* (Moore 1984). These results are in good agreement with the results reported here and suggest that there is a correlation between *in vitro* and *in vivo* grafting responses.

#### Acknowledgements

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- REYNOLDS R. S. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. J. Cell Biol. 17: 208—213.
- Fig. 1. Part of petiole in transverse section. Double-head arrows indicate the several tissue regions. Xylem and phloem rays radiate from the pith to the border of phloem (P) and cortex (Co). X=xylem, E=epidermis. x 125
- Fig. 2. Stem phloem. The crosses mark several sieve tubes and the small arrows point to companion cells. The large arrow indicates the direction of sieve tube degeneration.  $X=xylem,\,C=cambium,\,PR=phloem\,rays.$  x 620
- Fig. 3. Transverse section of a leaf, including a small vascular bundle. UE = upper epidermis, LE = lower epidermis, PP = palisade parenchyma,  $SP = spongy parenchyma, \times 230$
- Fig. 4. The vascular bundle of Fig. 3, at higher magnification. Leaf sieve tubes are small in diameter and develop thick nacreous walls. P = phloem, X = xylem.  $\times$  640

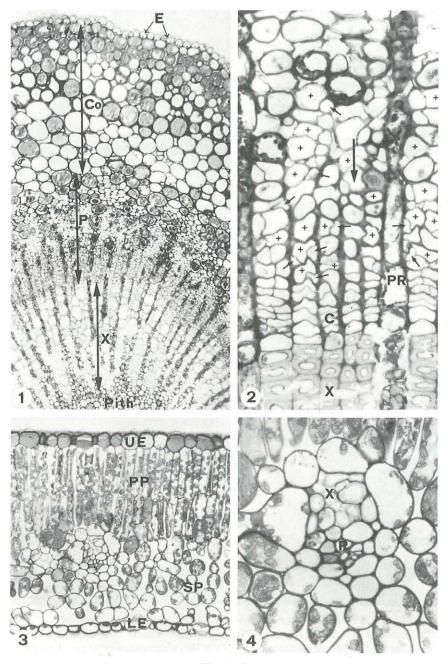


Fig. 1—4

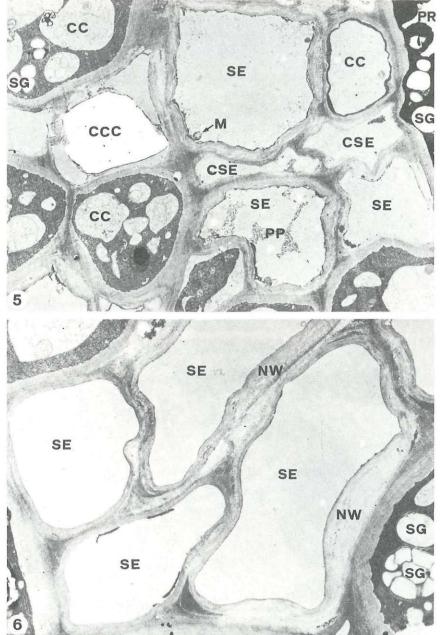


Fig. 5. Portion of stem phloem containing sections of several functioning sieve elements (SE), some crushing sieve elements (CSE), companion cells (CC) and a crystal-containing cell (CCC). The plastids in both the companion cells and the phloem rays (PR) bear starch grains (SG). PP = P-protein, M = mitochondrion. x 4,000

Fig. 6. Sieve elements (SE) of stem with apparently empty lumen found in the oldest part of secondary phloem. They are in all probability non-functioning. NW = nacreous walls, SG = starch grains. x = 6,500

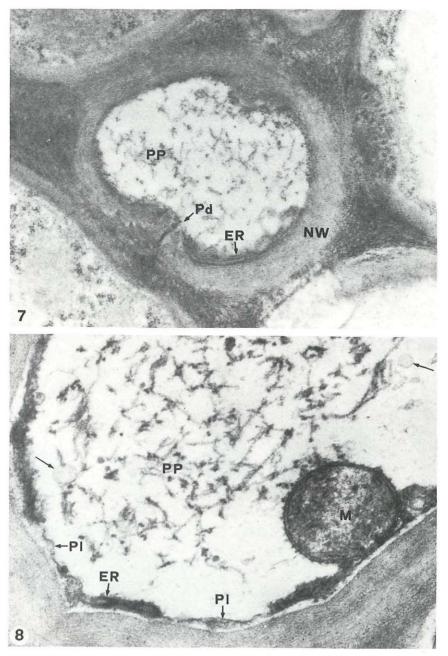


Fig. 7. Sieve element of a leaf vascular bundle, with thick nacreous wall (NW) and P-protein (PP) in the cell lumen. ER = endoplasmic reticulum, Pd = plasmodesma. x 22,000

Fig. 8. Portion of a sieve element of the stem. P-protein (PP) dominates the cell lumen. Arrows point to low-contrasted vesicles. ER = endoplasmic reticulum, Pl = plasmalemma, M = mitochondrion. x 50,000.

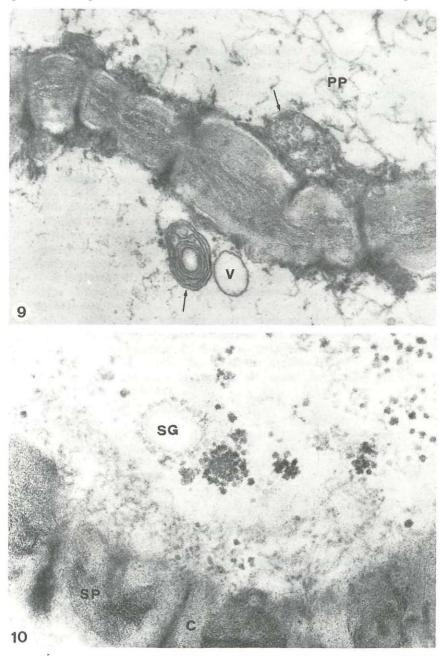


Fig. 9. Part of a lateral sieve area in stem. Multivesicular (upper cell) and multilamellar (lower cell) bodies indicated by arrows. PP = P-protein,  $V=vesicle. \ x \ 39,000$ 

Fig. 10. Portion of a disturbed sieve element from the petiole near a sieve plate (SP). Starch grains (SG), released from plastids, disorganize into numerous small segments which disperse throughout the lumen. C = callose.  $\times$  57,000

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## Recensio

SCHUBERT Rudolf, HANDKE Horst Herbert & PANKOW Helmut 1983. Niedere Pflanzen — Grundband. — In: Rothmaler Werner, Exkursionsflora für die Gebiete der DDR und der BRD, Band 1. — 8°, 811 Seiten, zahlreiche Abbildungen; geb. — Volk und Wissen, Volkseigener Verlag Berlin. — M 46,80. — Bestelln. 707-161-5.

Während die Gefäßpflanzen betreffenden Bände in der Reihe "ROTHMALER Exkursionsflora" seit über zwei Jahrzehnten in Verwendung sind, mehrere Auflagen erlebt und sich bestens bewährt haben, konnte der Band 1 für die Niederen Pflanzen (Kyrptogamen exkl. Farnpflanzen) erst jetzt vorgelegt werden. Dieser Umstand demonstriert schon die, durchaus verständlichen, großen Schwierigkeiten, die dem Konzept einer solchen Flora entgegenstanden.

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Jahr/Year: 1985

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<u>123-133</u>