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LIGHT MICROSCOPY OF ROBINIA MOSAIC CUCUMOVIRUS CRYSTALLINE INCLUSIONS

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In cells infected with robinia mosaic cucumovirus (RoMV) we established two basic types of crystalline virus inclusions: hollow crystals, of polyhedral and spherical to oval appearance, and ordinary polyhedral crystals. The former seem regularly present in the vacuole, and the latter in the cytoplasm. The optimal temperatures for development of RoMV crystalline inclusions were betwenn 15—20°C at the period of longer days. All forms of the crystals were more freuquent in relatively younger cells in which more intense filling of the hollow crystals took place.

Introduction

The first data concerning the cytopathic alterations under the influence of peanut stunt cucumovirus (PSV) were notified by Kraev et al. (1975). In the cytoplasm of infected cells the authors observed small virus aggregates of common appearance. Large crystalline inclusions of PSV, readily observable by light microscopy, were later described by Štefanac et al. (1988a, b) in cells infected by robinia strain of PSV (= robinia mosaic cucumovirus, RoMV). By their distinctive appearance these last inclusions were analogous to the hollow crystals of cucumber mosaic virus (CMV) situated in the vacuoles of infected cells (Francki et al. 1987, Christie and Edwardson 1977, Honda and Matsui 1968). In order to investigate the variability of the forms of RoMV crystalline inclusions and their origin, parallel microscopic and submicroscopis investigations were performed. This paper describes two principal types of light microscopic intracellular crystals including the effects of some external and internal factors on their frequency.

Materials and Methods

All investigations were done with same isolate of RoMV from *Robinia pseudacacia* used earlier (Štefanac et al. 1988a, b). Most observations and analyses were performed on infected cells of *Chenopodium quinoa* from the area of local lesions; only in a few cases *C. amaranticolor* and *Nicotiana megalosiphon* infected tissues were employed. Sections were done on living material using leaf epidermal and mesophyll cells, often close to vascular bundles. In the case of *N. megalosiphon*, hair cells were analysed. For the purpose of dissolving plastids, in order to facilitate observations of virus crystals, some sections were treated with 5% solution of detergent Triton X-100; sections were held in this solution for 5—10 min, and then rinsed in distilled water (Christie and Edwardson 1977).

For investigation of the influence of cell age on the crystal formation, *C. quinoa* leaves 7 days after inoculation were used. At that time in our experimental conditions well formed chlorotic lesions were present. First analysis was done from the basis towards the top of inoculated leaves. Each leaf lamina was divided lengthwise into four zones; in this way local lesion areas of 9 leaves of approximately the same age taken from different plants were examined. In an analyse done on leaves of different ages, inoculated leaves from 8 plants which developed satisfactory lesions were used. A hundred cells were checked by the first analysis in each zone of a particular leaf, and by the second in the lower lamina half of every single leaf.

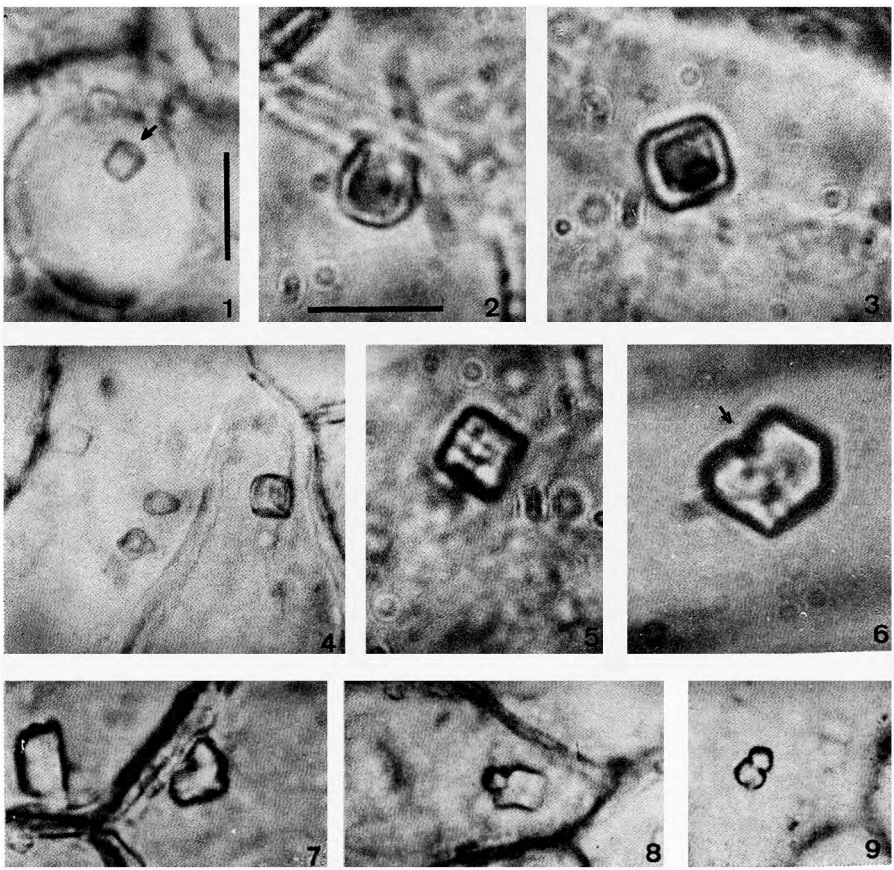
Results

Appearance and variability of RoMV crystalline inclusions

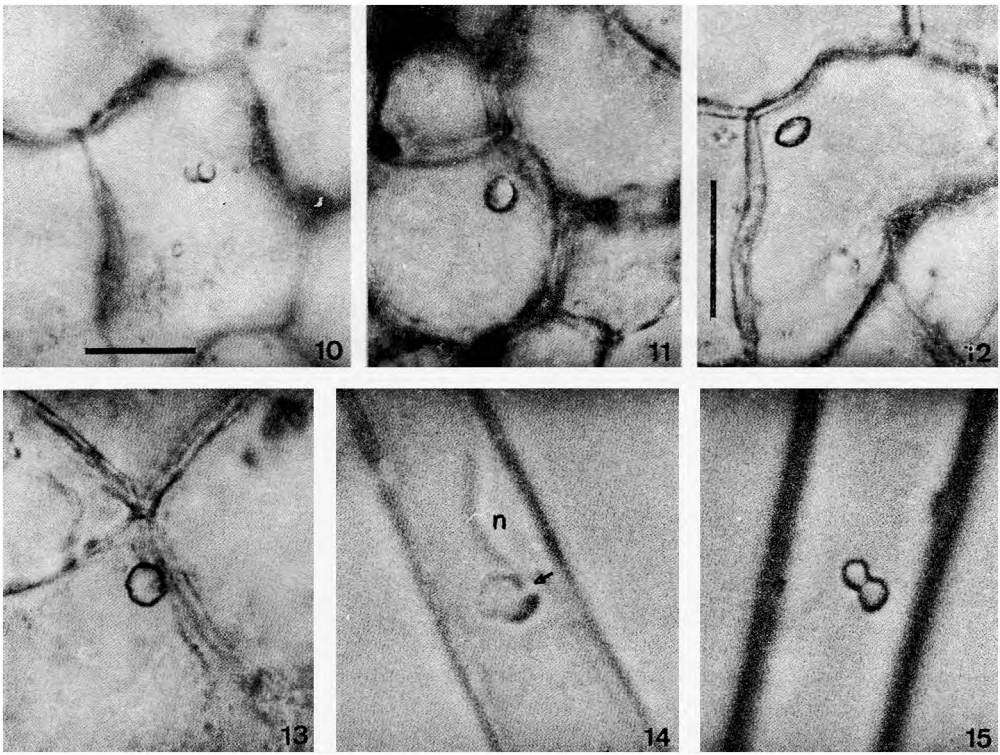
Inside RoMV infected cells two basic types of the virus crystalline inclusions were found. Besides the empty or hollow crystals described earlier (Štefanac et al. 1988b) we now also noticed crystals of polyhedral form which, in our opinion, at no stage of their development included a hollow central area. Therefore we refer to the last crystalline inclusions as ordinary crystals.

As known (Štefanac et al. 1988b), the RoMV hollow crystalline inclusions have, at the beginning of their formation, a large clear space connected with the surrounding area by an opening in the crystal wall. At the very beginning of their formation the hollow crystals are quite open and markedly empty of virus particles (Figs. 1, 2, 10); later they can appear to be more or less intensively (Figs. 4, 5) to almost completely filled (Fig. 6) with RoMV particles. Among the hollow crystalline inclusions the most common were those of polyhedral appearance (cf. Figs. 1—9), which at the same time showed the greatest variability. Spherical to oval forms (cf. Figs. 10—12) were far less frequent. Between the hollow crystals evidently of polyhedral form and those of spheroidal appearance transitional forms were noticed: sometimes the crystal gave the impression of an oval body, but in fact it was bordered with a number of planes (cf. Figs. 13—15).

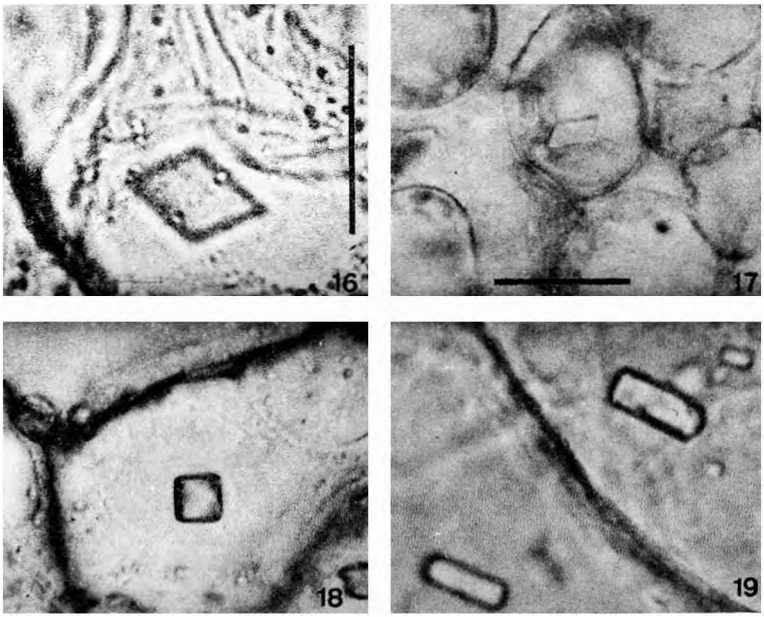
Thin plates of rhombic to rhomboidal forms (Figs. 16, 17) which showed poor birefringence belonged to the type of ordinary or conventional polyhedral crystals. These inclusions, however, were less frequent



Figs. 1—9. Different forms of hollow polyhedral crystalline inclusions of robinia mostic virus, including their aggregates (7—9), in live epidermal or mesophyll leaf cells of *Chenopodium amaranticolor* (1, 5, 7—9) and *C. quinoa* (2—4), and in hair cell of *Nicotiana megalosiphon* (6). Opening in the crystal wall arrowed. Bars = = 25 μm (one for 1, 4 and 9, the other for 2, 3 and 5—8).



Figs. 10—15. Aspects of hollow spherical and oval crystalline inclusions of robinia mosaic virus (10—12), including transitional forms towards hollow polyhedrtl crystals (13—15), and crystalline aggregates (10, 14, 15) in intact epidermal or mesophyll leaf cells of *Chenopodium amaranticolor* (10) and *C. quinoa* (11—13) and hair cells of *Nicotiana megalosiphon* (14, 15). Opening in the crystal wall arrowed. Contour of the nucleus (n). Bars = 25 μm (one for 10, 11, 14 and 15, the other for 12 and 13).



Figs. 16—19. Polyhedral crystals of robinia mosaic virus of the ordinary type in the form of rhombic (16) and rhomboidal (17) thin plates inside the intact tissue (17) of *Chenopodium quinoa* and the one treated by detergent Triton X—100 (16). The crystal of tetragonal form (18) and elongated prisms (19) of high birefringence, both inside live cells of *C. quinoa*, quite possibly belong to the ordinary polyhedral crystals. Bars = 25 μm (one for 16, the other for 17—19).

than those of the hollow polyhedral type. It is not excluded, particularly owing to electron microscopy (Bezić 1991, unpublished results), that the ordinary polyhedral crystals could also include many crystalline inclusions of tetrahedral form (cf. Fig. 18) and the form of elongated prisms (cf. Fig. 19) of high birefringence. In contrast to the delicate rhombic and rhomboidal plates, the last mentioned crystals were of the same frequency as plugged polyhedral inclusions of the hollow type which could be readily identified in light microscope because of the presence of a clear trace of a previous aperture on their surface (*cf.* Fig. 6).

In addition to single virus crystals of two types, their aggregates were also present. A particularly uncommon appearance characterised the aggregates of hollow crystals: Sometimes two hollow crystals, of an approximately analogous size, were completely connected by edges of their openings (Figs. 9, 15) so that any further filling of the established common hollow space with the virus was not possible. Sometimes, similarly, a more or less filled large hollow crystal was found with a small one attached to its opening (Figs. 7, 8), resembling a vessel with plug. Another time two hollow crystals were aggregated laterally by their walls (fig. 10), or one hollow crystal by part of its wall entered the clear space of the other (Fig. 14) which usually happened at the very beginning of their formation.

Location of crystalline inclusions inside the cell

Part of the RoMV crystalline inclusions described above was undoubtely located in the cytoplasm. The location of RoMV crystals in the cytoplasm was noted even earlier (Štefanac et al. 1988b) although it was ascribed to crystalline inclusions of the hollow type. During this study we noticed, however, that some RoMV crystals, particularly those of the hollow type, displayed a kind of oscillation which suggested that they were placed in the vacuole or inside the injured cytoplasm. That the ordinary RoMV crystals are normally placed in the cytoplasm and those of the hollow type only at the very beginning of their formation in damaged cytoplasm and later in the vacuole was confirmed by electron microscopy (unpublished results).

Factors that affect the freqency of crystals

Temperature and length of day. By summing up the data of the four years of investigation we established that the most favourable temperatures for growth, i.e. appearance of the RoMV crystalline inclusions were between 15—20°C. At temperatures below 15°C and at those higher than 30°C all forms of crystals were exceptionally rare.

The other essential factor in the formation of virus crystals was the length of a day. At the optimal temperatures mentioned and during the period of longer days, when infected plants grew best (in our conditions April, May and June), the largest number of all forms of the crystals was present. In September and October when temperature was again close to the optimal, but the days shorter, all forms of crystals were present: however, they were substantially less frequent than from April to July.

Relative age of cells. Searching for a correlation between the relative age of inoculated cells and the number of the crystals present two analyses were completed. In the first analysis, by examining the crystals from the basis towards the top of inoculated leaf lamina, the crystals were

most frequent in cells of the basal, i.e. youngest part and in the smallest number in cells of the top, i.e. oldest leaf part. Also, the cells of the oldest leaf part contained mostly quite open hollow crystals, while completely formed i.e. the filled ones, and ordinary polyhedral crystals prevailed in cells of the youngest part of the leaf, where conditions for multiplication of the virus were generally the most suitable.

In accordance with the above findings, in comparing the virus crystalline inclusions in leaves of different age, the largest number of inclusions was found in relatively younger leaves which showed advanced infection. In such leaves completely filled hollow crystals and/or ordinary polyhedral crystals predominated.

Discussion

The paper brings data concerning our additional light microscopy investigation of virus crystalline inclusions following infections with RoMV naturally present in black locust (Schmelzer and Milić 1965, Schmelzer 1971). In analyses, infected plants were used of the species *Chenopodium quinoa, C. amaranticolor* and *Nicotiana megalosiphon* which supported the formation of well visible virus crystals. It was found even earlier (Stefanac et al. 1988) that the appearance of RoMV crystalline inclusions was the same as that in the hollow crystals of cucumber mosaic virus (CMV). Since the hollow crystals of CMV have not been analysed in more detail (cf. Francki et al. 1987) our investigation of crystals of RoMV was directed to a better understanding of hollow crystals in general. We would like to add that up to now this specific form of virus crystalline inclusions has not been recorded with members of other groups of plant viruses.

The results of the study complete our earlier findings (Štefanac et al. 1988) that the RoMV hollow crystals obtain their basic aspect and size at the very beginning of their inception. Later on, such thin walled crystals can only be more or less filled with the virus. Also, besides the type of hollow crystals, we found in RoMV infected cells large ordinary virus crystals which appeared quickly and during their development did not include voluminous empty central space. The ordinary RoMV crystalline inclusions have been verified particularly following our electron microscopy investigation (unpublished data). Under the light microscope they may seem the same as completely filled crystals of the hollow type. In contrast to the data published earlier that the hollow RoMV crystals are situated in the cytoplasm (Štefanac et al. 1988) we have now established that they are placed in the central vacuole as the hollow crystals of CMV are (Francki et al. 1987). In the cytoplasm, however, ordinary polyhedral crystals were found located. These facts were also shown to be correct particularly owing to unpublished data of our electron microscopy investigation. Both types of RoMV crystalline inclusions were the most frequent at temperatures between 15—20°C and during the period of rather long days. The relatively younger cells comprised a larger number of filled hollow crystals than the older ones, since the conditions for multiplication of the virus in such cells were more propitious.

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SAŽETAK

SVJETLOSNA MIKROSKOPIJA KRISTALIČNIH UKLOPINA CUCUMOVIRUSA MOZAIKA BAGREMA

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U stanicama zaraženim cucumovirusom mozaika bagrema (RoMV) ustanovili smo dva osnovna tipa kristaličnih virusnih uklopina: prazne kristale poliedričnog i sferičnog oblika, i obične poliedralne kristale. Prazni se kristali, kako se čini, redovito nalaze u vakuoli, a obični poliedralni kristali u citoplazmi. Najpovoljnije temperature za razvitak kristaličnih uklopina RoMV bile su između 15—20°C u periodu dužeg dana. Svi oblici kristala bili su češći u razmjerno mlađim stanicama u kojima se također odvijalo intenzivnije ispunjavanje praznih kristala.

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