

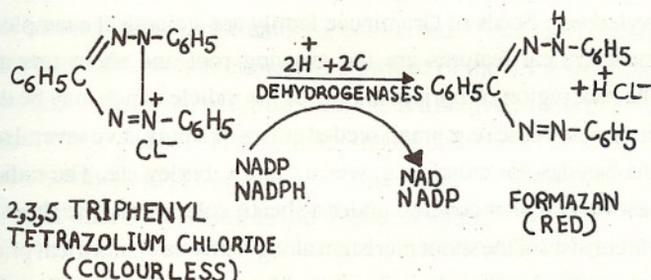
14. TETRAZOLIUM TEST FOR SEED VIABILITY AND VIGOUR

V.N. PATIL & MALAVIKA DADLANI

Germination test is the best indication of the potential of a seed lot to emerge under field conditions. However, it takes from days to weeks and in some cases even months to complete. Tetrazolium test, commonly known as the TZ test for seed viability have, therefore, been developed to furnish quick estimates of seeds' germinability. The test is very useful in processing, handling, storing and marketing large quantities of seed in a short time, testing dormant seed lots, vigour rating of the seed lots, supplementing germination test results and diagnosing the cause of seed deterioration.

Machanism of the Reaction

The TZ test is reliable and widely utilized. the method was pioneered by the German scientist Lakon during the mid nineteens (1939-1958), who recognised that all living tissues, which respire, are capable of reducing a colourless chemical 2,3,5 triphenyl tetrazolium chloride or bromide) into a red coloured compound formazan by H transfer reactions catalysed by the enzyme dehydrogenases



Formazan being non-diffusible, stains the living tissues red. Thus, the living parts of a viable seed should be stained red when incubated in the solution of this chemical.

The immense significance and practical utility of this test was realised by the seed scientists and technologists the world over and it was suitably modified and improved to assess the seed quality in terms of its viability.

Since the tissues within a seed could be at different states of viability they would be stained differently. Moore (1973) described the use of TZ staining more efficiently on the basis of the topographic pattern of the seed.

The accuracy of the tetrazolium test requires :

1. A sound knowledge of the seed and seedling structures,
2. An understanding of the mechanisms of the reactions leading to the staining of the seed.
3. Ability to interpret the staining pattern by critically examining the seed topography and by keeping other visible seed qualities such as tissue turbidity, abnormal or missing structures etc., in view and,
4. Experience gained through keen interest and natural skill.

Essential Seed Structures for TZ Test Evaluation

Seeds can be grouped into monocots, dicots and polycots on the basis of the presence of one, two or more than two cotyledons. Embryo structures or structure primordia are, relatively similar for embryos within each groups though they may differ in shape, size and differentiation/development of embryo structures. The critical features of the seeds of these groups are described below.

Monocotyledons : Seeds of Gramineae family are a classical example of this class. The most critical features are the growing root and shoot tips and the scutellum. The root region comprises mainly of the radicle which may be the only root primordia in some cases e.g. small seeded grasses or it may have several seminal root primordia besides the radicle e.g. wheat, maize, barley etc. The radicle lies below the mesocotyl region covered under a sheath coleorhiza. The shoot region or the plumule consists of the shoot meristem along with one or more leaf primordia near the base, enclosed within the coleoptile. Mesocotyl separates plumule from the point of attachment to the scutellum. The main embryo structures of maize - a typical monocot seed are shown in Figure 14.1.

Dicotyledons : In the dicot seeds with a low degree of differentiation the embryo is embedded in nutritive tissues and consists of a radicle and two cotyledons. In the seeds with a higher degree of differentiation, embryo occupies

a larger portion of the seed. The radicle is well recognizable part of the embryo while the shoot meristem with the primary leaf primordia generally lies compressed between the two cotyledons. The structure of a bean seed - a typical dicot seed with well developed embryo is given in Figure 14.2.

Polycotyledons : Not very common. This type of seed is found in Pinaceae and other conifers. The embryo is not very well differentiated except for a radicle and more than two cotyledons. Living nutritive storage tissues encircle the embryo while the shoot meristem is encircled by the base of the cotyledons.

Equipments required for conducting TZ test

(1) Staining dished *e.g.* watch glasses for small seeds or petridishes., (2) Beakers of 100 to 250 ml capacity for larger seeds, (3) Single edge razor blade, (4) Needles, (5) Forceps, (6) Magnifying devices, (7) Medicine dropper, (8) Dispensing bottle, (9) Germinating blotters, filter paper and paper towels, and (10) Oven or incubator.

Preparation of the staining solution

The chemical used for this test is a cream or light yellow coloured water soluble powder called 2,3,5,- triphenyl tetrazolium chloride.

Several concentrations of tetrazolium solution ranging from 0.1 to 1.0% are used with comparable results. Generally the 1.0% solution is used for seeds that are not bisected through the embryo, is bisected. Other low concentrations much as 0.2% and 0.5% are sometimes used instead of the 0.1% solution. To prepare a 1.0% solution, one gram of tetrazolium salt is dissolved in the distilled or tap water to make 100 ml. The pH of the solution should be around 7.0 for the proper staining to occur. Solutions of pH 4 or lower will not stain even viable embryos and solution of pH more than 8 will result in too intense staining.

If the pH of the water is not in the neutral range, the tetrazolium salt should be dissolved in phosphate buffer solution. The buffer solution is prepared as follows:

Solution A—Dissolve 9.078 g of Potassium dihydrogen phosphate (KH_2PO_4) in water to make 1000 ml.

Solution B—Dissolve 11.876 g of Disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) to make 1000 ml.

Take 400 ml of solution A and 600 ml of solution B and mix them together. In one litre of the buffer solution prepared as above, dissolve 10 g of the tetrazolium salt. This gives a 1.0% tetrazolium solution of pH 7.0

Store the solution in a dark or amber coloured bottle to prevent the reaction which also occurs in the presence of light. The solutions can be stored for several months in a refrigerator.

Preparation of the clearing solution

A lactophenol solution can be used as a clearing agent, if the pigment within the seed coat or lemma prevents clear vision after staining. By using the clearing agent the embryo is visible through the seed coat or lemma. It consists of: Lactic acid: 20 parts, Phenol: 20 parts, Glycerine: 40 parts and Water: 20 parts.

Temperature

Temperature influences the staining reaction. Staining will take place twice as fast at 30 as at 20°C and twice as fast at 40 as at 30°C. Temperature between 20°C and 40°C have no effect on accuracy of tetrazolium test, but staining proceeds faster at the higher temperature. The tests can be performed satisfactorily at room temperature.

Working Sample

At least 100 seeds should be tested in replicates of 50 or less. The seeds should be randomly selected from the pure seed component and counted in replicates before conditioning.

In order to ensure a contact of the tetrazolium solution with the embryo, some conditioning and preparatory steps may be essential. These depend on the type of the seed, permeability and thickness of the seed coat, location of the embryo etc.

Preconditioning

It is done to,

- allow complete hydration of all the tissues.
- prevent damage to cotyledons and embryo axes while cutting seeds

- initiate and activate the germination process, and to
- have proper penetration of tetrazolium solution.

Some kinds of seeds can directly be placed in water, while others must be moistened slowly. Slow moistening is generally practiced for large seeded legumes or for the seed samples which are dry, brittle or are aged to avoid tissue damage due to rapid intake of water. This can be done by keeping the seed on top of or in between the moist blotter or paper towels respectively.

Small seeded crops or seed with a permeable coat can be moistened by direct soaking without any adverse effects. The moistening time can be considerably reduced by putting the seeds at a slightly higher temperature (within 40°C) during imbibition.

Preparation for Staining

In addition to moistening, most kinds of seed require some preparatory steps before staining, to

- assure the adequate penetration of the staining solution into the seed,
- accelerate the rate of staining,
- facilitate the evaluation.

Thus, depending upon the kind of the seed, time available, degree of accuracy desired and the experience of the analyst, one of the following methods can be adopted:

(a) No moistening or preparation required (small seeded legumes with soft coats).

(b) Slow moistening without any preparation (large seeded legumes with soft coats).

(c) Piercing, puncturing or cutting of the seed coat (small seeded grasses).

(d) Cutting the seeds longitudinally through the midsection of the embryo and through part of the endosperm, leaving the two halves attached at the base or slitting

the seed completely, keeping only one half for the staining, discard the other.

(c) Cutting the seeds longitudinally slightly off-centre to avoid cutting into the embryo.

(f) Removing the seed coat (with forceps/needles/razor blades etc.) with minimum injury to the tissues. Sometimes, a thin membrane adheres to the cotyledons even after removal of the seed coat, this may be removed by a sliding motion after an additional 30 min. of soaking (dicots with hard seed coats).

Staining

Seeds can be stained in watch glasses, petridishes or beakers. Sufficient solution should be used to cover the seeds and to allow its absorption. As a rule of thumb, a concentrated (1.0%) solution can be used for legumes, cotton and grasses that are not bisected through the embryo and a dilute (0.25% or 0.50%) solution for grasses and cereals that are bisected through the embryo.

Suggested preparation method for staining, concentration of the solution, staining time at 35° C, and evaluation for a number of important Indian crops are given in Table 14.1 (for further details, refer to Moore, 1995). Care should be taken not to overstain seeds as this causes degeneration, making interpretations difficult and uncertain.

In general, seeds are placed in the solution and held at 35° C for complete colouration. After a period in the solution, the length of which varies according to the condition of the seed, species, temperature and concentration of the solution: the seed is removed from the tetrazolium solution, rinsed 2-3 times in water and then evaluated according to the staining pattern. During evaluation, the seed should be left in a little water to prevent these from drying. If seeds are not to be evaluated immediately, the seeds in a little water should be kept in the refrigerator. Whenever required, 2-3 drops of lactophenol clearing solution can be added to 100 seeds of small seeded grasses, after completely removing the tetrazolium solution. Small seeds are examined under a stereo microscope, while large seeds may be examined with magnifying lenses only. Seed coats of legumes must usually be removed before examination.

Tissue Characteristics

(i) Sound tissues

Staining proceeds gradually and uniformly from the exposed surfaces inward. Changes in the intensity of colour are gradual without distinct boundaries. The colour intensity is lesser but lustrous in sound tissues than the bruised, damaged or aged tissues. A gradual reduction in colour intensity from the surface to the interior of the seed indicates slow absorption and firm, sound unstained tissues indicate the lack of penetration of the tetrazolium solution.

(ii) Weak viable tissues

Such tissues tend to stain greyish red or brighter red than normal. The apical tips of leaves, coleoptiles and radicles tend to reveal earlier evidence of deterioration than do other areas. Weak and aged tissues also lose the turgidity of the sound tissues, they appear flaccid and develop mottled stain.

(iii) Weak non-viable tissues

Tissue colour is often mottled, it may be purplish, brownish or greyish red and its intensity may vary from abnormal dark to pale pink. Cut surfaces may appear off white, while the inner tissues may appear dark red. The unstained dead tissues look flacid, liquid-logged, blurred, chalky white and lack-lustre.

A definite boundary does not necessarily occur between the non- viable, abnormally stained, stained and unstained tissues. Both types of tissues are therefore, considered dead, regardless of colour differences.

Seed structure and the seed as a whole must be considered while evaluating staining pattern. Seed may be considered non- germinable even when there is only one fracture or one small broken or missing spot in a vital position such as the point of attachment of roots and cotyledons. Seed is considered dead when unstained areas include mesocotyl and seminal roots in grasses and tips of coleorhiza in wheat and rye. The areas of vital importance in interpreting the staining pattern in monocot are the plumule tips, the portion where embryo is attached to the scutellum and seminal root region. In legumes, injuries caused to the growing organs must be noted more carefully than the injuries of the other organs. Unstained radicle tips in legumes may not allow the radicle to grow. Improper staining in areas such as

radicle and hypocotyl development, cotyledons and in the plumule region indicate a non-germinable or abnormal seed.

The tetrazolium percentage is the percentage of normal germinable seeds to be expected when the seed lot is germinated under very favourable conditions. Properly conducted tetrazolium and germination test results are generally very close.

Interpretation of TZ-test results

The Seed Technology Laboratory, Mississippi State University developed detailed staining patterns of some of the major crop species for interpreting the TZ test results (Fig. 14.3-14.10). Most of the other crops can be evaluated taking one or the other of these staining patterns as the guide. However, for some crops it may be necessary to first standardise the staining. The standardization of the TZ methods for ragi (*Eleusine coracane* and khesari (*Lathyrus sativus*) have been reported (Agarwal and Kaur, 1975; Agrawal and Negi, 1977). If properly conducted, TZ test's result is in close agreement with the germination percentage (Agrawal *et al.*, 1973). However, discrepancies between the two results might arise due to several factors such as (Agrawal and Dadlani, 1987) :

- (i) Sample difference
- (ii) Improper germination testing
- (iii) Improper TZ testing
- (iv) Seed dormancy
- (v) Presence of hard seeds
- (vi) Presence of seed-borne organisms
- (vii) Chemical injury (such as fumigation injury and excess-treatment with mercurial fungicides etc, which may not inhibit the TZ staining but could affect normal germination).

For the convenience of the seed analysts, viable seed characters are coded by the Tetrazolium Committee of the ISTA (1985).

Terms used

Stained : Refers to viable (functional) tissues as established by staining characteristics and/or tissue turgidity.

Necrosis : Refers to non-viable (non-functional) tissues as established by abnormal staining characteristics and/or lack of tissue turgidity.

Evaluation codes for staining patterns of viable seeds of different species (for column 5 of Table 6.1)

(i) Embryo

- A. Completely stained.
- B. Completely stained except for the type of necrosis listed below:

Radicle

- 1. Distal tip
- 2. Distal 1/3
- 3. Distal 1/2
- 4. Distal 2/3
- 5. Entire

Root initial area

- 6. Primary root initial or surrounding area provided that at least two root initials are stained and are adjacent to a functional plumule.

Cotyledons

- 7. Superficial
- 8. Distal 1/4

15. Isolated superficial necroses, other than at junction with embryo and at centre of back of outer thickness or both cotyledonary lobes.

Scutellum

16. Necrosis involving no more than 1/3 of upper and lower ends.
17. Distal 1/4

Coleoptile

18. Distal 1/2

(ii) Nutritive storage tissue

- D. Completely stained.
- E. Stained except for small surface necroses that are not in contact with embryo cavity.

Advantage of TZ test

- (a) Quick and fairly accurate
- (b) Can also determine the viability of a dormant seed lot in a short time.
- (c) Seeds are not damaged (in dicots) and can be germinated.

Disadvantages of TZ test

- (a) Distinction between normal and abnormal seedlings difficult.
- (b) Cannot differentiate between dormant and non-dormant seeds.
- (c) Correct evaluation is possible only after prolonged experience.
- (d) Microorganisms harmful for seedling emergence remain undetected.

Tetrazolium test for vigour assessment

After differentiating viable seeds from the non-viable ones, the viable seed group can be reclassified into several vigour groups on the basis of intensity of stain and staining pattern of different seed parts. The simplest classification is to divide viable seeds into two groups. A seed lot having larger number of sound seeds will be considered more vigorous.

A better estimate of vigour can be obtained by colorimetric determination of formazan. Kittock and Law (1968) described a method of estimating seed vigour on the basis of colour intensity of the stained embryo or seed. The method is described below:

1. Prepare tetrazolium solution as described earlier.
2. Put 100 seeds in a petridish lined with moist filter paper and keep at $25 \pm 1^\circ\text{C}$ overnight.
3. Excise the embryonic axes and add 1 ml of tetrazolium solution to 25 axes in quadruplicate.
4. Incubate in dark at 30°C for 4 hours. The incubation time varies with the crop species. However, for maintaining uniformity, duration should be kept constant for a crop under study.
5. Drain out the excess solution and wash in distilled water.

6. Soak the axes in 10 ml of methyl cellosolve for 4 to 6 hours with occasional stirring till the extraction of red coloured formazan is complete i.e. the axes become colourless.
7. Decant the extract and read the intensity of the colour at 480 nm in a spectronic 20 colourimeter using methyl cellosolve as the blank.

Higher the intensity of the extracted formazan, greater is the vigour.

Table 14.1 Tetrazolium testing guide for some important crop species (adapted from Moore, 1985)

Crop	Moistening period(h)	Preparatory method after moistening	Staining time(h)	Staining evaluation (refer to the text)
1	2	3	4	5
Cereals				
Barley	6-18	(a) Cut the seed longitudinally through the mid-section of the embryonic axis	3-24	B6
Maize	18	— do —	3-24	B5, B17
Oat	6-18	— do —	2-24	B6
Paddy	18	— do —	6-24	B4
Pearl Millet	6-18	— do —	6-24	B4
Ragi	6-18	— do — or cut laterally	6-24	B4
Sorghum	6-18	(a)	6-24	B4, B17
Wheat	6-18	— do —	3-24	B6, B17
Pulses				
Chick pea	18	(b) Cut through the seed coat near midsection	6-24	B4, B11, B18
Cowpea	18*	— do — entire seed length	18-24	B3, B13, B18
Green Gram	18*	— do —	18-24	B3, B13, B18
Lathyrus	18*	— do — & (c) Sever a distal end of cotyledon	6-24	B3, B13
Lentil	18*	(b)	6-24	B3, B13, B18
Pea	18-24	— do —	6-24	B4, B13, B18
Pigeon pea	18	(b) & (c)	6-24	B3, B13
Soybean	18*	(b) or no seed coat preparation needed	6-24	B3, B13, B18
Oilseeds				
Castor	18	(b) & (c)	6-24	A, D
Groundnut	18*	(b)	6-24	B2, B8, B18
Linseed	18*	Treat with alum to remove seed coat sliperiness & (b)	6-24	B2, B9

1	2	3	4	5
Mustard	18	(d) Puncture the seed coat, cut as in (b) including 1/2 of circumference.	18-24	B2,B16
Safflower	18-24	(e) Cut longitudinally through the midsection of distal end.	6-24	B2,B14
Sesame	18	— do —	6-24	B1,B8
Sunflower	6-18	— do —	6-24	B2,B14
Fibre crops				
Cotton	18*	— do —	6-24	B3,B7,B10
Jute	18	— do —	24-48	A,C
Sunn hemp	18	(f) Cut through the coat near midsection of entire cotyledon length & (c)	18-24	B3,B13
Forage				
Alfa alfa	6-18	— do —	6-24	B3,B11
Berseem (Trifolium)	18	— do —	6-24	B3,B13
Guinea grass	6-18	(g) Cut through the midsection of distal end and expose the embryonic axis.	6-24	B4
Vegetables				
Amaranthus	18	(b)	18-24	B2,B9
Beet root	18	Puncture the seed coat and remove the cap covering embryo.	24-48	B2,B9
Bitter gourd	6-18	Remove the wings & (g)	6-24	B2,B12
Brinjal	18	Puncture and (h) Cut laterally or longitudinally through the entire depth.	6-24	A,C
Broad bean	24*	(b) & (c)	16-24	B4,B13,B18
Carrot	18	(c)	16-48	A,C
Chillies	18	(h)	6-24	A,C
Cucumber	6-18	Remove the Wings & (g)	6-24	B2,B12
Dolichos bean	18	(f)	6-24	B3,B13,B18
Lettuce	6-18	(e)	6-24	B2,B17
Luffa	6-18	Remove the wings & (d)	16-24	B2,B16
Onion	6-18*	(h)	6-24	A,D

1	2	3	4	5
Pumpkin	6-18	Remove the wings & (g)	6-24	B2,B12
Radish	18	(d)	6-24	B2,B16
Roundgourd	6-18	Remove seed coat sliperiness by drying & (g)	6-24	B2,B12
Tomato	18	Puncture & (h)	18-24	B2,B14
Flowers				
Aster	6-18	(e)	6-24	B2,B14
Begonia	18	(e)	18-24	A
Canna	18	Remove hard seed- edness & (e)	24-48	A,D
Colcus	18	Treat with alum to remove seed coat sliperiness & (b)	18-24	B2,B11
Dahlia	6-18	(e)	6-24	B2,B14
Gladiolus	18	Severe through the distal end of the seed	24-48	A,C
Rose	18	Severe wings & (e)	24-48	
Others				
Citrus	18	Remove seed coat sliperiness by drying. Remove or puncture the inner coat.	6-24	B2,B12
Coffea	18	(g)	24-48	A,C
Pine	18	Cut off centre through the coat & nutritive tissues to expose the embryo outline.	24-48	A,D
Rubber	18	— do —	2 at 40°C	—
Tobacco	24	(b)	24-48	A,C

Note :

* = Slow moistening recommended.

Preparatory methods as indicated in col. 3 are as follows :

a = Cut the seed longitudinally through the midsection of the embryonic axis.

b = Cut through the seed coat near midsection.

c = Severe a distal end of the cotyledon.

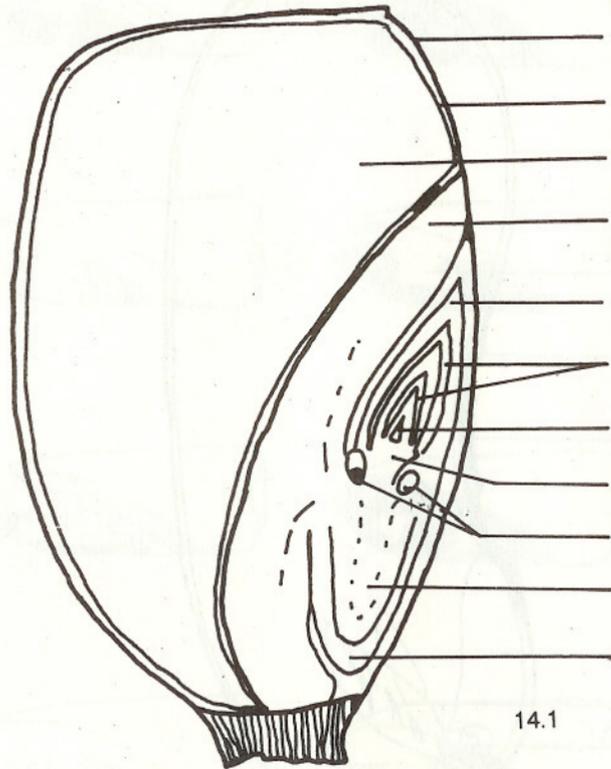
d = Puncture the seed coat, cut as in (b) including 1/2 of circumference.

e = Cut longitudinally through the midsection of distal end.

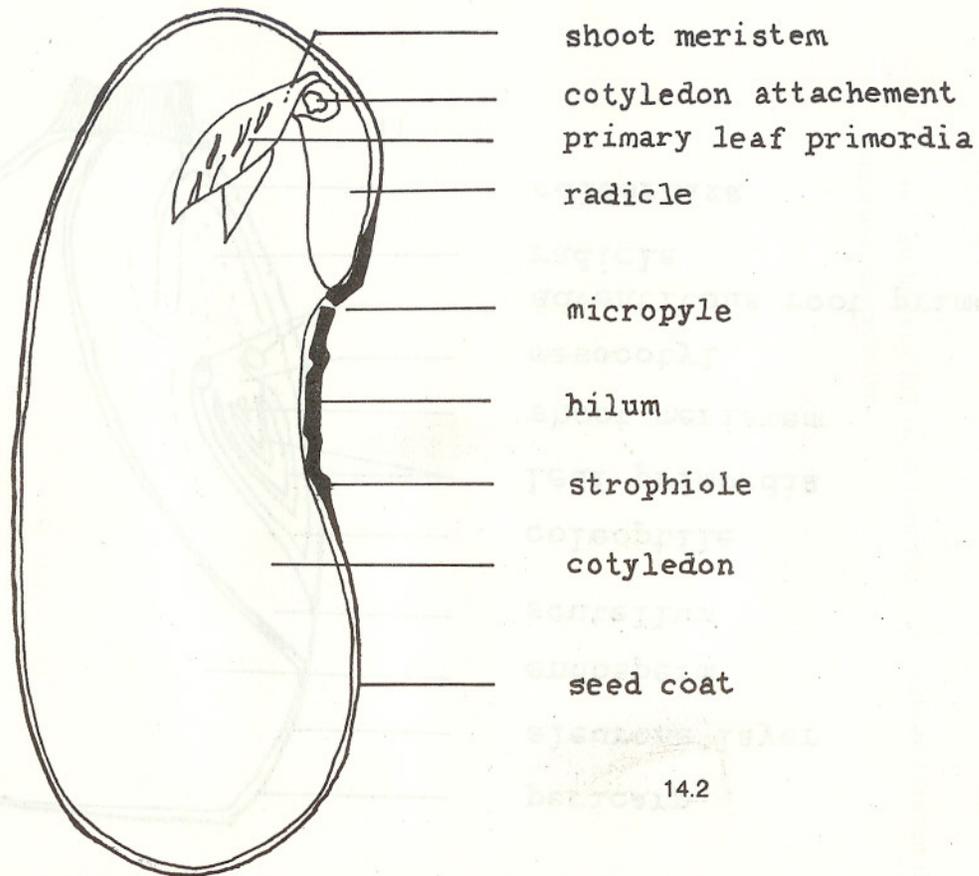
f = Cut through the seed coat near midsection of entire cotyledon length.

g = Cut as in (d) and expose the embryonic axis by spreading the cut surfaces slightly apart.

h = Cut of seed laterally full depth from the centre outward between the radicle and the cotyledons.



pericarp
 aleurone layer
 endosperm
 scutellum
 coleoptile
 leaf primordia
 shoot meristem
 mesocotyl
 adventitious root primordia
 radicle
 coleorhiza





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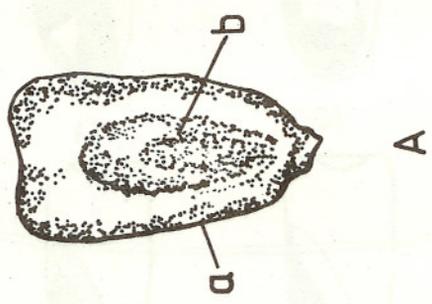
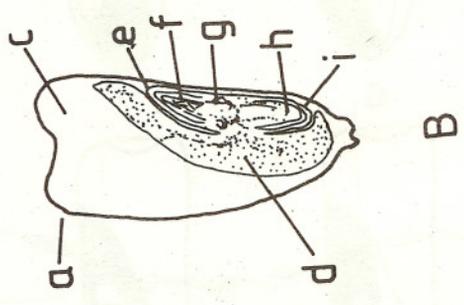
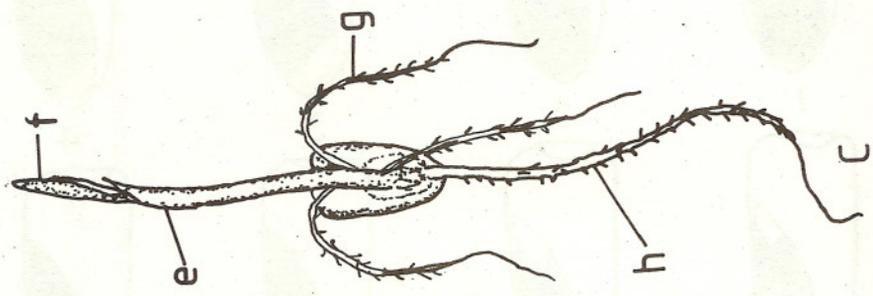
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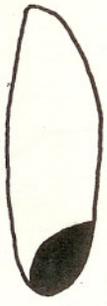
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14.3a



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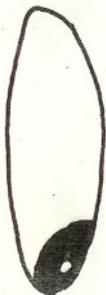
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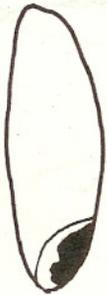
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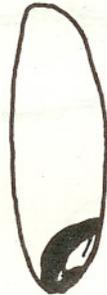
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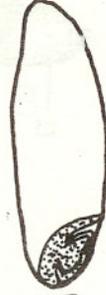


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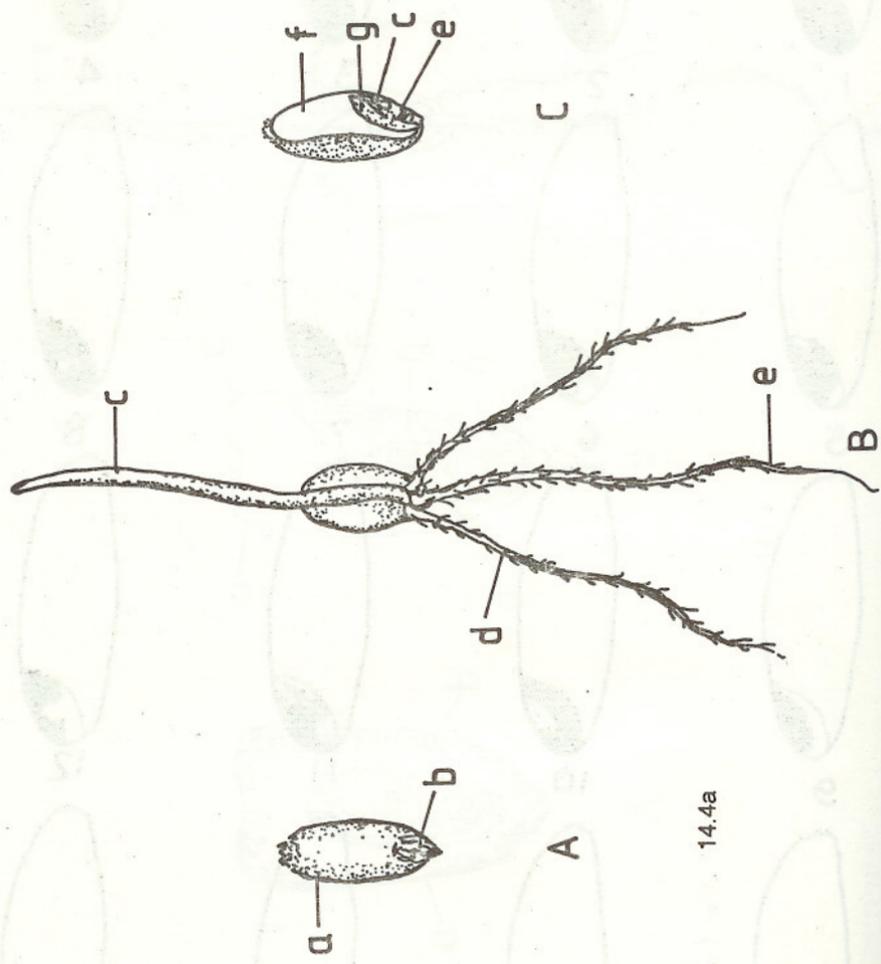
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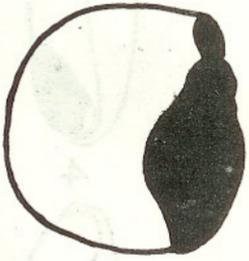
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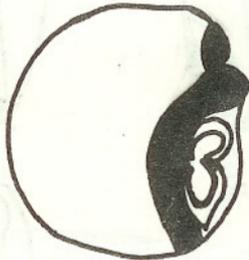
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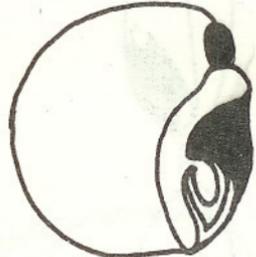
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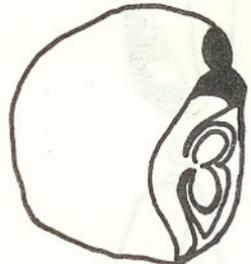
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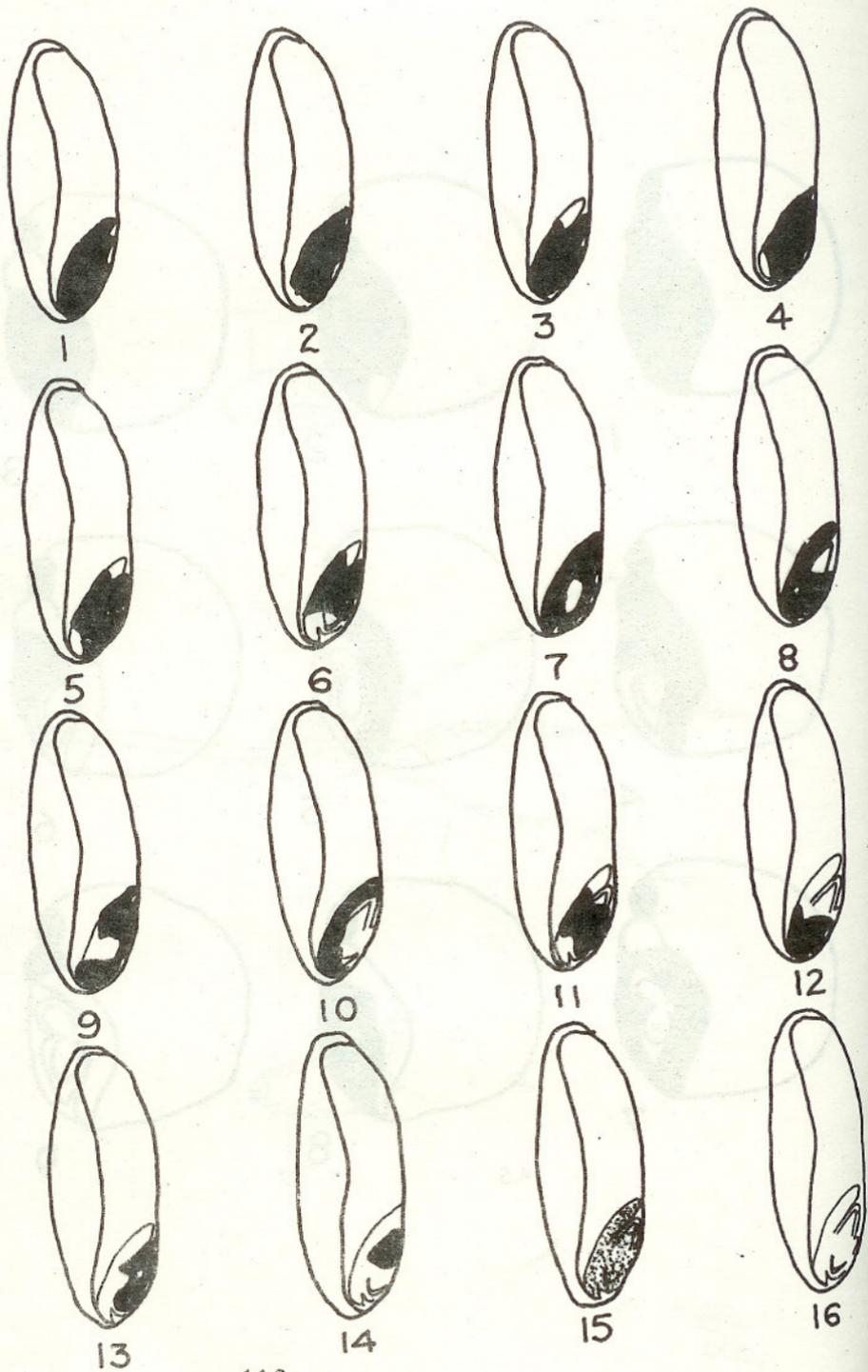


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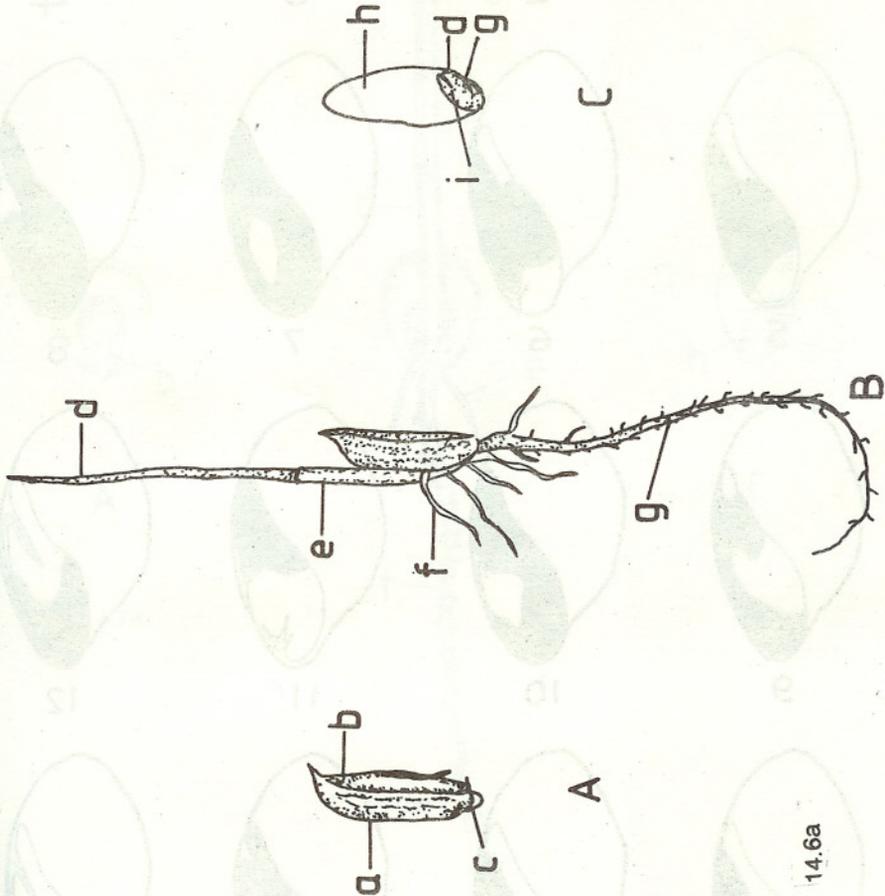


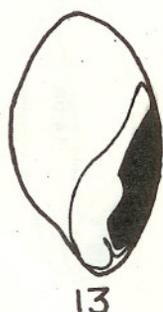
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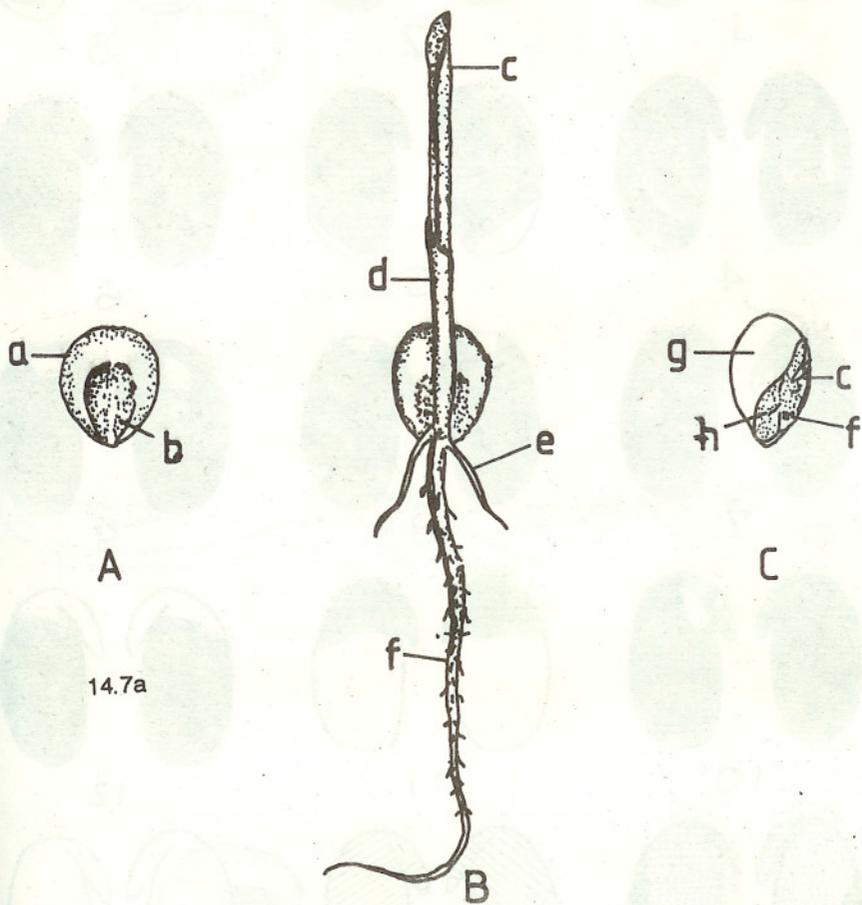


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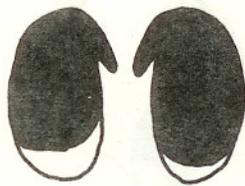
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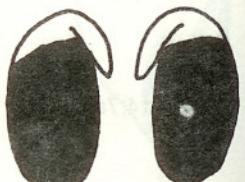
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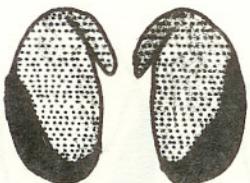
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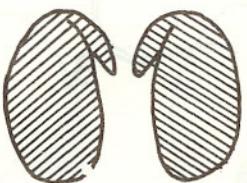
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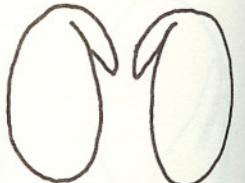
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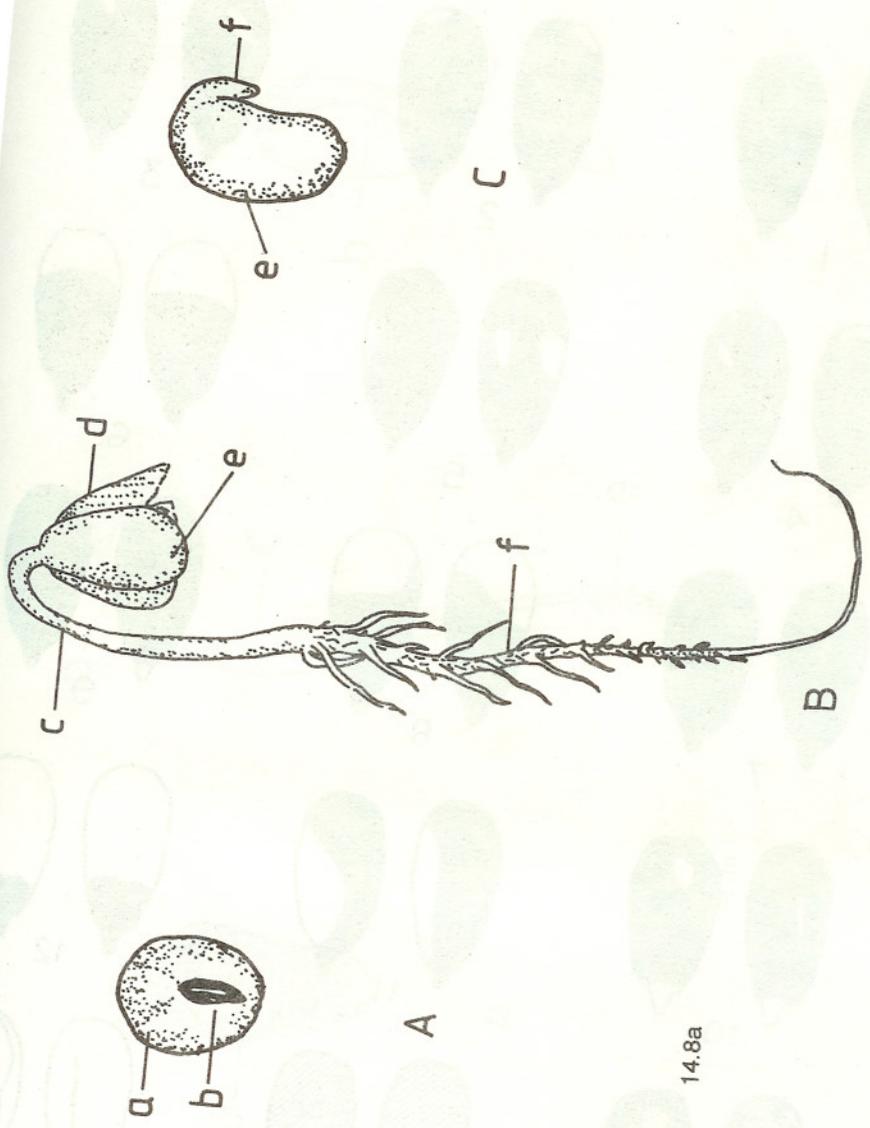


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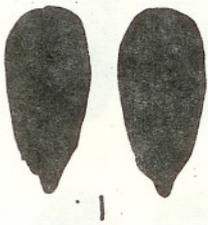


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14.8



14.8a



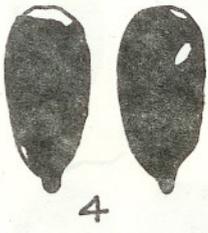
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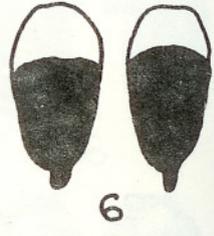
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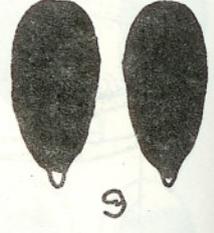
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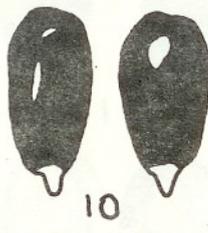
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11



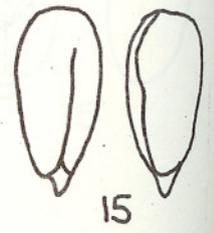
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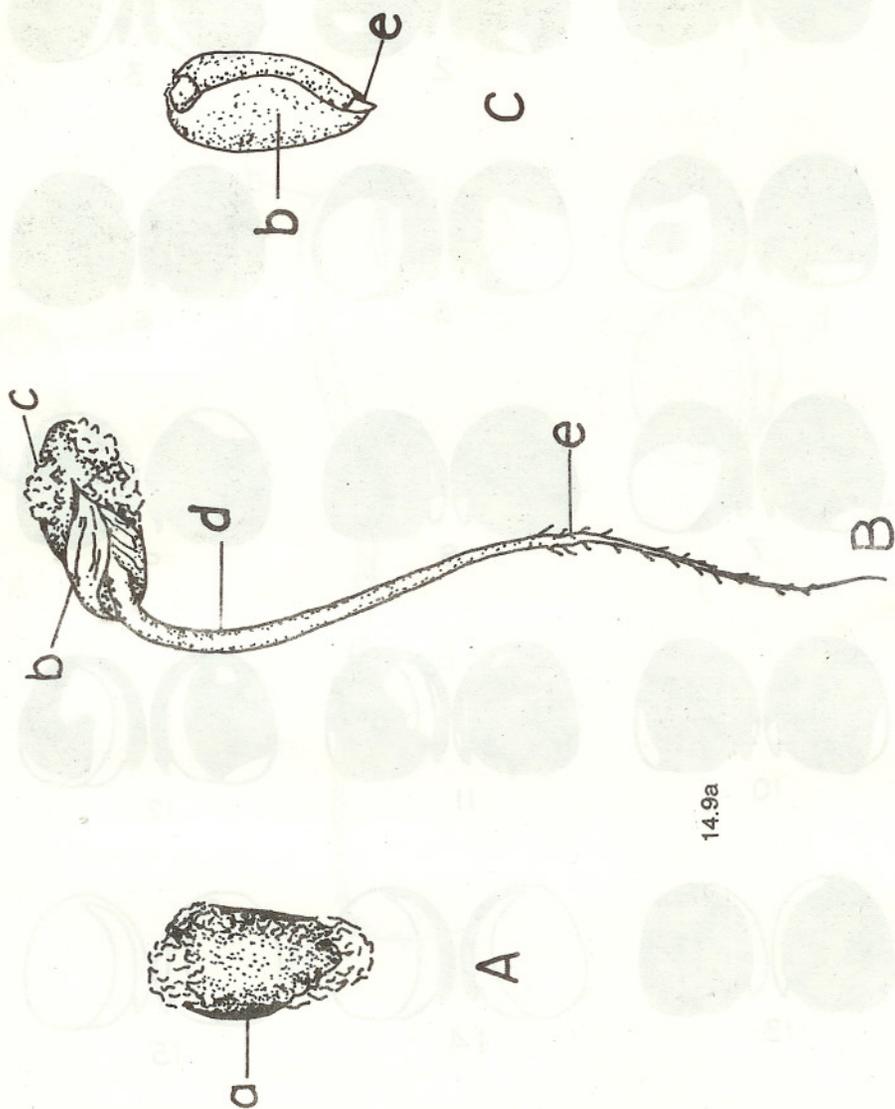


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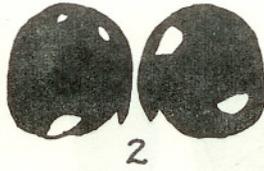
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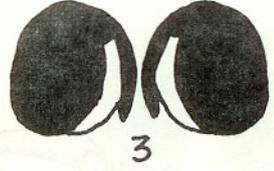
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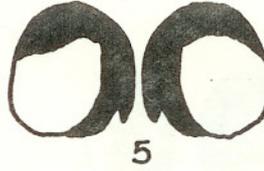
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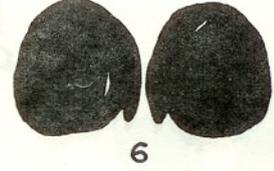
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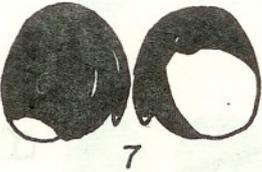
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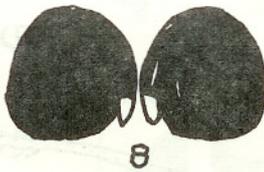
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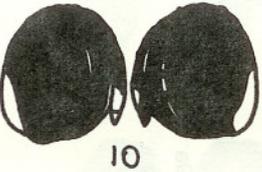
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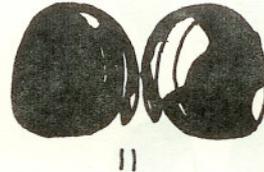
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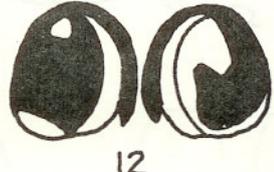
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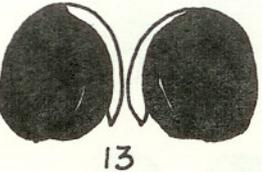
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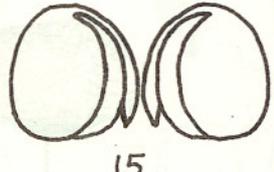
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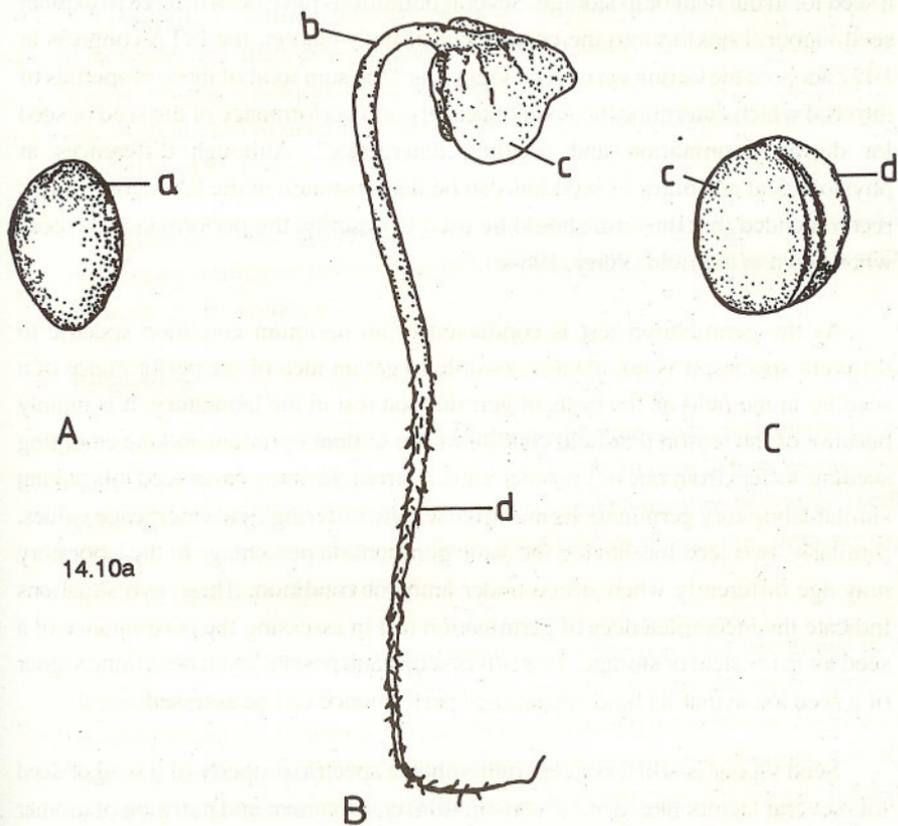


14



15

14.10



14.10a