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# HISTO-CYTOLOGICAL STUDY OF APICES FROM *IN VITRO* PLANTLETS OF DATE PALM (*Phoenix dactylifera* L.) DURING A CRYOPRESERVATION PROCESS

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**KEY-WORDS**: Date palm, *Phoenix dactylifera*, cryopreservation, histo-cytology, apex.

**SUMMARY**: An histo-cytological study was performed on apices of *in vitro* plantlets of date palm submitted to a cryopreservation process. A prerequisite 24-hour preculture on standard medium induced the recovery of cellular activity and the initiation of starch synthesis in some samples. This starch synthesis intensified during the pregrowth treatment. Cells which survived in liquid nitrogen showed characteristics of cells under osmotic stress. The first cell divisions were observed 4 days after thawing. In some samples, the cell multiplication first gave rise to a callus. Each surviving meristem regenerated a single shoot within two to three months after thawing.

## INTRODUCTION

A cryopreservation process was developed recently for date palm apices sampled on *in vitro* plantlets (1, 2), in order to ensure their long-term preservation. The survival rates varied between 11.8 and 48.2%, depending on the variety. A better understanding of the phenomena occurring during the cryopreservation process may help to improve these results.

The success of a cryopreservation process implies that the samples go through a series of successive steps: pregrowth, cryoprotective treatment, prefreezing, immersion in liquid nitrogen, thawing and recovery. Today, the majority of the work on cryopreservation mainly concerns the development of procedures (3). Only a limited amount of data on the effect of these various treatments on qualitative aspects of the cryopreserved samples are available in the literature. Only a few authors have studied the case of meristems (4, 5, 6, 7).

During this work, an histo-cytological study was carried out at each sucessive step of the cryopreservation protocol, in order to observe the modifications at the cellular level, to understand the mechanisms of adaptation to low temperatures and also to indentify precisely those steps which may be considered as the "key steps" on which the successful regeneration depends.

## MATERIALS AND METHODS

### Plant material

The apices submitted to the cryopreservation process were sampled on *in vitro* plantlets of date palm (*Phoenix dactylifera* L. var. Bou Sthammi Noir) multiplied according to the method described by Ferry *et al.* (8).

### Methods

- Cryopreservation

The apices were cryopreserved according to the process developed by Bagniol and Engelmann (1, 2): after dissection, the apices were cultured for 24 hours on standard medium (0.1 M sucrose). They were then transferred onto a sucrose-enriched medium (0.5 M) and after 24 hours in culture, submitted to a cryoprotective treatment for 2 hours at 0°C, in a liquid medium containing 0.5 M sucrose and 10% dimethylsulfoxide (DMSO). The apices were then frozen at $1°C.min^{-1}$ to -30°C and immersed in liquid nitrogen, where they were stored for 3 to 5 days. After thawing (± 2 min in a 40°C water-bath), they were placed onto a medium containing 0.5 M sucrose and 1 $g.l^{-1}$ activated charcoal. They were then transferred every 24 hours to media containing activated charcoal, with progressively lower sucrose concentrations, until the standard sucrose concentration (0.1 M) was reached.

- Histo-cytological studies

The samples were fixed for 48 hours at 4°C in a phosphate buffer (pH 7.2) containing 2% paraformaldehyde, 1% glutaraldehyde and 1% caffeine. They were then dehydrated by successive transfers in alcohol baths with progressively increased alcohol concentrations. Inclusion was carried out in Kulser 7100 resin. Three µm thick sections were cut using an automatic microtome (Historange 2218, LKB). The samples were treated with periodic acid-Schiff reaction and naphthol blue black. This double staining technique allows specific characterization of the polysaccharide compounds which stain red and the soluble and non-soluble proteins which stain blue black (9).

Five to ten apices were prepared for examination after each of the following steps: dissection, 24-hour culture on standard medium, 24-hour culture on medium with 0.5 M sucrose, cryoprotective treatment, freezing to -30°C, immersion in liquid nitrogen, 2, 4, 10, 15 and 30 days of recovery on standard medium. The observations described correspond to the average behaviour of the samples studied.

## RESULTS

- Histological structure of sampled apices (Fig. 1)

Figure 1 corresponds to a longitudinal section of an apex sampled for cryopreservation. It consisted of the apical dome and two differently aged foliar primordia (only one primordium shown on Fig. 1). The cells showed the characteristics of meristematic cells: they had a high nucleoplasmic ratio, a dense cytoplasm, small vacuoles. No polysaccharide reserves were present. A gradient of differentiation was visible in the meristems, from the superficial layers to the central region, where the cells had a lower nucleoplasmic ratio and more accuentuated vacuolisation.

- Histological structure of apices after 24 hours culturing on standard medium (Fig. 2)

Recovery of cellular activity could be observed: several mitotic figures as well as numerous divisions were visible. Starch grains were present in some cells (not shown in Fig. 2).

- Histological structure of apices after cryoprotective treatment (Fig. 3)

In some of the samples studied the synthesis of starch increased in intensity, but others did not show any starch synthesis. Starch grains were located mostly in the cells of the superficial zone. The cells retained a very meristematic appearance.

- Histological structure of apices after freezing in liquid nitrogen (Fig. 4-6)

After freezing in liquid nitrogen, the apices showed cellular heterogeneity (Fig. 4). Some cells conserved their meristematic characters. This was the case in the cellular layers corresponding to the meristem itself, whereas in the underlying zone, where the cells were more vacuolated, some were damaged, showing broken cell walls. Finally, cells with cytoplasm contracted away from the cell wall were observed in some samples (Fig. 4 and 6). Many nuclei displayed a particular appearance, being intensely and uniformly stained (Fig. 5). Starch grains were visible inside some cells, more often located in the superficial zone.

- Histological evolution of apices during recovery (Fig. 7-11)

Two days after thawing, the samples showed a cellular zonation: meristematic cells surrounding a central zone of differentiated or harmed cells. The first mitoses occurred in the superficial layer 4 days after thawing (Fig. 8). However, not all the apices observed at this stage showed mitotic figures. From this moment on, thickening of the cell wall could be observed in some cells of various areas. From the 6th day onward, meristematic or non-meristematic cells had a sinuous aspect (Fig. 9). Up to the 7th day, the outline of the original apex remained recognizable. Afterwards, in most cases, the initial apex was no longer distinguishable, hidden by the proliferation of meristematic cells of the apical dome and of the foliar primordia (Fig. 10). Intense synthesis of starch occurred inside the cells of the superficial and underlying zones (Fig. 11).

- Histological structure of a non-recovered apex, 6 weeks after thawing (Fig. 12)

The samples which did not produce any callus 6 weeks after thawing did not develop further. In such cases, the histological examination showed that most cells had a condensed and poorly stained cytoplasm and sligthly visible nuclei.

- Macroscopic evolution of apices during recovery (Fig. 13-15)

Regrowth of the apices which had survived after cryopreservation was visible 7 days after thawing. Proliferation occurred from the basal part of the explants and led to the formation of a callus which had a limited development (Fig. 13). Each sample which had formed a callus gave rise during the following weeks to a single shoot (Fig. 14 and 15).

## DISCUSSION/CONCLUSION

The results obtained showed that, contrarily to what was observed with carnation (6), date palm meristems do not withstand freezing in liquid nitrogen as intact structures. It seemed that the more meristematic the cells were, the more they resisted. The same phenomenon was observed with pea (4) and potato meristems (5). In the case of pea, the authors showed that the surviving cells were located mainly on the surface of the apex. During the steps which precede freezing, gradients may be established both for the outflow of water and the penetration of the cryoprotectants. In the case of date palm, as in that of other plants, the central cells as well as the ones located at the base of the explant are more vacuolated and their dehydration may be more difficult or they may be less tolerant to the necessary level of dehydration. This may explain their incapacity to withstand freezing. The superficial cells, which are more meristematic, are more likely to reversibly dehydrate since they have no or only a few vacuoles. The cells which do not withstand freezing show characters similar to that of cells which do not withstand dehydration: detachment of the plasmalemma and breakage of the cell wall (10). After prefreezing to -30°C, many cells had a heavily stained nucleus, the nucleolus of which was no more visible. This type of nuclei has been observed notably in cells under osmotic stress (11).

During culture prior to the cryoprotective treatment, obvious starch synthesis occurred in some cells of the samples. This synthesis intensified when the medium contained more sucrose. Uragami (12) observed an increase in sucrose, glucose and fructose concentration in axillary buds of *Asparagus* after a two-day preculture on a sucrose-enriched medium. The sucrose of the culture medium is thus absorbed by the cells in large quantities. According to Crowe *et al.* (13), sugars, dissoluted or attached to the

membrane components through covalent bounds, would stabilize the lipid bilayer, thus protecting the cells during dehydration. The accumulation of polysaccharide compounds is, among the Angiosperms, a mechanism of resistance against dehydration (14). Therefore, the meristems which did not show any starch synthesis may be those which were unable to survive after cryopreservation. The aptitude to withstand dehydration seems thus to be the key factor determining the resistance of the cells to freezing in liquid nitrogen.

The initial structure of the meristem persisted up to the sixth day after thawing. From this moment on, the cell walls displayed a sinuous aspect which could be an effect of a change in the volume of the sample induced by dehydration. The cell wall may bend to accomodate this. This phenomenon may also be linked with the protective effect of DMSO. Indeed, Morisset *et al.* (15) have shown in carrot cells that, in the presence of DMSO, the microfilaments of the cytoskeleton are less numerous. This partial depolymerization renders the cells less rigid, which gives them this sinuous aspect.

During this study, we observed that after a transitory callogenesis, a single shoot was regenerated from each apex within two to three months in culture. This mode of recovery was also observed in *Chrysanthemum* shoot-tips (7). In order to really understand the reorganization and the recovery growth pattern of the meristems, it would be necessary to precisely follow the histo-cytological evolution of the explants during a longer period after thawing.

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**Figure 1**: Structure of a control sample. ad: apical dome; fp: foliar primordium.

**Figure 2**: Apex after a 24-hour culture on standard medium. Structure of the apical dome. Arrows indicate the figures of mitosis and the cell divisions.

**Figure 3**: Sample after cryoprotective treatment. Arrows indicate the cells containing starch grains. Note the localization of these cells in the superficial zone.

**Figures 4 to 6**: Sample after prefreezing and immersion in liquid nitrogen. ad: apical dome; fp: foliar primordium; pn: intensely and uniformely stained nuclei; sc: cells with retracted cytoplasm. Arrows on Fig. 4 indicate cells with retracted cytoplasm.

**Figure 7**: Sample two days after thawing. ad: apical dome; fp: foliar primordium; ec: disrupted cells.

**Figure 8**: Sample 4 days after thawing. The arrow indicates a figure of mitosis. Note wall thickening. s: starch grains.

**Figure 9**: Sample 6 days after thawing. Note the sinuosity and wall thickening of the cells located in the foliar primordia.

**Figure 10**: Sample 15 days after thawing. Note the cell proliferation inside the meristem and the foliar primordia.

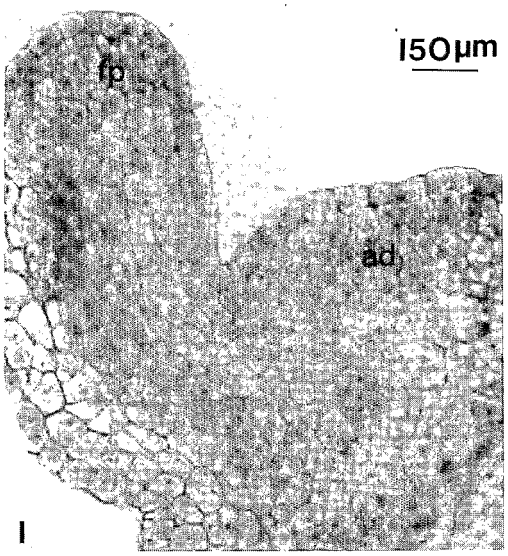
**Figure 11**: Richness in starch content in the underlying zone of the meristem, 15 days after thawing. s: starch grains.

**Figure 12**: Sample having shown no morphological sign of regrowth, 6 weeks after thawing. ad: apical dome; fp: foliar primordium.

**Figure 13**: View of a living sample, 7 days after thawing.

**Figure 14**: Shoot development from a cryopreserved apex, 3 months after thawing.

**Figure 15**: Plantlet obtained from a cryopreserved apex, 5 months after thawing.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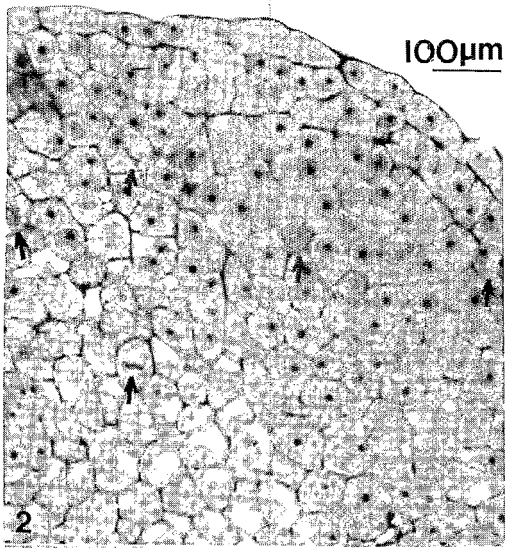
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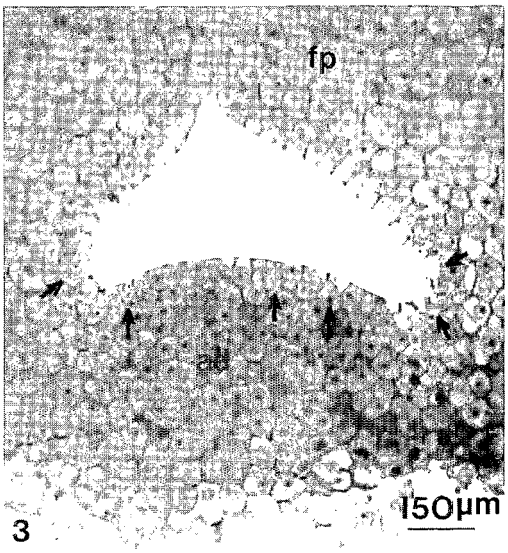
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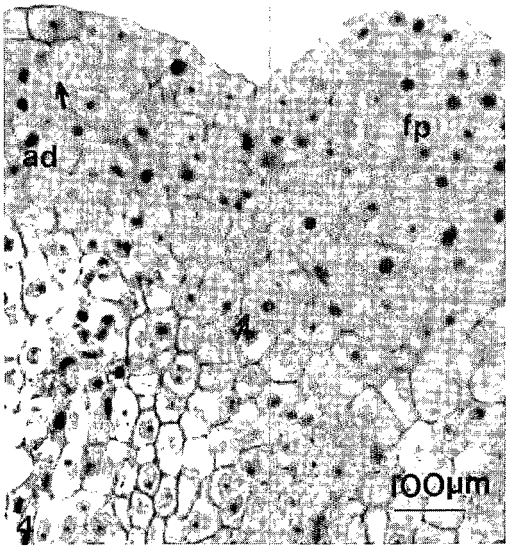
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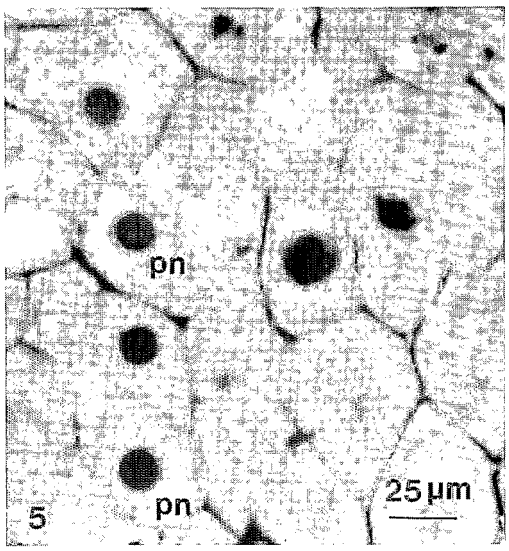
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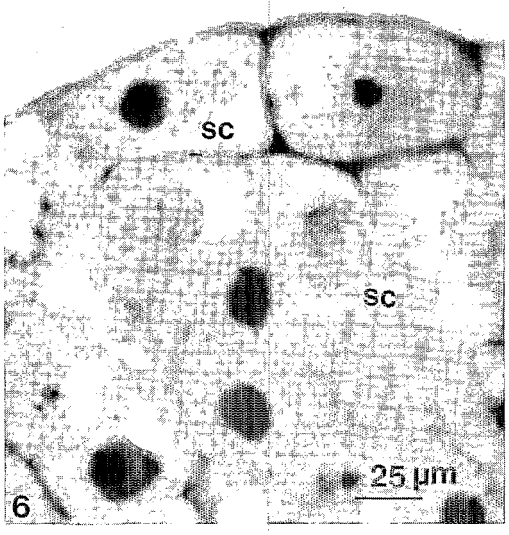
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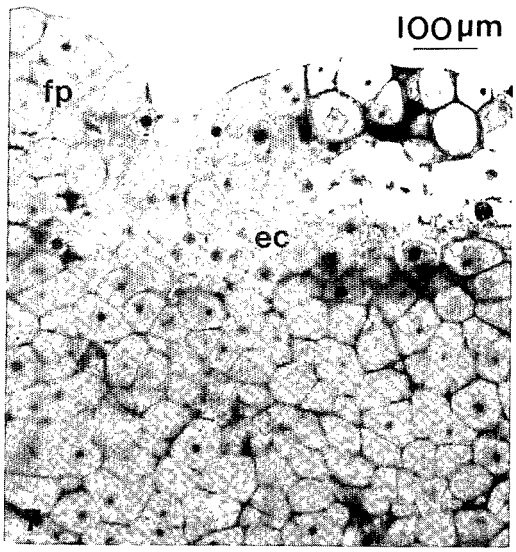
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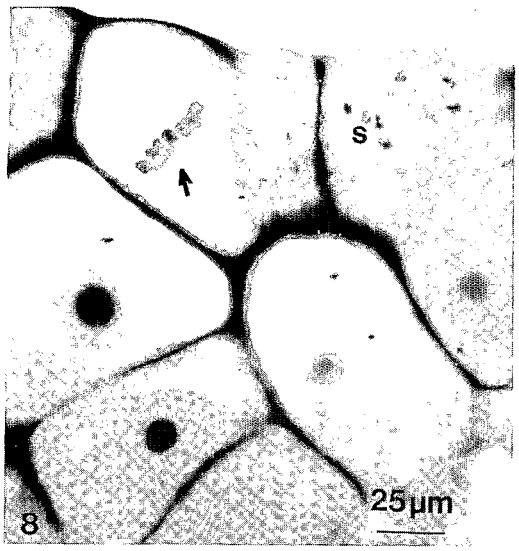
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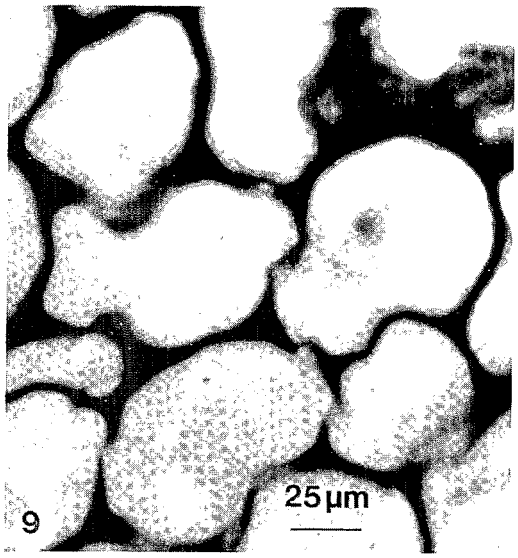
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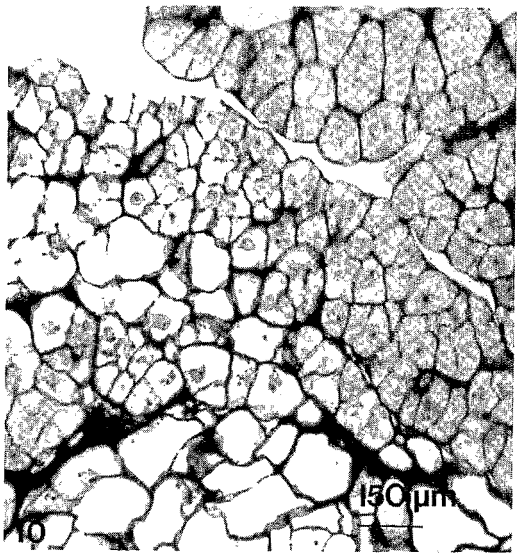
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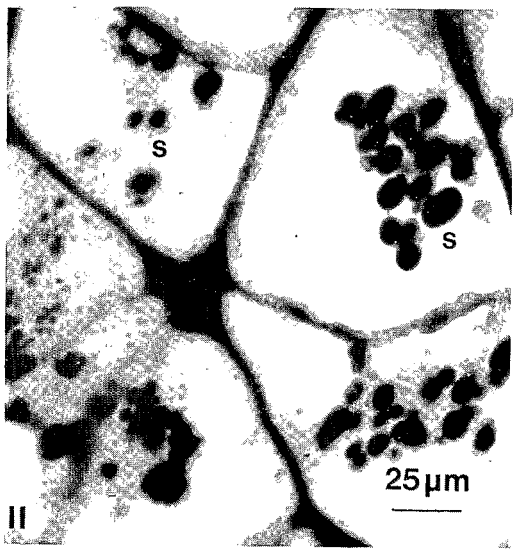
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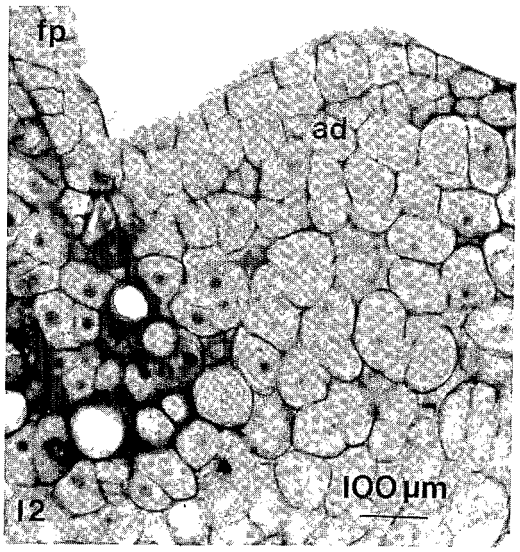
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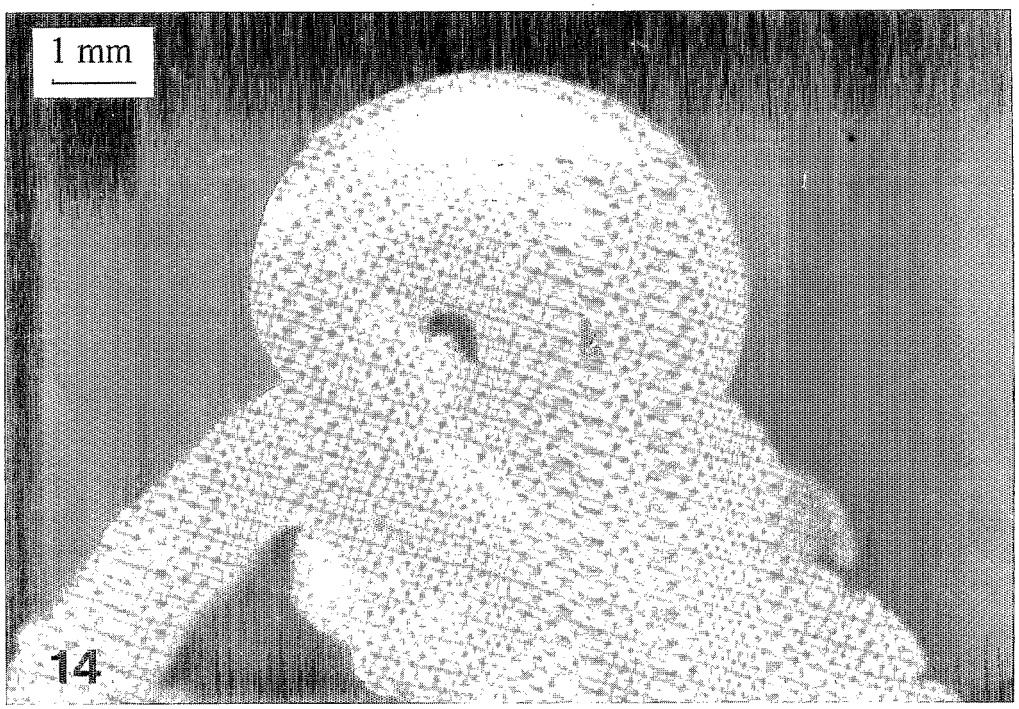


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