BIOLOGIA PLANTARUM (PRAHA)

15 (1) : 50—56, 1973

On the Media Improving Freeze-sectioning of Plant Material

Dedicated to the 100 anniversary of the birth of Professor Němec

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Received March 14, 1972

Abstract. Using a semiconductor freezing microtome, there is no difficulty in getting an ample supply of quite suitable sections of Ca formol fixed root tips of *Vicia faba*, applying Holt's sirup or plain 0.88 M sucrose as media for keeping the material, getting the sections and treating them after sectioning, but the protoplasts shrink and often fall out from the cells. No improvement in this respect was revealed using — at different concentrations — sucrose and arabic gum alone. The changes in the proportions of the ingredients of the sirup were also ineffective. A series of experiments were therefore performed with plain sucrose, but none of the factors tested was efficient enough to improve the results as compared with the standard procedure, the gradual transfer being the only exception. Since this makes the procedure more laborious and time consuming, certain other media were tested, 0.88 M ethanol and 0.88 M dimethylsulfoxide yielding the best results. Attempts are being made to understand some points of the present experience in connection with the results of other authors and on the basis of general theory respectively.

Passing through the active work of scientists one or two generations ago, one could be surprized by the limited number of papers, devoted solely to the methods and techniques. This holds good even in the case of professor Němec, who was not only well informed and highly interested in microtechnique, but was — as known generally — a master in this field. Many technical suggestions are scattered throughout his numerous papers but many remained unpublished. It is fresh in the present author's memory, that professor Němec regarded it as a debt and therefore, he paid great attention to the edition — under his guidance — of a handbook of microtechnique (Němec 1962). In this respect, the present paper could be taken as a continuation of professor Němec's intensions.

In spite of the possibility of making freehand sections of plant objects, attention recently has been paid again to sectioning vegetable material in frozen state (see *e.g.* Läuchli 1966a, b, Gahan *et al.* 1967, Knox 1970).

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Using a semiconductor freezing microtome there is no difficulty in getting enough quite suitable sections of the material we are interested in, applying Holt's sirup (HOLT *et al.* 1960, 0.88 M sucrose containing 1% of arabic gum) or plain 0.88 M sucrose after Ca formol fixation, but, regularly, the protoplasts shrink and fall out from the cells (BENEŠ 1971). It is the purpose of the present work to improve the mentioned procedure in this respect. For this reason 3 series of experiments will be performed: A. some previous experiments will be repeated and, then, ingredients of Holt's sirup will be used either separately at different concentrations either mixed in various proportions; B. a series of factors will be tested using plain 0.88 M sucrose; C. different chemicals will be tried as substitutes for Holt's sirup. Moreover, an attempt will be made to elucidate some points concerning the effect of different media for keeping the material, getting the sections and treating them after sectioning.

Material and Methods

The objects of the present work were root tips of broad bean *Vicia faba* L. taken from 3 days germinated seeds and Ca formol fixed as given before (Beneš 1971). After fixation the material was washed and kept in an ice cold* medium (see later) for 1 to 4 days. For sectioning, the ordinary sliding microtom was used equipped with a semiconductor freezing table Tesla Cryotom, which enables one to operate at 1 of 3 temperature choices: choice 1 -5 °C, choice 2 -12 °C, choice 3 -20 °C (DOČKÁLEK 1967, POSLT 1967; according to information from the manufacturer the differences up to ± 3 °C from the given values may appear depending on the cooling water, the electric power supply, room temperature *etc.*). A non-concave knife was adjusted in a suitable position which was maintained constantly. Before sectioning, the knife was equipped with 2 metal baskets filled will dry ice. To start with, a piece of filter paper moistened with the medium used was placed on the surface of the freezing table. Then, the power supply was switched on. During several minutes, necessary for the table to reach the adjusted temperature the portion comprising the 2nd and 3rd mm was dissected from the root tip (see BENEŠ 1971). Then the dissected piece was placed vertically on the freezing table, the apical end downwards. Just enough medium was added to surround the object. After 5 min freezing the whole sample was cut into 50 μm transverse sections, each of which was carefully but quickly transferred by means of a fine hair brush into the cooled medium. The use of epprouvetts provided with Silon tissue (sheet) at the end immersed in the medium markedly facilitates the handling of the sections. The sections were placed for 1 h in the given medium ice cold. Then they were washed by immersion in H_2O of laboratory temperature and, thereafter, they were kept in another lot of H_2O of laboratory temperature for 30 min. Finally, the sections were picked up onto slides and mounted in Gama medium. (If only a few more or less damaged sections were available, the best of them were picked up, the sample on the slide thus being not quite representative in this case.) After some time, the slides were framed with Canada balsam.

If not otherwise stated, the same medium of the same temperature was used throughout the whole procedure, *i.e.* for keeping the material, freezing, sectioning and handling the sections, commonly, 0.88 M sucrose, ice-cold. The standard freezing table temperature choice was 2 for both freezing and sectioning. The other particular technical details are given together with the results achieved.

The course of the sectioning and the state of sections before and after transfer to the medium and in H_2O was checked. Under the 160× magnification, the sections from the mentioned part of the root tip *i.e.* the beginning of the zone of elongation were evaluated, in particular the cells from the central part of the cortex. The general state of the sections and the shrinkage and loss of protoplasts from the mentioned cells were considered.

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* The temperature 0 to + 5°C as measured in the medium used is denoted here as ice-cold, cooled, cold *etc.*

Results

In order to prevent the shrinkage and loss of protoplasts from the cells which appears during the standard procedure with Holt's sirup, 3 series of experiments were undertaken: A. some previous experiments, were repeated and extended and, then, the ingredients of Holt's sirup were used either separately at different concentrations either mixed in various proportions; B. a series of factors was tested using 0.88 M sucrose; C. different chemicals were tried as substitutes for Holt's sirup.

A. In the first series of our experiments, the sections from H_2O, Holt's sirup and its ingredients were compared, using all possible choices of freezing table temperature. It was confirmed that sectioning in Holt's sirup is quite easy, but the protoplasts shrink and sometimes fall out of the cells. This does not appear in H_2O, but only few sections that are not fully damaged are available. Sections from 0.88 M sucrose are like those from Holt's sirup, whereas the results with 1% arabic gum are about the same as with H_2O. The temperature choice is not critical.

The separate ingredients of Holt's sirup were then tested at different concentrations. Lowering the concentration of sucrose to 0.22 M and 0.11 M prevents the shrinkage and loss of protoplast, but the sectioning itself is difficult and many sections are damaged. With 0.44 M sucrose cutting is easy, but the protoplasts shrink. The use of 2, 4 and 8% solutions of arabic gum leads to a slight improvement in sectioning, but the whole object shrink. The temperature choice is not again decisive. Thus, if not otherwise stated, all the following experiments were performed under constant temperature conditions, namely, at choice 2 of the power supply.

As regards both the sectioning and the shrinkage of protoplasts, no improvement was observed using media containing the ingredients of Holt's sirup at proportions other than customary, namely 0.44 M saccharose + 2% arabic gum; 0.22 M saccharose + 4% arabic gum; 0.11 M saccharose + 8% arabic gum.

B. With 0.88 M sucrose, the following particular factors were tested in order to obtain better sections (only the points differing from the standard procedure are mentioned): 1. The knife was not cooled. 2. The material was frozen at one and cut at another temperature (freezing at choice 1 for 5 min, then, the power supply was switched to choice either 2 or 3 and, after 5 min, sectioning at the given choice; similarly, freezing at 3 and sectioning at either 2 or 1; for comparison, objects were cut after a total of 10 min freezing at either 1 or 3). 3. The sections were transferred to ice cold sucrose 0.44 M or 0.22 M or 0.11 M; to uncooled (18 to 20 °C) and warm (37 °C) 0.88 M sucrose; to cold, uncooled and warm H_2O. Unfortunately, none of the factors tested was proved decisive in preventing protoplasts shrinkage and loss.

To eliminate the sudden changes of concentration, the material after fixation was washed in H_2O and then transferred to sucrose solutions of increasing concentrations, *i.e.* 0.11 M; 0.22 M; 0.44 M and 0.88 M; about 12 hours each, all treatment at ice — bath temperature. This procedure resulted in a substantial improvement of the quality of the sections: practically no shrinkage and loss of protoplasts was observed.

C. While searching for embedding medium, giving the desired effect without time-consuming gradual transfer and — moreover — trying to ob-

tain data enabling one to explain the effect of sucrose on sectioning and on shrinkage of protoplasts, different substances were tested under the standard conditions. 1. Some simple sugars and sugar derivatives were tried, namely l-arabinose (Lobachemie), d-galactose (Lachema), d-glucose (Lachema), d-fructose (Lachema), lactose (Lachema, satur. solution), maltose (Lachema), d-manit (Lachema), d-sorbit (Lobachemie), inositol (meso, inakt. Lachema, satur. solution), d-glucuronic acid (BDH); apart from the mentioned exceptions, 0.88 M solutions were used. The application of all these substances was ineffective in obtaining better sections. However, it is of interest that closely related substances like manit and sorbit behave differently: sectioning is easy with sorbit, but difficult with mannit. 2. Several polysaccharides and some other macromolecular substances were tested: dextran m.n. 11 400 (kindly supplied by Dr. Lieblová, prepared in Res. Inst. for Natur. Drugs), dextran partially degraded (obtained through the generosity of Dr. Šterzl, the sample from the Inst. of Haematol. and Blood Transf., m.w. about 200 000) dextrin (commertial, not otherwise designed), Ficoll m.w. 700 000 (Pharmacia, Uppsalla; obtained from Dr. Mareš), pectin (from apple, 81% esterification; a gift from Ing. Čurdová), polyvinylalcohol (PVA; Sloviol R 20%, Záv. W. Piecka, Nováky; diluted 1 : 5 with H_2O), polyvinylpyrrolidone (PVP; Lachema, imported). 4% solutions were used throughout, with the exception of pectin, which was 2%. The objects shrank in many cases. The sectioning was facilitated — more or less — but the sections were destroyed during further treatment, so that only remainders of them were seen on the slides. 3. NaCl and KNO_3 solutions (0.22 M; 0.44 M; 0.88 M) were tried. Mostly, the sectioning was facilitated in comparison with plain H_2O, but the sections were no better as compared with the standard procedure with sucrose. 4. Very promising results were obtained when applying 0.88M solutions of the following solvents: acetone, dimethylformamide (Lachema), dimethylsulfoxide (Lachema), ethanol, glycerol, glycol (ethyleneglycol), methanol, n-propanol, isopropanol. Especially with ethanol and dimethylsulfoxide the results were excellent, both as regards the sectioning and the quality of sections.

Discussion

Recently, much attention has again been paid to the technique of sectioning in frozen state *i.e.* to the sectioning of objects embedded in ice.

Freeze sectioning is a rather old technique, used since 1873 (KAVINA 1932). Many problems, in which the present day workers are interested, have remained open since that time, the use of different "embedding" media being one of them. While the freeze-sectioning technique is broadly used in the case of animal material, its application is not quite common in botanical microtechnique, in spite of its doubtless utility and repeated recommendations by some authors (*e.g.* KISSER 1928, KAVINA 1932, SOLGER in KRAUSE 1926).

The employment of low temperature in contemporary microtechnique is multilateral. Since the present work concerns the sectioning in a frozen state, freeze drying, freeze substitution and some other techniques like freeze etching *etc.* are out of the focus of our interest, unless some questions are common, *e.g.* the relation between the rate of freezing and cell damage. Following Pearse's views (PEARSE 1960), cold microtome (cryostat) and cold knife techniques are distinguished, dealing with the latter in the present work. But there is much in common for both again. Even ultrathin sectioning is possible in frozen state. Moreover, some problems of handling and sectioning material in frozen state are connected with physiological or pathological studies — both general and applied — of low temperature effects on living organisms (see *e.g* MERYMAN 1966, WOLSTENHOLME and O'CONNOR 1970).

The work of HOLT *et al.* 1960 represents the beginning of our studies. Holt was inspired by the biochemists' use of sucrose and some other substances in the homogenization and fractionation procedures. He was interested both in the preservation of the enzyme activity together with the satisfactory morphology of the material and in the facility of its sectioning. It seems, however, that he did not devote himself to more systematic studies in this field. For instance, a proper description of the sectioning procedure is lacking in the mentioned paper. The fact that a solution of sugar with arabic gum was used as a medium facilitating sectioning a long time ago would be of interest, see SOLGER in KRAUSE 1928.

The sectioning in frozen state was studied closely by Chayen and his coworkers (see CHAYEN *et al.* 1969), who elaborated the "controlled temperature freeze sectioning technique", used also with plant material. The mentioned work of GAHAN *et al.* 1967 may be regarded as an extension of these studies.

CHANG and HORI (1961) developed the somewhat laborous "section freeze substitution technique" mainly to avoid the effect of defreezing. This part of the procedure is overlooked sometimes.

Regarding it as suitable for enzyme localization studies the method of freeze sectioning was extensively tested by MELNICK 1964, 1965 a, b, MELNICK and HEIZER 1965, in particular the effect of the rate of freezing and of protective substances. The latter question was the focus of interest to RICHTER 1968a, b, ROTTENBURG and RICHTER 1969.

The mentioned papers — see *e.g.* the discussion following the paper by MELNICK 1965b — reflect all the complexity of sectioning in frozen state. Many points must be taken into consideration: storing the material, the freezing process, sectioning, handling the sections. Such papers exist, where *e.g.* storing the material, the freezing process and the sectioning procedure are described in detail, but the way of handling sections is not given at all. Two attitudes may be distinguished: 1. the physical and technical and 2. the cytological and biochemical. Neither of them should be neglected. In the view of the present author, it would be quite difficult to give proper data *e.g.* on the temperature of the object under treatment. But this seems to be unnecessary. It is regarded as more useful to give enough details of the procedure, to enable one — with regard to the purpose of such a work — to reproduce it safely in any laboratory designed for ordinary microtechnique.

The 1st of the 2 aims of the present work was reached: the shrinkage of protoplasts and their loss from some cells may be prevented either by gradual transfer to sucrose or by the use of some other media like ethanol. Concerning the 2nd aim, attempts will be made to understand some points of our experience in connection with the results of the above-mentioned authors and on the basis of general theory respectively.

It was found in our previous work (BENEŠ 1971) that arabic gum may be omitted from Holt's sirup without any deleterious consequences. This was confirmed even here. The experience of Lojda (personal communication) corresponds to our findings. Arabic gum alone has not the desired effect and the changes of the proportions of the ingredients in the sirup were also ineffective. Thus a series of experiments were performed with plain sucrose but none of the factors tested was efficient enough to improve the results as compared with the standard procedure the gradual transfer being the only exception but this makes the procedure more laborious and time-consuming. For this reason and — mainly — in order to understand the effect of sucrose some other media were tested for comparison.

It is necessary to mention that the results described here are valid for the material fixed as given above. With the same objects but unfixed — as far as tested — the results were different.

The shrinkage and loss of protoplasts does not seem to be caused by the fixation. We may prevent the appearance of these two shortcomings transferring the objects to sucrose gradually or employing some of the tested solvents diluted properly with H_2O.

It was observed in our early studies (BENEŠ 1958) that unfixed broad bean root tip, if allowed to freeze and then defreeze, fixed afterwards and, subsequently processed by the standard paraffin procedure, revealed considerable protoplast shrinkage. In this connection it seems worthwhile raising the question as to whether ice is formed within the cells at all under the conditions of the mentioned and of the present work (MAZUR 1970). If the medium out of the cells only gets frozen then the protoplasts may shrink by a process similar to plasmolyse since the cell surrounding solution becomes more concentrated in this way. Since the semipermeability of cell membranes need not be destroyed — in spite of being altered — by formol fixatives (FEDER 1960), it seems possible to extend the forgoing proposal to the findings of the present work. While in the mentioned previous work the freezing or freezing and defreezing proceses are undoubtedly responsible for the shrinkage of protoplasts this seems unlikely for the present findings: *e.g.* the gradual transfer to sucrose and the use of ethanol, dimethylsulfoxide *etc.* prevents shrinkage in spite of the freezing or freezing and defreezing procedures being the same as in the standard process. The medium used and the method of its application are regarded as decisive for the protoplast shrinkage under the conditions of the present work assuming the permeability barrier is not destroyed as mentioned above. By preventing the tissue from undergoing great concentration differences either by the gradual transfer to the embedding media or by the application of easily permeable chemicals no shrinkage appears. This explanation based on common rules of plasmolysis would be valid for cells bearing central vacuoles. It was specified above that the cells of the middle part of the cortex — which are vacuolized — will be mainly taken into consideration in the present study. It is necessary to bear in mind the processes corresponding to sineresis and similar phenomena in the case of non-vacuolized meristematic cells.

Some points like the specific cryoprotective effect of some substances tested were omitted from the discussion. Even other cases like the section damage by the after-sectioning treatment will not be commented on here. Attention will be paid only to some aspects of the sectioning process itself. However, the success in the sectioning procedure alone das not imply the success of the treatment as a whole.

Closely related substances like manit and sorbit used in the same concentrations and under the same conditions behave quite differently with regard to the facility of sectioning. It follows that this cannot be explained cryoscopically. Besides, the solutions used are quite concentrated. Thus, the search for the eutectic points and the phase diagrams of the substances used for preparing the media seems to be more promising. But other important physical data like the dependence of the mechanical properties of ice on its temperature, the influence of different ingredients on such properties (comparing low and high molecular weight substances) *etc.* are lacking. Further steps in this direction are beyond the scope of the present work.

The search for suitable media and their proper application seems to be a more promising road towards progress in the use of frozen sections than the control of temperature conditions only (*c.f.* WALTER and MÜLLER 1969). The existence of commercially obtainable media for freeze sectioning favours this view (see *e.g.* the Cryoform embedding media in 1970 Catalogue of IEC — Microtomes and Microtome Cryostats). The use of small molecule chemicals quickly penetrating into the tissue seems to be more promising than the conventional application of macromolecular substances (in plant material, *c.f.* LEWIS and SHUTE 1963, KLEIBER and MOHR 1967).

The cytological and biochemical aspect has been somewhat neglected in the present work in spite of its being useful for solving the problems raised (MOHR and STEIN 1970). Taking *e.g.* the enzyme localization into consideration, the inhibitory effect of some media becomes a matter of discussion but on the other hand events leading to the intensification of hydrolytic enzyme reaction should not be neglected. It is known that media, regarded as "inert", influence enzyme reactions (see *e.g.* LISÝ *et al.* 1971). But, fortunately, different media may be compared in particular cases.

Acknowledgement

The skilled assistance of Mrs. Soňa Blažková is gratefully acknowledged.

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