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The control of seed germination in *Trollius ledebouri*: The breaking of dormancy

A. Hepher* and J.A. Roberts
Department of Physiology and Environmental Science, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, Leics, LE12 5RD, UK

Abstract. Seeds of *Trollius ledebouri* exhibit low germination when maintained on moistened filter paper. Dormancy can be overcome by the application of gibberellins A_4+A_7 or by testa removal. Germination is characterised by a change in the anatomy of the seed and by specific alterations in the protein complement of the endosperm tissue. These anatomical and biochemical changes are also exhibited by isolated endosperm tissue maintained in the absence of the embryo. The observations described are discussed in relation to the interaction between the endosperm and the embryo in the control of seed germination in *T. ledebouri*.

Key words: Dormancy (seed) – Endosperm breakdown – Germination (seed) – Gibberellin and seed dormancy – *Trollius*.

Introduction

Studies of seed dormancy primarily center upon the regulation of germination of weed species, and species that have come under cultivation in relatively recent times, such as certain trees and ornamentals. Additional to the commercial implications of these investigations, the results from such research can furnish valuable information at a more fundamental level. The transition from a quiesent seed, to a vigorously growing seedling is the most dramatic event of a plants life cycle. That such a developmental change may be arrested in dormant seeds and yet the capacity for germination retained, provides a mechanism for studying the regulation of the germination process itself.

The *Trollius* genus is a member of the Ranunculaceae and consists of 20 species of perennial herbs found in Europe, Asia and North America (Royal Horticultural Society 1951). Problems have been encountered in germinating *Trollius* sp. for many years (Anon. 1941), and its dormancy remains the only factor restricting its commercial potential.

This paper describes the process of germination in *Trollius ledebouri* and how it can be stimulated, and examines the interaction between the embryo and the endosperm during germination.

Material and methods

Plant material. Seeds of *Trollius ledebouri* cv. Golden Queen (hereafter referred to as *Trollius*) were obtained from Colegrave Seeds Limited, Banbury, Oxon, UK, and stored in an atmosphere of 30–35% relative humidity at 4°C.

Standard germination procedure. Seeds were arranged in 5·5 arrays on one layer of Whatman 182 germination paper (Whatman, Springfield Mill, Kent, UK) in plastic Petri dishes to which 5 ml distilled water had been added. The dishes were then stored in plastic boxes containing wet blotting paper to reduce evaporative losses. The boxes were maintained at 20°C in constant light from white fluorescent lamps (5 μmol , $m^{-2}s^{-1}$). Scoring of germination was carried out over a period of 20 d and both testa rupture and radicle protrusion recorded.

Gibberellin treatment. A mixture of the gibberellins (GAs) A_4 and A_7 was obtained from ICI, Jealotts Hill Research Station, Bracknell, Berks., UK. This was dissolved in a minimum quantity of 100% ethanol before diluting to final concentration with distilled water. The pH was then adjusted to 5.5 with 0.1 M sodium hydroxide. A solution of 10^{-4}M $GA_{4+7}$was found to be the highest aqueous concentration attainable without subsequent reprecipitation.

Seeds were placed for 24 h on one sheet of Whatman No. 1 paper (12.5 cm) to which 4 ml GA_{4+7} solution had been added. These were blotted dry and transferred to Petri dishes

* *Present address:* Department of Botany, University of Durham, Durham DH1 3LE, UK

Abbreviation: GA = gibberellin

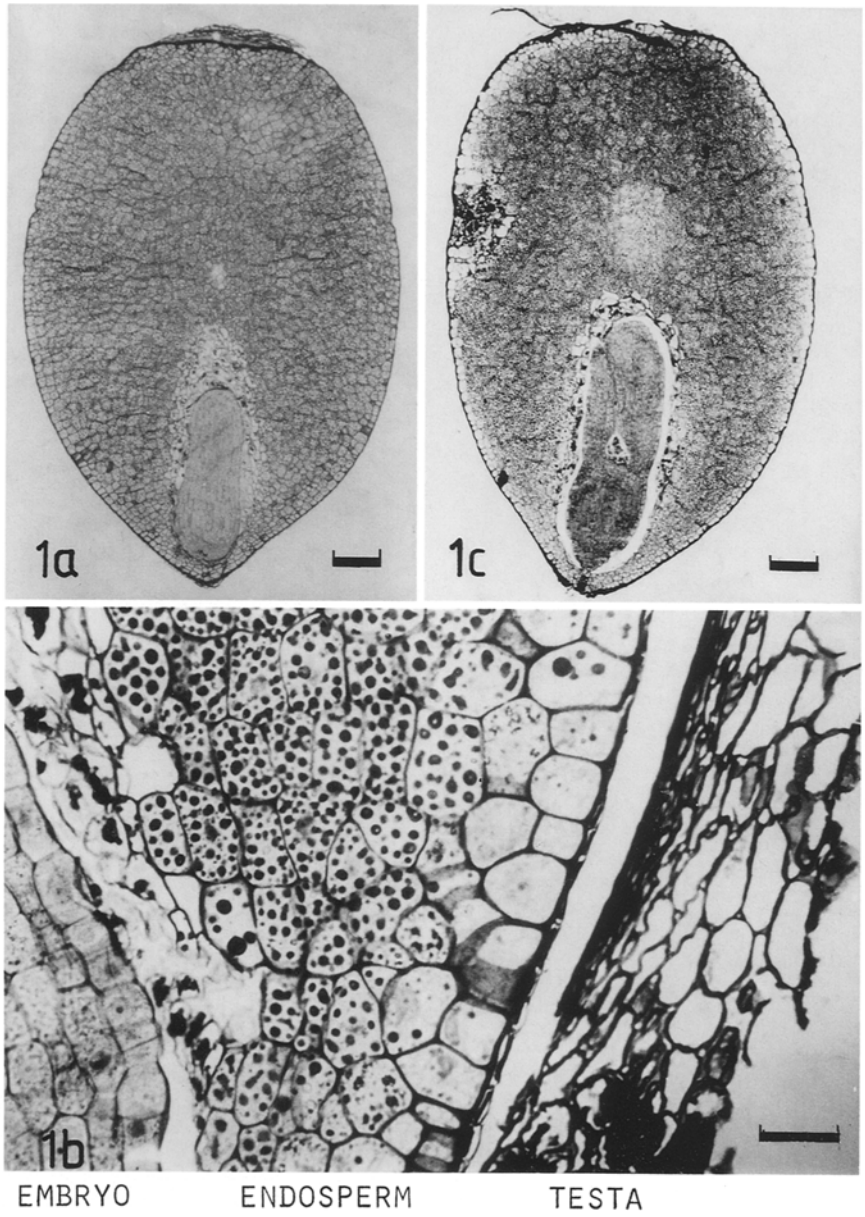

Fig. 1 a-c. Light micrographs of *Trollius* seed tissue. Seeds were soaked in water for 1 d (**a**) and 97 d (**c**) before being fixed (after testa removal to aid resin penetration), sectioned and stained with Coomassie blue and counterstained with toluidine blue. ×33; bar = 200 µm. **b** Light micrograph of part of an intact seed (imbibed for 1 d) showing the embryo, endosperm and testa tissue. Section stained with Coomassie blue and counterstained with toluidine blue. ×300; bar = 50 µm

containing moistened Whatman 182 filter paper as described previously.

Testas and embryos were removed with the aid of a dissecting microscope at a combined magnification of ×35. A minimum of 18 h imbibition at 20° C was found necessary to ensure consistently successful removal of the testa.

Seed anatomy. For examination of the fine structure of *Trollius* seed, testas were generally removed before fixing to aid resin penetration. They were then fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 48 h at room temperature and dehydrated in an alcohol series. Tissue was infiltrated with "LR white" resin (medium grade; London Resin Co., Basingstoke, Hampshire, UK) on a clinostat for three weeks at 20° C. The resin was changed twice during the first 24 h and fresh resin was used for polymerisation. Infiltrated seeds were placed separately into size No. 1 gelatin capsules (Agar Aids, Stansted, Essex, UK) and polymerised for 22 h at 60° C. Sections, 2.5 µm thick were cut on an ultramicrotome (O_mU_2; Reichert-Jung, Slough, UK), stained with Coomassie blue (7% acetic acid, 40% methanol, 0.1% Coomassie brilliant blue R) and counterstained with 0.1% toluidine blue (aqueous) to visualise both cell walls and any proteinaceous material. Observations and photographs were recorded using a photomicroscope II (Carl Zeiss, Oberkochen, FRG).

Germination time courses. Seven stages of germination and seedling establishment were defined: dormant; testa rupture (TR); advanced testa rupture; radicle protrusion (RP); advanced radicle protrusion (RP+); hypocotyl elongation (HE); and advanced hypocotyl elongation (HE+). Seeds previously soaked for 3 d in distilled water were placed on filter paper containing 10^{-4} M GA_{4+7} and sampled after 2 d and thereafter when the stages TR, RP, RP+, HE and HE+ were prevalent (3,

5, 6, 8 and 10 d, respectively). In addition, seeds were sampled immediately before GA treatment (time 0). On each sampling date the embryos of 50 seeds were excised and their lengths measured and five replicates of five endosperms from seeds at the requisite developmental stage weighed fresh and after 2 d at 98° C. The protein content of five replicates of five endosperms was also measured using the method of Lowry et al. (1951) and compared with a standard curve from 0 to 250 $\mu g \cdot ml^{-1}$ bovine serum albumin (BSA) prepared with the same reagents at the same time.

Statistical note. All measurements of endosperm length were log-transformed to account for non-homogenous errors and subjected to an analysis of variance. The value at "Time 0" from the table of means was subtracted from subsequent data and $\sqrt{2}\times$ the standard error of the difference for within treatments used as the error term.

Results

Seed anatomy. In the unimbibed state each *Trollius* seed weighed approx. 1.3 mg and was approx. 1.5 mm long and 1.0 mm wide. Imbibition was complete after 18 h, by which time fresh weight had increased by 40% (Hepher 1983). A median section of a freshly imbibed seed (Fig. 1 a) showed a small, rudimentary embryo at the micropylar end, surrounded by endosperm tissue. After staining with Coomassie and toluidine blue the endosperm was seen to be composed of cells containing large numbers of protein bodies (Fig. 1 b). Additionally the endosperm stained black with Sudan IV, indicating the presence of lipid, and gave the positive red formazan staining reaction with triphenyl tetrazolium chloride characteristic of living tissue (Mackey 1972). The testa was a deeply pigmented, multi-layered structure and was occasionally incomplete at the micropylar end of the seed.

Seed germination. When *Trollius* seeds were placed on moistened filter paper germination was slow with 18% germination recorded after 190 d at 20° C (Fig. 2). Although the majority of seeds did not germinate, they were not totally quiescent since embryo growth occurred in the absence of germination (Fig. 3). This increase in size and differentiation of the embryo could be seen in sections taken from seeds after prolonged soaking (Fig. 1 c), in which protein-body depletion in the outer cells of the endosperm was also evident.

Germination of *Trollius* seed was stimulated by testa removal or treatment with GA_{4+7} (Fig. 4). Removal of the testa resulted in some germination after 4 d, and this steadily increased to 90% after 30 d. The majority of the remaining seeds showed signs of fungal contamination. Seeds exposed to

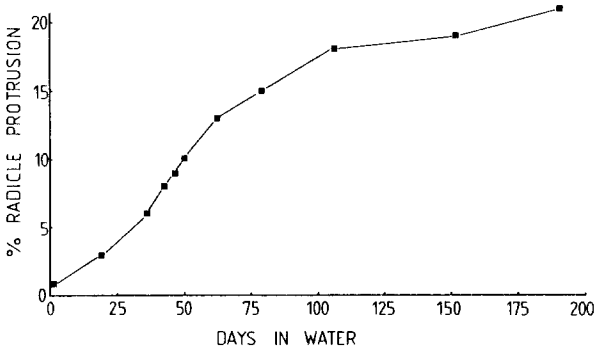

Fig. 2. Germination of *Trollius* seeds maintained on moistened filter paper

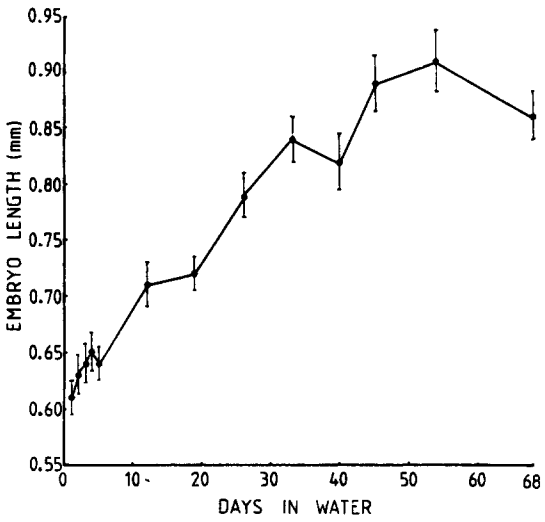

Fig. 3. Embryo growth of *Trollius* seeds maintained on moistened filter paper

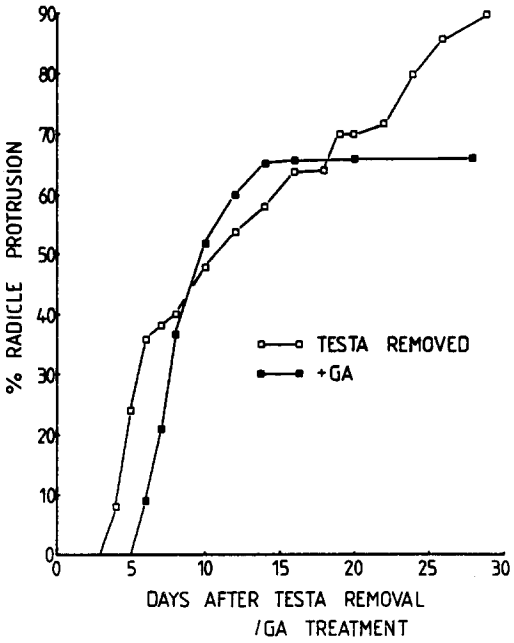

Fig. 4. Germination of *Trollius* seeds in the presence of 10^{-4} M GA_{4+7} or after testa removal

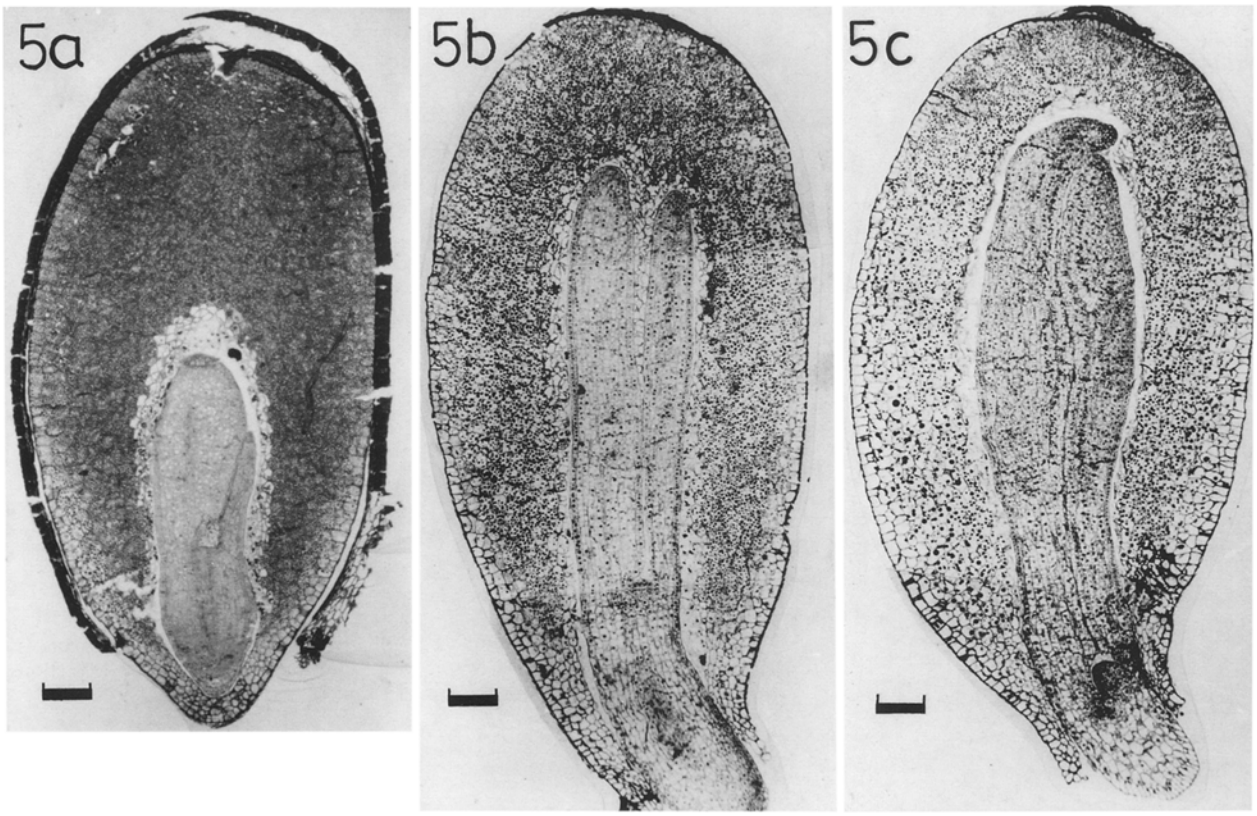

Fig. 5a-c. Light micrographs of *Trollius* seeds after GA treatment. Sections stained with Coomassie blue and counterstained with toluidine blue. Stages shown are **a** testa rupture, **b** radicle protrusion (testa removed prior to fixing), and **c** advanced radicle protrusion (testa removed prior to fixing). ×33; bar = 200 μm

GA_{4+7} exhibited radicle protrusion 6 d after treatment and germination attained a plateau of approx. 65% within 14 d.

Following treatment with GA_{4+7} the first sign of germination in *Trollius* was a rupture of the testa at the micropylar end of the seed (Fig. 5a). This was correlated with an increase in endosperm fresh weight of approx. 35% (Fig. 6) although the dry weight of the tissue remained constant. Concomitant with the swelling of the endosperm was the enlargement of the embryo, which came to occupy the space left by the hydrolysis of the storage tissue. This process continued with the development of the vascular system, the appearance of a well-defined epidermis and an increasing degree of organisation of the shoot apex (Fig. 5b, c). After testa rupture, endosperm dry weight began to fall while the protein content of the endosperm decreased steadily throughout germination (Fig. 6).

Studies on isolated endosperm tissue. All endospermic changes described so far took place during germination of intact seeds. The role of the endosperm in the dormancy of *Trollius* was first examined by studying the changes in endosperm tissue isolated from the embryo, in the presence or absence of GA_{4+7}. Seeds were imbibed in sterile distilled water for 24 h, after which time both the testa and the embryo were removed. The isolated endosperm tissue was then incubated in either sterile distilled water or 10^{-4} M GA_{4+7} for 2 or 4 d at 20° C prior to sectioning. In all sections examined, protein-body depletion was apparent in the area adjacent to the original position of the embryo and in the peripheral region of the endosperm (Fig. 7). These observations were similar to those seen during germination of intact seeds. Although this breakdown appeared to be accelerated by the presence of GA_{4+7}, no qualitative differences were apparent between gibberellin-treated endosperm and controls in water. The enhanced breakdown of endosperm in the presence of gibberellin was also demonstrated by measurement of the long axis of the tissue after excision. Increase in endosperm length was significantly greater over controls in water at all dates tested after 2 d from testa and embryo removal (Fig. 8).

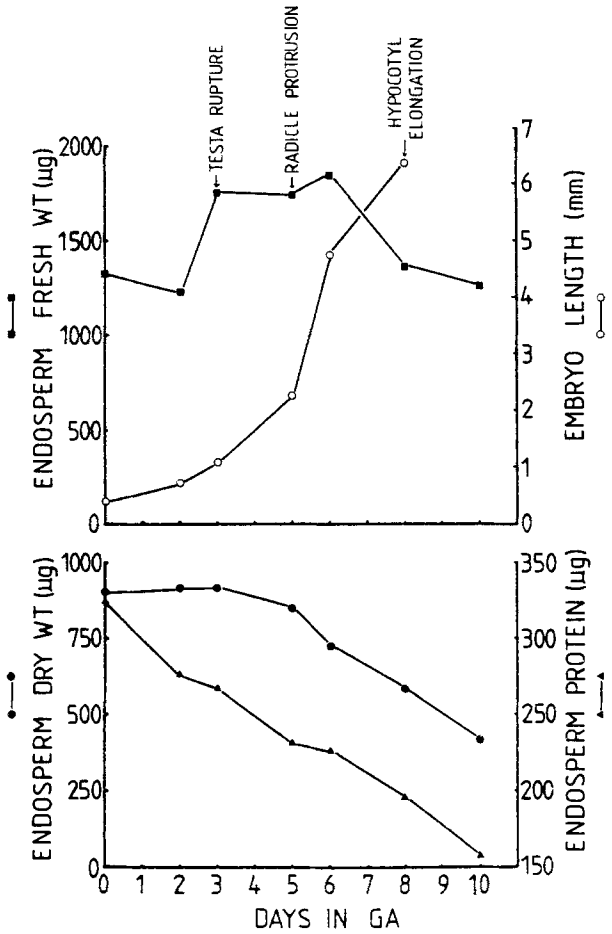

Fig. 6. Time course of endosperm fresh weight, dry weight, protein content and embryo growth of *Trollius* seeds after treatment with 10^{-4} M GA_{4+7}

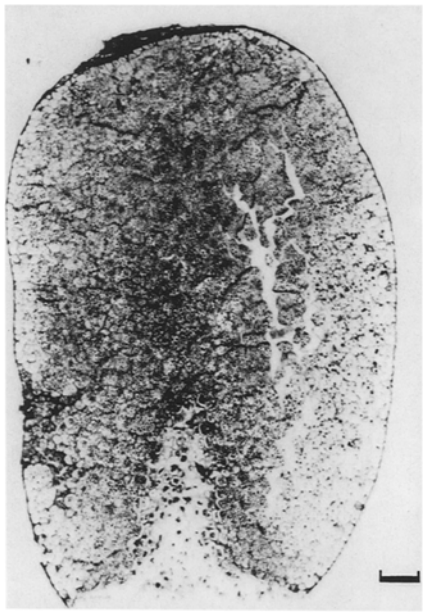

Fig. 7. Light micrograph of an isolated endosperm after 4 d incubation in water from testa and embryo removal. Sections stained with Coomassie blue and toluidine blue. ×25; bar = 200 µm

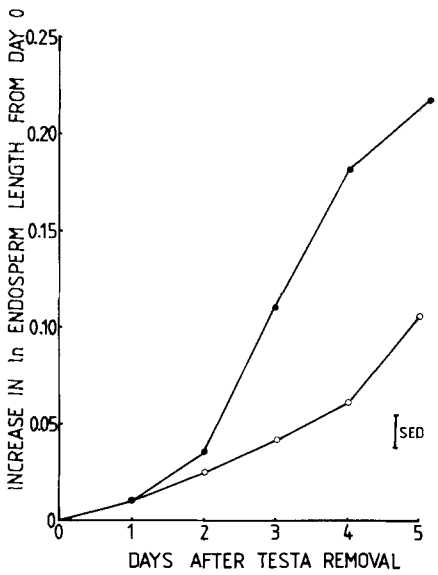

Fig. 8. Cumulative increase in length of isolated endosperm tissue soaked in either water (○) or 10^{-4} GA_{4+7}(●). Seeds were imbibed for 1 d prior to testa and embryo removal. *Bar* represents standard error of the difference for within treatments

Discussion

Seeds of *T. ledebouri* have a small rudimentary embryo, surrounded by a living protein-and-lipid endosperm which occupies the bulk of the seed. This organisation is typical of the Ranunculaceae (Atwater 1980).

Germination of *T. ledebouri* is stimulated by the application of GA_{4+7}. Promotion of germination in a closely related species, *T. europaeus*, has been previously reported using $3{\cdot}10^{-3}$ and $3{\cdot}10^{-4}$ M concentrations of an unspecified gibberellin, presumably GA_3 (Kallio and Piiroinen 1959). In the same study, acid scarification of the seeds prior to GA application increased the germination response and this was assumed to have occurred as a result of increased GA uptake. No attempt was made to investigate this observation in our study, as it was found that testa removal per se was sufficient to stimulate germination.

Germination of *Trollius* is characterised by the endosperm absorbing water, presumably as a consequence of reserve breakdown, and subsequently rupturing the testa. At the same time the rudimentary embryo enlarges and occupies the space left by the degraded endosperm tissue. This process continues after radicle protrusion until the

endosperm has been completely absorbed and the remaining testa shed from the cotyledons. During this period the protein bodies in the endosperm swell and coalesce before being degraded, an observation reported for many other species (Ashton 1976). Changes in the protein complement of the endosperm have also been assessed (qualitatively) by gel-electrophoresis analysis of the tissue (Hepher 1983). In addition to the general trend of a decline in endospermic polypeptides during germination, increases in the intensity of certain bands were also apparent. These latter changes may be the consequence of protein breakdown or they might represent the synthesis of enzymes involved in the degradation of the storage reserves. The pattern of endospermic protein breakdown was similar when germination was promoted by either testa removal or application of GA_{4+7}.

Endosperm breakdown also occurs in the absence of the embryo. This is apparent from both the anatomical studies presented in this paper and sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the endosperm proteins (Hepher 1983). Although this process may be accelerated in the presence of GA, no qualitative differences are observed between GA-treated and untreated isolated endosperm tissue. The anatomical and biochemical changes observed in isolated endosperm tissue are similar to those that take place during the germination process. This finding is important since artefactual changes have been reported to occur in seed components studied in isolation (Khan et al. 1972; Ford et al. 1976).

The autonomous breakdown of isolated endosperm tissue has been reported in several other species including lettuce (Halmer and Bewley 1979) and the well-studied castor-bean system (Canvin and Beevers 1961; Huang and Beevers 1974). Additionally, endosperm tissue isolated from celery seeds which are structurally similar to *Trollius*, having a small eccentric embryo surrounded by a living protein-and-lipid endosperm, has also been shown to break down, but, in contrast to *Trollius*, only in the presence of exogenous GA (Jacobsen et al. 1976; Jacobsen and Pressman 1979).

A number of hypotheses that might account for the dormancy of *Trollius* seed have been investigated. Embryo excised from dormant seeds and cultured in vitro rapidly expanded and differentiated into apparently normal plantlets (Hepher 1983) and thus the seed dormancy exhibited by *Trollius* is unlikely to be a consequence of embryo dormancy such has been reported for apple (Come 1980/81). Equally, impermeability of the testa to either water or oxygen can be discounted as imbibition is complete within 18 h and seeds soaked in hydrogen-peroxide solution or imbibed in elevated levels of atmospheric oxygen remained dormant (Hepher 1983).

One hypothesis that would account for the observations presented in this paper is that the seed contains a germination inhibitor, the loss of which is prevented or retarded by the testa and whose action is overcome by gibberellin treatment. Such an indirect inhibitory role for the seed coat has been suggested for *Avena fatua* (Black 1959) and Charlock (Edwards 1968). If this hypothesis is correct, the question remains as to the site of synthesis and action of the putative inhibitor. Although the embryo is an obvious candidate, and considerable work has been carried out investigating "embryo dormancy" in many species, the independence of endosperm breakdown in *Trollius* indicates that the capacity of the storage tissue to degrade itself could have a crucial role in the regulation of dormancy in this seed. In the subsequent paper, the nature of this role is examined and the relationship between the embryo and the living endosperm in the control of germination in *Trollius* developed with a view to producing an intergrated model for the control of seed dormancy in this species.

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