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The Cytomorphological Effects of Low Temperatures Studied by Temperated Fixatives

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Souhrn

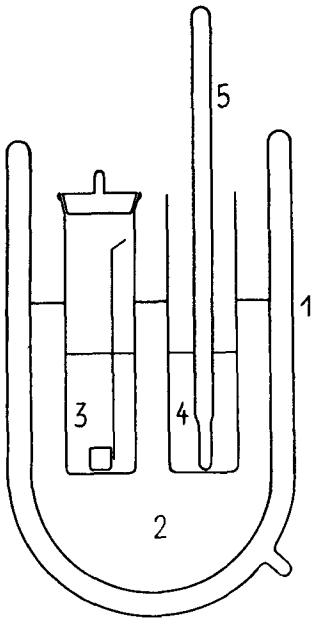
V této práci je sledován mechanismus cytomorfologického působení nízkých teplot na listy 4 dny naklíčené pšenice. 1. Odstřižené špičky listů byly ponořeny v pletivovém košíčku do tekutého dusíku a bez rozmrznutí co nejrychleji nafixovány. 2. Stejným způsobem byly zpracovány další objekty, avšak po vyjmutí z tekutého dusíku byly ponechány volně na vzduchu rozmrznout a teprve pak nafixovány. 3. Další objekty byly nafixovány přímo, tj. bez zmrazení tekutým dusíkem. Fixáže byly vytemperovány na —76, —27, +27 a +56° C. Fixace v Carnoyově alkoholoctové směsi 20 min, v Lisonově směsi 2 hod. Odvodnění a zalití bylo provedeno na vibrátoru. Řezy byly barveny kyselým fuchsinem podle Drawerta a Regaudovým železitým haematoxylinem. Výsledky uvádí tabulka (popsány chloroplasty špičky prvního zeleného listu).

	— 76° C	— 27° C	+27° C	+58 °C
Z tekutého dusíku přímo do fixáže	normální	normální	nepravidelné útvary a normální	nepravidelné útvary a normální
Z tekutého dusíku 10 min. rozmrznout, pak do fixáže	nepravidelné útvary	nepravidelné útvary	nepravidelné útvary	nepravidelné útvary
Přímo do fixáže	deformované a normální	deformované a normální	normální	normální

Stejným způsobem zmrazené, avšak různě rozmrznuté objekty dávají odlišné výsledky. Z toho vyplývá, že k destrukci chloroplastů dochází při rozmrzání tkáně. Je však nutno připustit, že v případech, kdy zmrazení je pomalé, pracuje-li se za vyšší teploty (asi — 20 °C), anebo je-li objekt příliš veliký, mohlo by dojít k destrukci při zmrazování. Destrukce chloroplastů nenastává současně s destrukcí jádra, ani nemusí být destruovány všechny chloroplasty současně.

Summary

The mechanism of the cytomorphological effects of low temperatures was studied by the fixation of young leaves of *Triticum vulgare* with temperated alcoholic fixatives. Some specimens were plunged into liquid nitrogen, allowed to thaw and fixed; other objects were plunged into liquid nitrogen and fixed without thawing; control objects were fixed directly. At various temperatures of fixatives (—76, —27, +27, +58° C) different results were observed on the objects pretreated in the same manner. It was thus demonstrated that the destruction processes occur, in this case, during thawing.



Scheme 1. The arrangement of baths for temperating of fixatives. 1 — Dewar flask, 2 — Water bath or cooling mixture, 3 — Alcoholic fixative, 4 — Ethanol 96%, 5 — Thermometer.

Introduction

The effect of temperature on fixation processes has been very little studied hitherto (e. g. fixation of mitochondria by Regaud's fluid in a refrigerator, fixation of glycogen by Lison's fluid, cold acetone in histochemical procedures etc.). Having developed the technique of cutting in a frozen state, various methods of handling frozen sections were studied (SOLGER 1926). New attempts appeared when freezing—drying and freezing—substitution were developed (BELL 1956). Starting with the results of GENÈVES (1955) and the author (1958), cytomorphological effects of low temperatures with temperated alcoholic fixatives have been studied in this work.

Material and Methods

Leaf tips of wheat, *Triticum vulgare* VILL. (Stupická vouska), germinated for 4 days in Petri dishes on filter paper moistened by tap water, at +22° C, with 12 hours illumination, were used. The height of shoots was 2—3 cm. The tips (coleoptile +1st green leaf) 1 cm. long were cut off, placed in metallic gauze baskets (diameter and height of each 1 cm.) and stoppered with wool.

The specimens were then prepared in three ways:

1. The specimens were submerged in liquid nitrogen for 3 min, then directly transferred (without thawing) into temperated fixatives.

2. Other specimens were submerged in liquid nitrogen for 3 min, after removal the baskets with objects were placed for 10 min. on a table, having been allowed to thaw first and then fixed with the temperated fluids.

3. Control specimens were fixed with the temperated fluids without preliminary freezing.

For temperating of fixatives water baths were used for temperatures of +58 and +27° C, mixtures of CCl_4 + solid CO_2 for —27° C and C_2H_5OH + solid CO_2 for —76° C in one litre Dewar flasks. Fixation for 2 hours at +58° C was carried out in a water bath with automatic regulation of temperature. For arrangement see scheme 1. The temperature varied ±2° C from average values. The fixatives, always 20 ml. in well-stoppered bottles, were freshly prepared and temperated 30 min. before use (Carnoy: ethanol absol. 3 parts + glacial acetic acid 1 part, fixation for 20 min. Lison: saturated solution of picric acid in ethanol 96% 85 parts + formalin 10 parts + glacial acetic acid 1 part, fixation for 2 hours). After fixation the specimens were placed for 10 min. in ethanol absol. I at laboratory temperature (+20° C) being removed from the metallic baskets. Dehydration and clearing was done on a shaker (c.f. HRŠEL 1952): ethanol absol. II. 30 min., III. 30 min., ethanol—benzene 30 min., benzene I. 30 min., benzene II. 30 min., benzene—paraffin at 37° C 30 min. and embedding in pure paraffin overnight at +56° C. When mounted, the longitudinal sections 4 μ were deparaffinised and stained with buffered acid fuchsin (DRAWERT 1936/7): 0·1% solution of Acid Fuchsin S (Lachema) in HCl—phosphate buffer pH 1·25, 30 min., ethanol 70% 30 sec., ethanol 96% 30 sec., isopropanol I. 1 min., II. 3 min., xylene I. 1 min., II. 3 min. or with Regaud's haematoxylin.

Results

The chloroplasts in the tips of young wheat leaves were studied.

	— 76° C	— 27° C	+27° C	+58° C
From liquid nitrogen directly to fixative	Normal	Normal	Irregular formations and normal	Irregular formations and normal
From liquid nitrogen fixed after 10 min. thawing	Irregular formations	Irregular formations	Irregular formations	Irregular formations
Directly to fixative	Deformed and normal	Deformed and normal	Normal	Normal

"Irregular formations" are clumps of material, probably from destroyed chloroplasts. The borders of "deformed chloroplasts" are irregularly shaped and they are sometimes stuck together.

During temperation, some changes in the composition of fixatives occur (darkening of Lison's fluid at +58° C, partial freezing of Carnoy's fluid at —76° C), but the table of results demonstrates that these processes are not responsible for the cytomorphological changes observed.

Discussion

The cytomorphological method is one of the techniques for studying cold death. We do not know the mechanism of cold injury (SMITH 1954, MERYMAN 1956, 1957). In interpreting our results, we must have in mind the velocity of the thermal changes, the minimum temperature reached, the time of exposure to low temperatures, the phase reversal, etc. We must distinguish the processes during freezing and during thawing. For this reason many works

are not comparable (BUGAEVSKY 1955, 1957, LEVITT 1957). I hope, the work of GENÈVES and the present paper are to some extent comparable.

The statements of GENÈVES that the specimens undergo the cytomorphological changes which are observed after plunging into liquid air, when being frozen and not when thawed, and that plunging into liquid air is an unsuitable technique of fixation, are not supported by the present work. In the present work the results obtained with chloroplasts of wheat leaves support the conclusion that when the specimens have been frozen by plunging them into liquid nitrogen the cytomorphological changes occur during thawing. This statement is supported by the experiences of many authors working with freezing—drying and freezing—substitution (authors's results in this field c.f. BENEŠ and HRŠEL 1958).

Other cases are to be distinguished when the specimens are frozen slowly, to a higher temperature (—20° C) and when the specimen is large. In this case the changes may occur during the freezing.

In the present work, destroyed chloroplasts were observed with a normal nucleus in the same cell. Normal and destroyed chloroplasts were also found together in the same tissue. The two following problems have been touched on here:

1. Are all of the cellular constituents of any kind (chloroplasts) in the same tissue altered by cold simultanously?

2. Is the mechanism and time of cold destruction the same for various kinds of cell constituents (chloroplasts, nucleus)?

A comparison of cytological and biochemical data would be very interesting in connection with the problem of the preservation of active chloroplasts in a frozen state (SMITH et BENITEZ 1954, BISHOP et al. 1955, CLENDENING et al. 1956). Some cytological studies were made in connection with diagnosis of frost resistance (GENKEL and OKNINA 1954).

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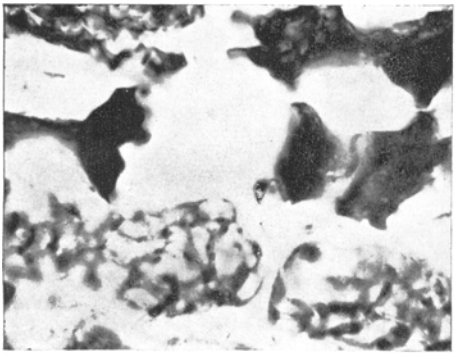
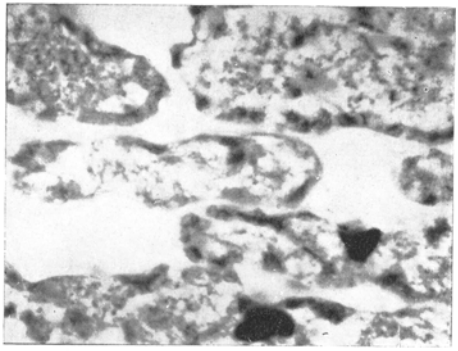
THE CYTOMORPHOLOGICAL EFFECTS OF LOW TEMPERATURES

Triticum vulgare VILL., chloroplasts in leaf cells, fixation with temperated Carnoy's acetic alcohol, staining with buffered acid fuchsin.

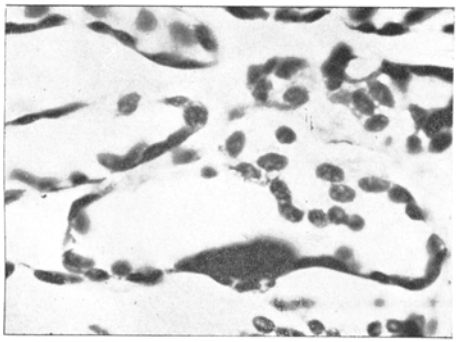


1a.

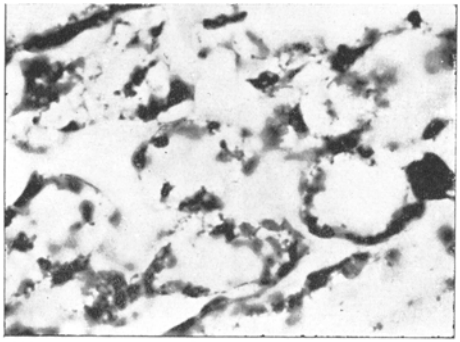


1b.

Fig. 1a, b. After plunging into liquid nitrogen 10 min. thawed and then fixed with Carnoy at a) − 27° C, b) + 27° C.

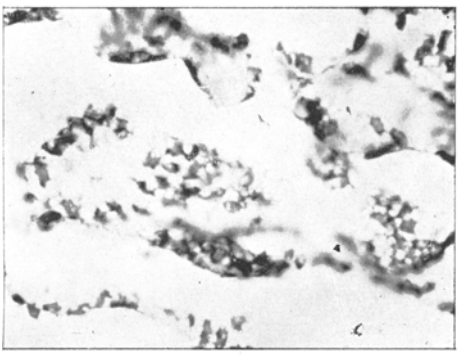


2a.

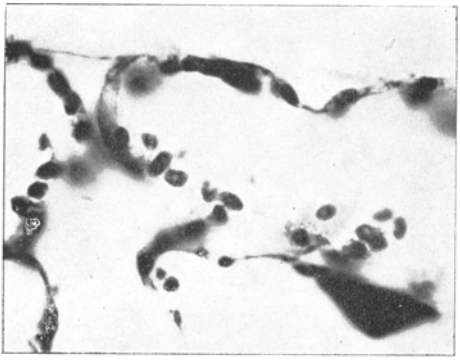


2b.

Fig. 2a, b. After plunging into liquid nitrogen, without being thawed immediately fixed with Carnoy at a) —27° C, b) +27° C



3a.



3b.

Fig. 3a, b. Directly fixed with with Carnoy at a) —27° C, b) +27° C. Magnif. 1500 : 1. Photomicrographs by J. Kubec.

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Цитоморфологическое действие низких температур, изучаемых с помощью фиксаторов различной температуры

КАРЕЛ БЕНЕШ

Резюме

Исследовался механизм цитоморфологического действия низких температур на листья 4-дневных проростков пшеницы. 1. Отрезанные верхушки листьев в металической корзинке погружались в жидкий азот и без оттаивания как можно быстрее фиксировались. 2. Таким же образом обрабатывались и следующие объекты, но после погружения в жидкий азот они оставлялись при комнатной температуре до оттаивания, и только после этого фиксировались. 3. Следующая серия объектов фиксировалась прямо, т. е. без замораживания в жидком азоте. Фиксаторы доводились до температуры —76, —27, +27 и +56° С. Фиксация в спиртово-уксусной смеси Carnoy-a 20 мин., в смеси Lison-a 2 часа. Обезвоживание и заливка производились на вибраторе. Срезы окрашивались кислым фуксином по Drawert-у и железным гематоксилином по Regaud. Результаты представлены в таблице (наблюдения хлоропластов верхушки первого зеленого листа):

	—76° С	—27° С	+27° С	+58° С
Из жидкого азота прямо в фиксатор	нормальные	нормальные	неправильные образования и нормальные	неправильные образования и нормальные
Из жидкого азота 10-мин. оттаивание и потом фиксатор	неправильные образования	неправильные образования	неправильные образования	неправильные образования
Прямо в фиксатор	деформированные и нормальные	деформированные и нормальные	нормальные	нормальные

Одинаковым способом замороженные, но различным образом оттаявшие объекты дают неодинаковые результаты. Отсюда вытекает, что разрушение хлоропластов происходит при оттаивании тканей. Необходимо однако допустить, что в случаях медленного замораживания, в опытах при высшей температуре (—20° С), или если объект слишком велик, разрушение может наступать и при замораживании. Разрушение хлоропластов наступает неодновременно с разрушением ядра, и не все хлоропласты одновременно разрушаются.