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IMPROVEMENT OF THE STRAW TECHNIQUE FOR THE PRESERVATION OF FUNGI IN LIQUID NITROGEN

J. A. STALPERS, A. DE HOOG, AND IJ. VLUG

Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, The Netherlands

ABSTRACT

A method for cryopreservation of fungi in straws is described in detail; for large scale application special equipment had to be developed for which technical diagrams are provided.

Key Words: preservation, fungi.

The storage of fungi in liquid nitrogen has generally been recognized as the most reliable method for long term preservation. For culture collections which are recognized as patent deposits it has become a necessity because of the obligations for the maintenance of patented strains under the Budapest Treaty.

The disadvantages of cryopreservation including the risks connected with the necessity of a constant supply of liquid nitrogen (LN), the expensive, space-consuming and sometimes dangerous use of glass vials and the lack of good alarm and automatic filling systems, have been strongly reduced by recent developments. The use of polypropylene straws instead of glass vials (Elliott, **1976**; Elliott and Challen, **1979**) is both cheap (for the whole CBS collection 360,000 straws (12-fold) are necessary which cost about 140 US $) and safe (when a glass vial is improperly sealed and LN has leaked in, thawing may cause an explosion resulting in flying glass, but a straw only splits) and the latest version of the Union Carbide alarm and filling system is relatively reliable.

Because of the small size of the straws the consumption of LN drops drastically when compared with an equal number of glass vials. The estimated annual LN consumption for one vivostat (filled) is about 5000 liters; the whole CBS collection, which can be stored 8-fold in three vivostats, would require 15,000 liters. Of course the consumption depends a great deal on how many times the vivostats are opened and how much is added.

As all the equipment for handling the straws had to be developed, it is considered useful to describe the method in detail and to provide technical diagrams.

Preparation of straws. — After testing many specimens of several trade marks of straws with diameters of 3 and 4 mm, Elliott and Challen's (**1979**) conclusion was confirmed: the straws with the best overall score with respect to resistance to autoclaving, heat-sealing and storage in LN were 4 mm polypropylene Sweetheart Winfield drinking straws, produced by Sweetheart International Ltd. (straw code 0175050). Straws with diam of 3 mm with the same qualities were not found and the 3 mm size proved too small for easy handling when agar plugs are used. However, the size may be useful when broth cultures are used and thus the equipment was designed for both 3 and 4 mm straws.

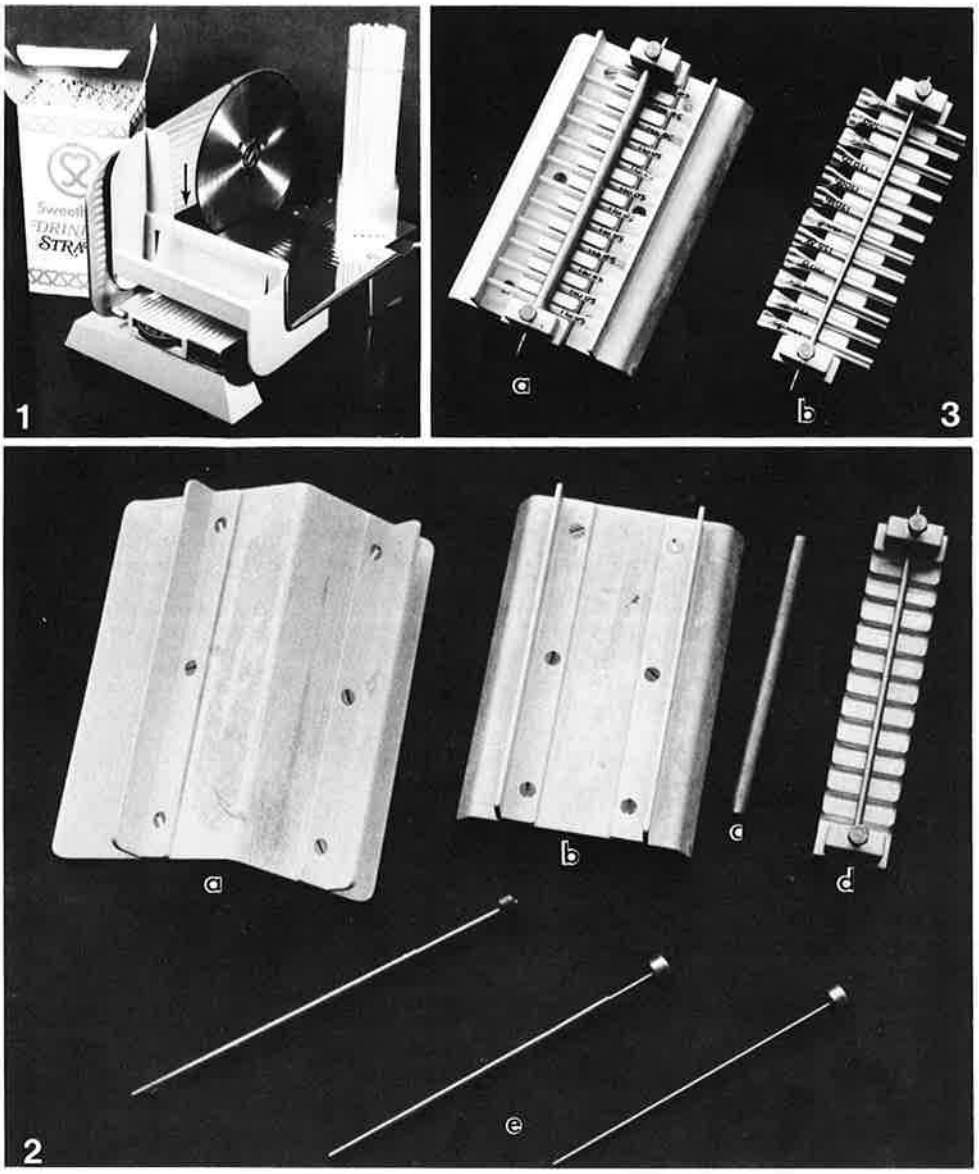
Straws of about 200 mm long are fixed in a mold (52 × 32 × 47 mm) and cut into pieces of 47 mm using an ordinary cutting device (Graef, type EH-170 T), from which a part of the protective shield had to be removed (FIG. 1, arrow).

For each strain 12 straws are prepared. The straw holder (FIG. 3, DIAGRAMS 1, 2) is placed in an adjusting frame (FIG. 2b, DIAGRAM 3) and the straws are lined up (FIG. 3). The pin is put through both clamping pieces and screw-tightened for fixing. When straws of 3 mm are used,

TABLE I

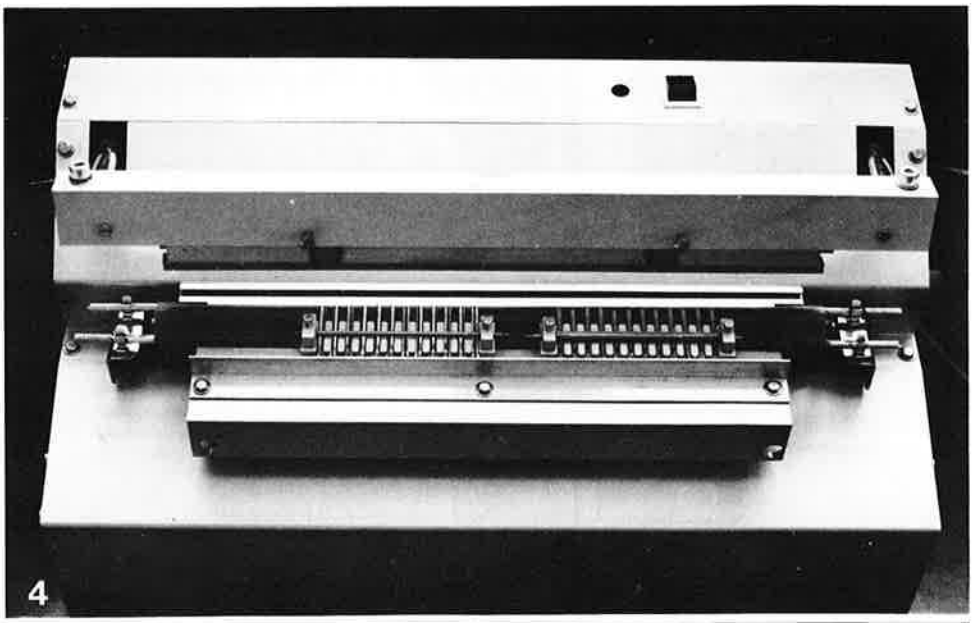
NUMBER OF STRAINS IN THE VARIOUS CLASSES OF FUNGI PRESERVED IN LIQUID NITROGEN.

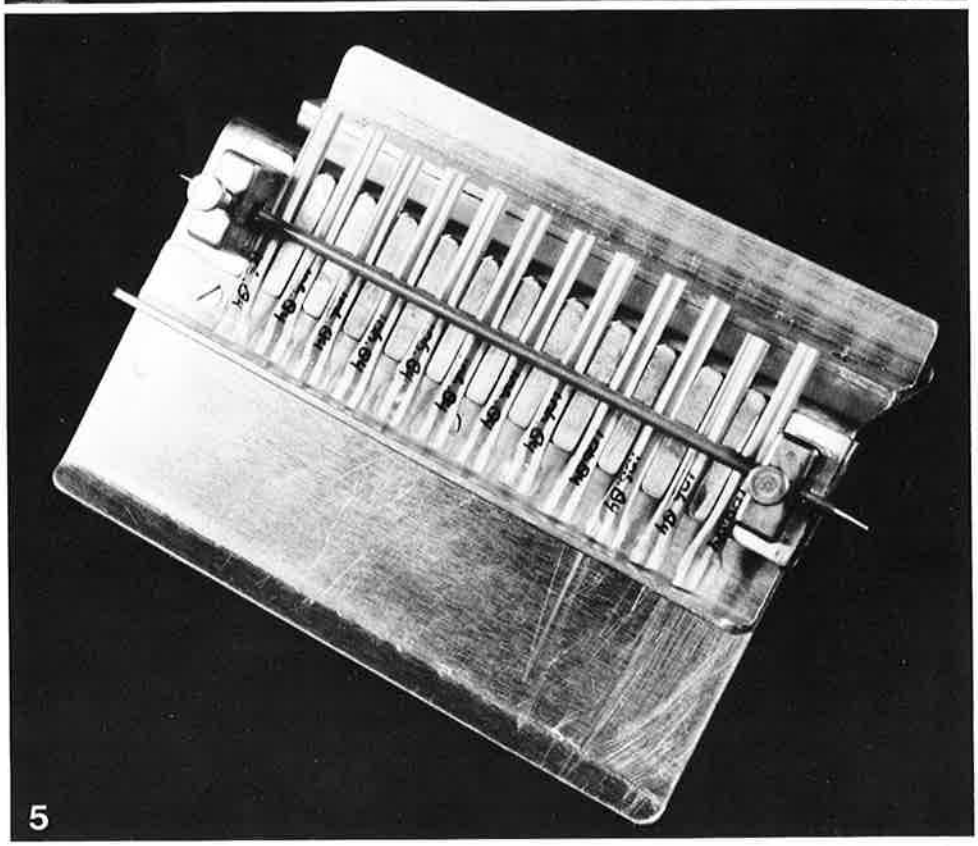
Class of fungi	Number	Failures	% success
Basidiomycetes	706	23	96.9
Ascomycetes	903	16	98.2
Zygomycetes	298	1	99.7
Oomycetes	34	5	85.3
Total	1941	45	97.7



FIGS. 1–3. Equipment for straw preservation technique. 1. Cutting device with partly removed protective shield (arrow). 2. Equipment for handling straws: a. support; b. adjusting frame; c. adapting tube for 3 mm straws; d. strawholder with pin; straws; e. corkborers with pins. 3. Strawholders: a. strawholder with adapting tube and 3 mm straws in adjusting frame; b. strawholder with 4 mm straws.

an adapting tube with a diameter of 4 mm and a wall thickness of 1 mm is placed on the straws and the pin is stuck through the tube and the clamping pieces. The straws (maximum three strawholders at a time) are then heat-sealed with an Automaster sealer (AM 400, Audion Electron, Amsterdam), which has a seal of 5 mm wide (FIG. 4). Each straw is hand-labeled with a CBS accession number using an alcohol-resistant microwriter (black, Staedtler Lumocolor 318). All 12 straws are then put into a bacteriological screw cap bottle (28 ml, 82.5 × 28 mm, A. Gallenkamp & Co. Ltd., London) and sterilized in an autoclave at 120 C for 20 min.





FIGS. 4, 5. Equipment for straw preservation technique. 4. Sealing device with 2 strawholders. 5. Support for filling straws with strawholder.

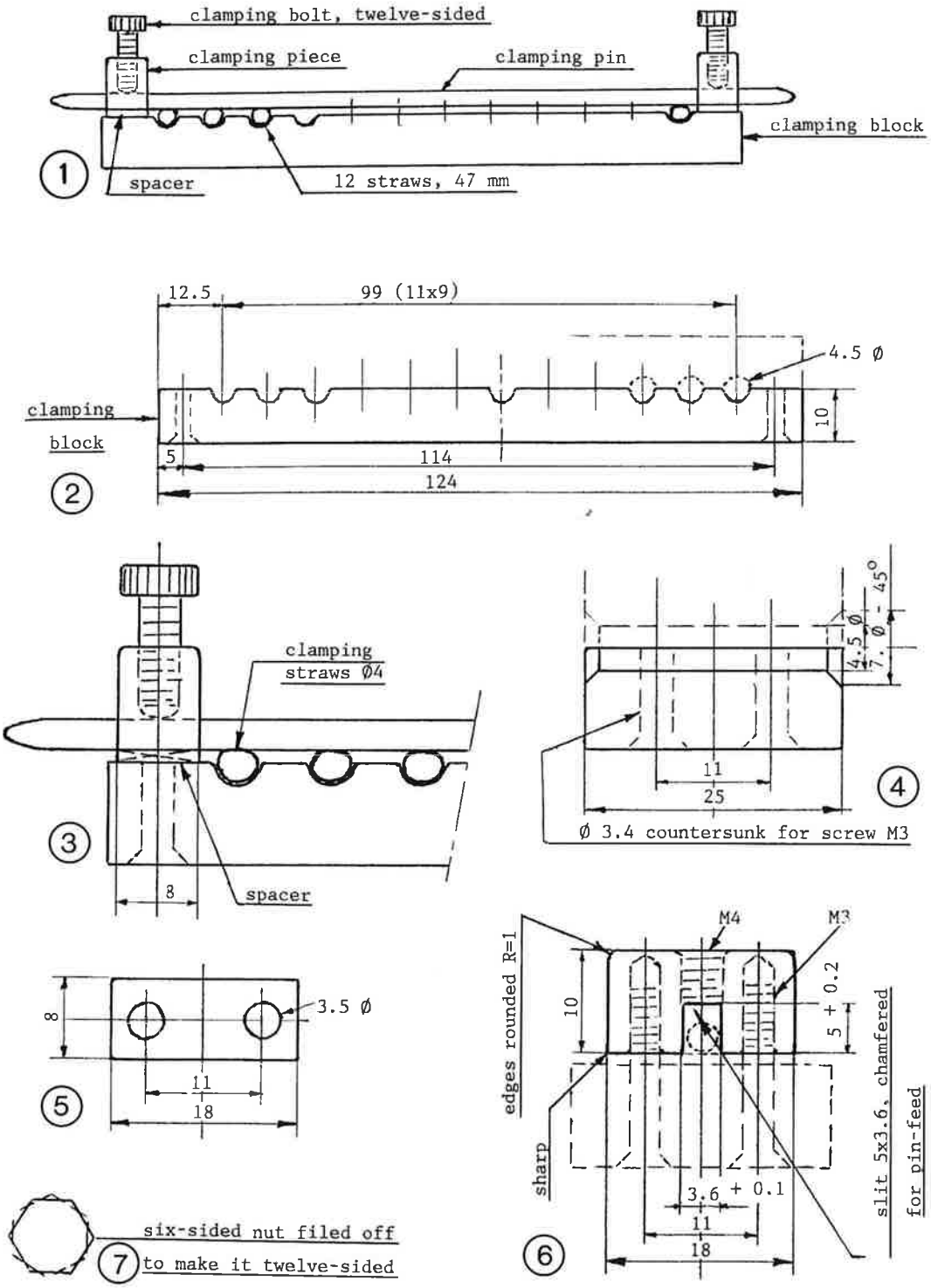


DIAGRAM 1. Strawholder for straws; 1. general overview; 2. clamping block; 3. detail of gutter; 4. clamping device front; 5. clamping device side; 6. spacer; 7. clamping bolt, surface view.

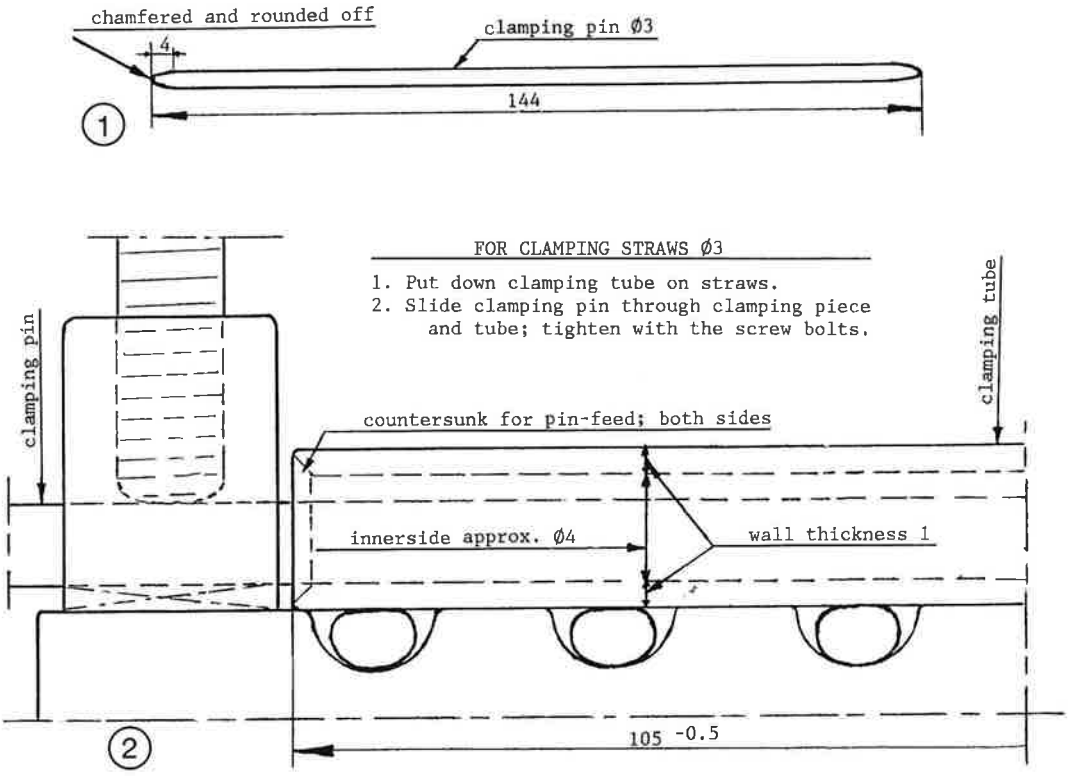


DIAGRAM 2. Strawholder for straws; 1. clamping pin; 2. clamping tube to adapt for straws of 3 mm. Material: 1× block, stainless steel or aluminium, 124 × 25 × 10 mm. 2× clamping piece, stainless steel or brass, 18 × 10 × 8 mm. 1× pin, stainless steel, diam 3 mm, length 144 mm. 1× clamping tube, stainless steel or brass, inside diam. approx. 4 mm, wall thickness 1 mm, length 105 mm. 2× spacer, stainless steel, 18 × 8 mm, thickness 1 mm. 4× screw, stainless steel or brass, countersunk head, M3 × 15. 2× screw bolt, stainless steel or brass, M4 × 12.

Handling of straws.—All manipulations are carried out in a Biohazard recirculating laminar flow cabinet (CLF 406, Clean Air, Woerden, The Netherlands) using sterilized instruments. After lining up the straws, the straw holder is placed on a support (FIG. 2a, DIAGRAM 4) at angles of 30 or 45 degrees dependent on the preference of the technical assistant. The straws are then filled half full with a cryoprotectant. The standard is 10% aqueous glycerol, but some fungi require 10% dimethylsulfoxide (DMSO in water) (Hwang *et al.*, **1976**) or an aqueous solution containing 8% glucose and 10% DMSO (Smith, **1983**). From agar cultures in Petri dishes 5–8 plugs of 2.8 mm diam (for 3 mm straws 1.8 mm diam) are punched out with a cork borer with pin (DIAGRAM 5) and transferred into the straw. When all straws are filled the series is heat-sealed. Compared with the first sealing, the sealing time will be longer as a consequence of autoclaving and the possibility of some moisture being present at the inner side of the straw at the place of sealing. All straws are checked for leakage and if necessary resealed. Then labels of 7 × 5 mm with the CBS accession number and provided with an ultra low temperature resistant rubber glue (Nederlandsche Speciaal Drukkerijen, Delft, The Netherlands) are glued on the upper seal. Finally, the straws are placed in a drawer, cooled with a rate of −1 C/min to −40 C in a plasma freezer (Forma Scientific, U.S.A., model 8070) and stored in liquid nitrogen.

For each strain 12 straws are prepared; 8 are stored in their final unit, one is stored separately for a viability check within a week and the others are stored in the first row of the same drawer in

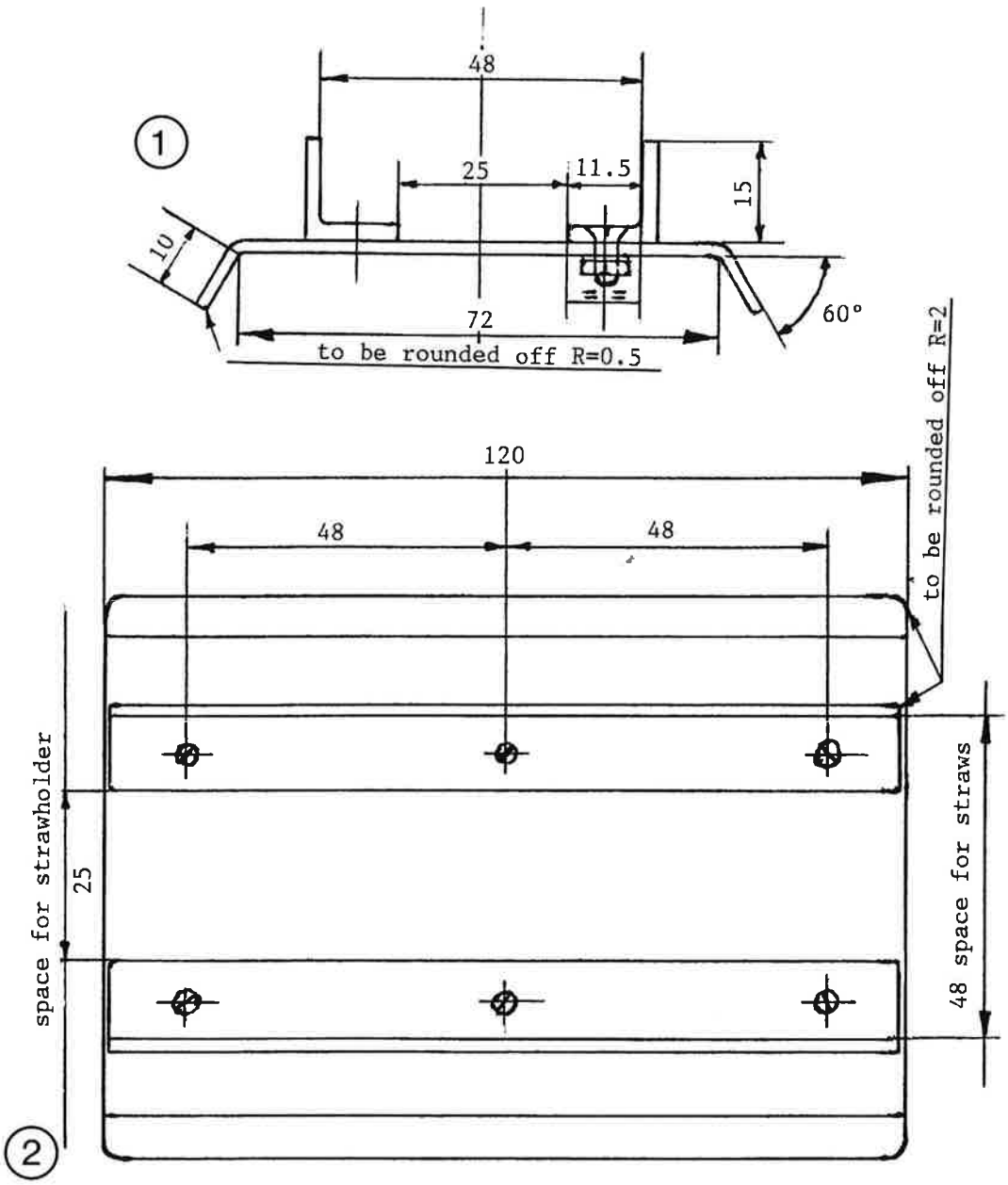
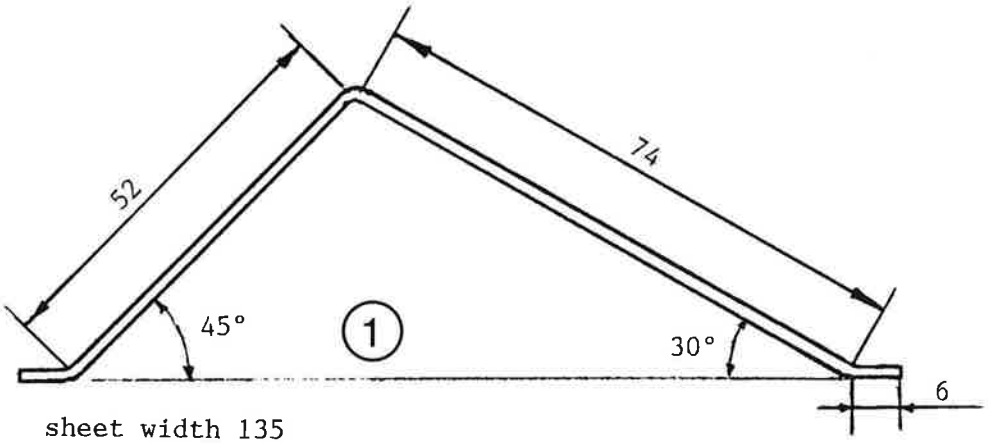


DIAGRAM 3. Adjusting frame to line up straws; 1. side view; 2. surface view. Material: 1× stainless steel, 120 × 92 mm, thickness 1.5 mm. 2× stainless steel, 15 × 13.5 × 2 mm, length 119 mm. 6× countersunk screw M3, stainless steel or brass, length 8 mm. 6× nut M3, stainless steel or brass. 6× spring washer 3.2, phosphor-bronze.



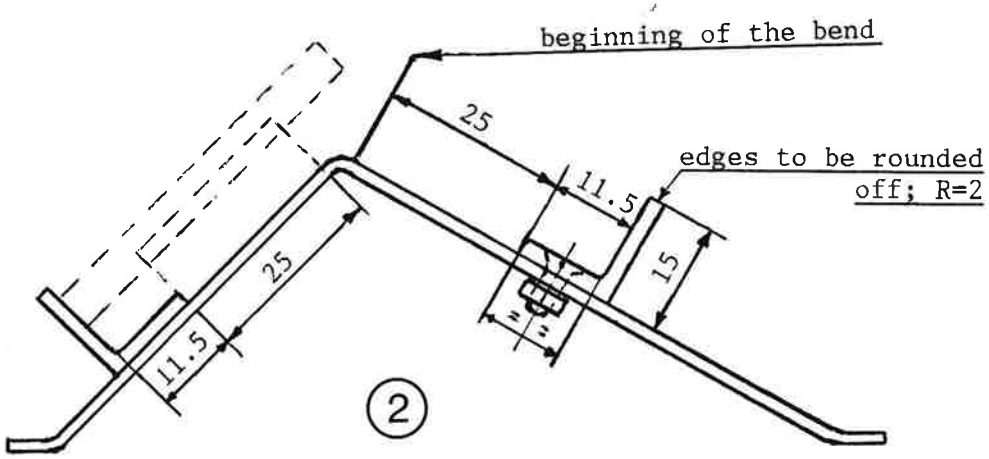


DIAGRAM 4. Support for filling straws. Material: 1× stainless steel, 135 × 138 mm, thickness 1.5 mm. 2× stainless steel, 15 × 13.5 × 2 mm, length 134 mm. 6× screw M3, countersunk head, stainless steel or brass, length 8 mm. 6× nut M3, stainless steel or brass. 6× spring washer, 3.2, phosphor-bronze.

which the first 8 can be found. Of these, one is to be checked after a year and the remaining straws are extras.

Thawing takes place in a water bath for five minutes at 30–35 C (Oomycetes: 20 C). Then the straws are rinsed in ethanol 96%, opened and plated on suitable media.

The method has been applied to about 2000 strains and the results are summarized in TABLE I. This table is slightly optimistic, especially with regard to the Oomycetes, where the method has been modified after the results of the original method were disappointing (more than 50% failures). As the new method is not yet in use for a long time, and the number of unfinished tests is thus relatively large, the percentage of success

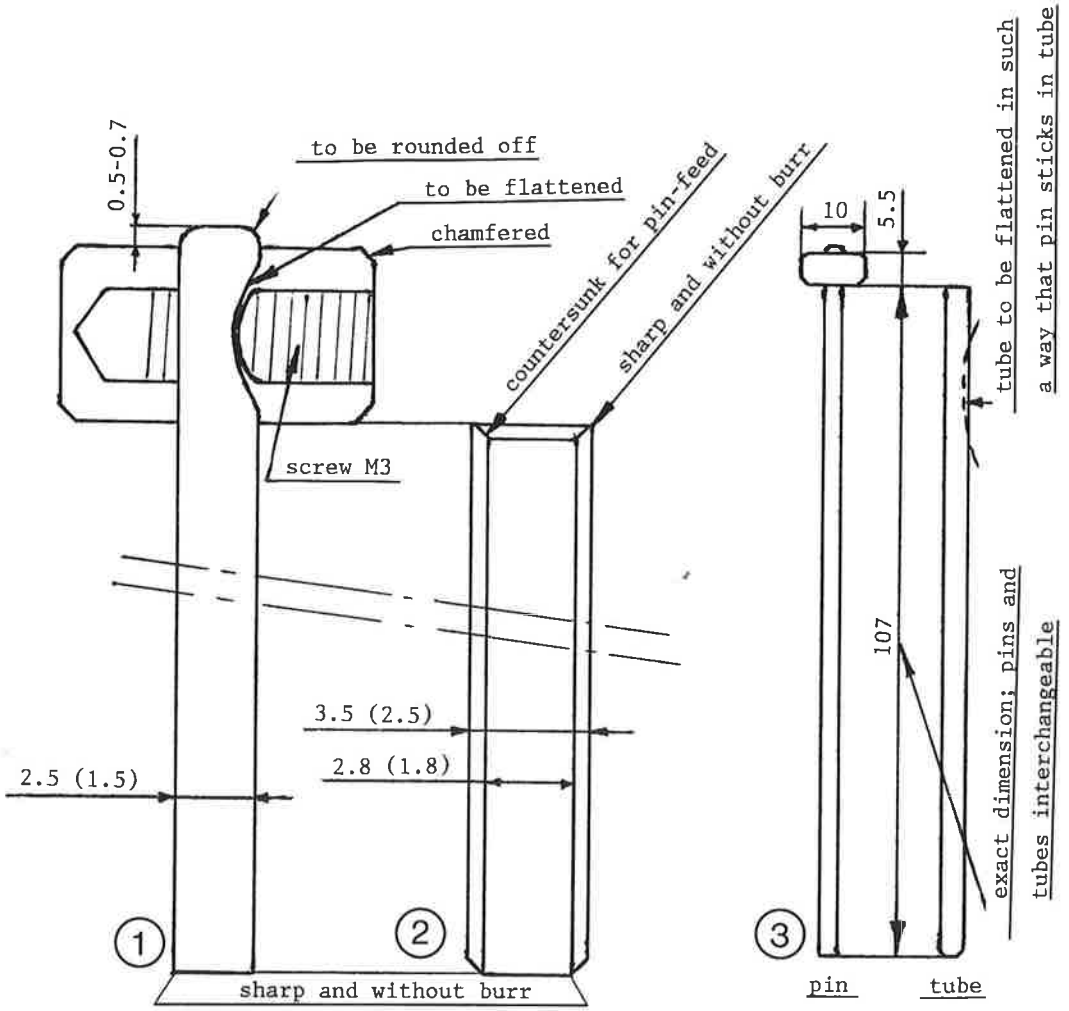


DIAGRAM 5. Cork borer with pin for cutting plugs of 2.8 (1.8) mm diam; 1. pin and tube; 2. pinhead; 3. detail tube. Material: 1× pin, stainless steel, diam 2.5 (1.5) mm, length 113 mm, with brass knob, secured by brass screw M3. 1× tube, stainless steel, internal diam 2.8 (1.8) mm, thickness approx. 0.7 mm, length 107 mm.

will probably decrease to an estimate of about 70%. Moreover the strains were generally recently isolated (up to two years in culture, except for about 500 Basidiomycetes which were up to 12 years in culture). Even if these positive effects are eliminated, our results are still comparable with or better than those obtained with glass vials.

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