A cytochemical investigations of dry and germinating *Iris pseudoacorus* seeds

B. GABARA and R. MODRZEJEWSKI

Laboratory of Plant Cytology and Cytochemistry, Institute of Biochemistry and Physiology,
University of Łódź, Łódź, Nowopółudniowa 12/16, Poland.

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Abstract:

The composition and distribution of storage substances such as proteins, lipids, carbohydrates and phosphates and also some enzymes in dry *Iris pseudoacorus* endosperm and their changes during germination were investigated with light microscope.

INTRODUCTION

Recently, many workers have applied various biochemical, cytological and physiological methods to determine seed components and their changes during germination (Horner and Arnott, 1966; Engleman, 1966; Smith and Flinn, 1967; Paulson and Srivastava, 1968; Srivastava and Paulson, 1968).

The chemical composition of maturing and dry *Iris pseudoacorus* endosperm has been studied cytochemically (Olszewska et al. 1968) and biochemically (Konopska, 1969).

The purpose of this work was to determine the composition and localization of the reserve substances such as proteins, phosphates, lipids, carbohydrates and enzymes in dry *Iris pseudoacorus* endosperm and their changes during germination.

MATERIAL AND METHODS

Dry and germinating *Iris pseudoacorus* seeds (germination period 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 15 days) were used as material. Fresh hand sections were made from the dry and germinating seeds. Cytochemical and cytoenzymatical tests were carried out as in table 1.

Table 1
Cytochemical and cytoenzymatical test methods

Component	Fixative	Method	Control
Total proteins ¹	Carnoy	Mercuric-bromphenol blue	pepsin extraction
Protein bound NH ₂ ¹	ethanol 70%	Ninhydrine-Schiff	pepsin extraction
Albumins and globulins ⁵	Carnoy	Mercuric bromphenol blue	1 M NaCl in phosphate buffer, pH 7.3
Inorganic phosphates ¹	Fo-Ca Baker	Molybdate methods I and II	—
insoluble ³	Fo-Ca Baker F-NaOH with Mg ⁺⁺ and Ca ⁺⁺	Lead nitrate method	citrate buffer extraction
soluble ³		Lead nitrate method	citrate buffer extraction
Inorganic polyphosphates ²	Carnoy	Toluidine blue	TCA 10%
Neutral lipids ¹	Fo-Ca Baker McManus	Sudan III and IV Oil Red 0 Nile blue	pyridin extraction
Polysaccharides ⁴ acid and neutral	Carnoy	PAS with Jensen extraction	acetylation and deacetylation after Mc Manus and Cason ¹
Starch ¹	Carnoy	PAS iodine-potassium iodide	—
Acid phosphatase ¹	Fo-Ca Baker, unfixed	Lead nitrate method Coupling azo dye technique, Fast Blue RR salt	medium without β -glycerophosphate 10^{-2} M NaF
Esterase ¹	unfixed	Indoxyl acetate method	10^{-2} M NaF
Succinic dehydrogenase	unfixed	Tetrazolium Nitro BT	water 90°C

1. Pearse, 1961; 2. Keck and Stick, 1957; 3. Poux, 1965 a; 4. Jensen, 1962; 5. Konopska, 1969. Moreover, a polarization equipment (Zeiss Jena) was applied.

OBSERVATIONS

Iris pseudoacorus seeds are composed of an external brown pigmented seed coat surrounding endosperm tissue in which the embryo is embedded. (Fig. 1. in text).

Dry seeds. In mature dry *Iris* seeds, the endosperm cells are filled with a large amount of oval bodies (2.5—17 μ in diameter), staining with mercuric-bromphenol blue. This indicates that they are protein bodies. Two types of protein bodies: simple ones-amorphous in structure (Pl I, fig. 1.) and complex ones containing inclusions embedded in the amorphous substance are visible in the endosperm of *Iris* seeds. Among

the complex protein bodies, the following may be distinguished: a) protein bodies with a small light zone ($0.3\text{--}0.7\ \mu$ in diameter) usually known as globoid (Pl. I, fig. 2), b) protein bodies with a large globoid ($0.5\text{--}3\ \mu$ in diameter); sometimes, small globoids are visible near the large one (Pl. I, fig. 3); c) protein bodies with many small globoids (Pl. I, fig. 4). Some protein bodies contain as much as 20 globoids. None of the above mentioned inclusions are birefringent under polarized light.

Fig. 1. Schematic representation of longitudinal section of dry *Iris pseudoacorus* seed

The simple and the first two types of complex protein bodies occur most frequently in the endosperm cells adjacent to the seed coat. In the deeper layers of the *Iris* seeds there are only complex protein bodies with numerous globoids (Fig. 2 in text).

After pepsin digestion, the contents of the protein bodies disappears (Pl. I, fig. 5) and the limiting membrane is visible. Similar pictures are obtained after extraction with 1 M NaCl (Pl. I, fig. 6, 7, 8). During the action of 1 M NaCl the swelling of globoids may also be observed (Pl. I, fig. 7). The sensitivity of all protein bodies types to 1 M NaCl indicates that their amorphous substance contains albumins and globulins, i.e. simple proteins.

All the protein bodies forms described above give a positive reaction with Ninhydrine-Schiff. This shows that these proteins contain free NH_2 groups.

Protein bodies give a positive reaction to inorganic phosphates. The reaction product is localized in the globoids only (Pl. I, fig. 9, 11) after Fo-Ca Baker fixation. This fixative dissolves soluble phosphates in the

Fig. 2. Schematic representation of localization of all protein bodies types in endosperm cells of *Iris pseudoacorus* seed

protein bodies amorphous substance. However, after fixation in F-NaOH with Mg^{++} and Ca^{++} , these phosphates may be observed not only in the globoids but also in the amorphous substance (Pl. I, fig. 10, 12).

In the globoids the Keck and Stick reaction demonstrates the presence of inorganic polyphosphates (Pl. I, fig. 13).

Staining with Oil Red O or Sudan III and IV reveals the presence of lipids in the endosperm of *Iris* seeds. The lipids constitute some kind of coat enveloping the protein bodies (Pl. II, fig. 1). After McManus fixation some of the proteins are dissolved and the fats take form of the typical lipid droplets (Pl. II, fig. 2). The lipids in the *Iris* seed cells are stained pink with Nile blue, this indicates their neutral character.

In spite of many tests, no starch was observed in the endosperm of *Iris* seeds.

Acid phosphatase activity is localized in the globoids of protein bodies (Pl. II, fig. 4, 5). Moreover, this enzyme is present in the cytoplasmic granules (0.3—0.8 μ in diameter) which are probably spherosomes. These granules are accumulated in the cytoplasm around the protein bodies (Pl. II, fig. 4). The spherosomes are also characterized by indoxyl esterase activity (Pl. II, fig. 6, 7). This enzyme is present in the larger vesicles (1.5—2.5 μ in diameter) as well. The reaction product accumulates in them in form of crescents (Pl. II, fig. 6). Moreover, numerous plasmodesmata (Pl. II, fig. 7) demonstrate intensive indoxyl esterase activity, especially in the endosperm cells adjacent to the seed coat. Here, the accumulation of the reaction product is so great that whole region of the middle lamella becomes indigo in colour (Pl. II, fig. 8).

The few spherical mitochondria (0.2—0.3 μ in diameter) show a positive reaction to succinic dehydrogenase (Pl. II, fig. 3).

The endosperm cell wall of mature *Iris* seeds is very thick. It has a large number of simple pits and demonstrates the presence of PAS positive substances (Pl. II, fig. 9). After extraction with 0.5% ammonium oxalate a large layer of hemicellulose may be seen (Pl. II, fig. 10). A thin layer of cellulose is visible after the removal of the hemicellulose (Pl. II, fig. 11). Therefore, the endosperm cell wall is especially rich in hemicellulose with a slight addition of pectin substances.

Germinating seeds. During germination of *Iris pseudoacorus* seeds very distinct changes take place in the protein bodies. On the first day after soaking the swelling of globoids may be seen (Pl. III, fig. 1, 2). Throughout the germination of the seeds, the swelling of globoids increases (Pl. III, fig. 3). Parallel to the swelling of globoids the digestion of amorphous protein takes place. The consecutive stages of this process in all protein bodies types of *Iris* endosperm are presented in plate III and in Fig. 3 (in text).

As the amorphous protein disappears, the protein bodies limiting membrane becomes visible (Pl. III, fig. 4, 5, 6, 7). In the case of the second

EXPLICATIONS OF FIGURES

Plate I

Protein bodies in endosperm cells of dry *Iris pseudoacorus* seeds. ($\times 1200$; fig. 11, 12 $\times 2400$)

Fig. 1—8. Carnoy fixed, Bromphenol blue stained

Fig. 1. protein bodies amorphous in structure

Fig. 2. protein bodies with small globoid

Fig. 3. protein bodies with large globoid. Note a few protein bodies with small globoid adjacent to larger one

Fig. 4. protein bodies with many small globoids embedded in amorphous protein

Fig. 5. protein extraction. Note many limiting membranes of protein bodies

Fig. 6—8. 1 M NaCl digestion. The limiting membrane and disappearance of amorphous protein and inclusion of protein bodies may be seen

Fig. 9, 11. Fo-Ca Baker fixed. Insoluble inorganic phosphates in globoids

Fig. 10, 12. F-NaOH with Mg and Ca fixed. Soluble inorganic phosphates in amorphous protein

Fig. 13. Carnoy fixed. Inorganic polyphosphates in the globoids of protein bodies

Plate II

Endosperm cells of dry *Iris pseudoacorus* seeds. ($\times 1200$)

Fig. 1. Fo-Ca Baker fixed. Oil Red O stained. Lipids coating the protein bodies

Fig. 2. McManus fixed. Oil Red O stained. Protein bodies are dissolved and lipids appear in form of droplets

Fig. 3. mat. unfixed. Succinic dehydrogenase activity in only a few mitochondria

Fig. 4—5. mat. unfixed. Acid phosphatase activity inside globoids and in numerous spherosomes around protein bodies

Fig. 6—7. mat. unfixed. Indoxyl esterase in spherosomes and in plasmodesmata

Fig. 8. mat. unfixed. Indoxyl esterase activity in distinctly seen in plasmodesmata of the first layer of endosperm cells

Fig. 9. Carnoy fixed. PAS (control section) $\times 600$

Fig. 10. Carnoy fixed. PAS (0.5% oxalate ammonium extraction) $\times 600$

Fig. 11. Carnoy fixed. PAS (17% NaOH extraction) $\times 600$

Plate I

Plate II

Plate III

Plate IV

Plate V

Plate III

Changes in protein bodies during germination of *Iris pseudoacorus* seeds. Carnoy fixed, Bromphenol blue stained. $\times 1200$

- Fig. 1—2. first day of germination
- Fig. 3. second day of germination
- Fig. 4—5. third day of germination
- Fig. 6. fourth day of germination
- Fig. 7. fifth day of germination
- Fig. 8. sixth day of germination
- Fig. 9. eighth day of germination
- Fig. 10. tenth day of germination
- Fig. 11. twelfth day of germination
- Fig. 12. fifteenth day of germination

Plate IV

Endosperm cells of germinating *Iris pseudoacorus* seeds. $\times 1200$.

Fig. 1—4. F-NaOH with Mg and Ca fixed, inorganic phosphates, Fig. 7—11. Fo-Ca Baker fixed, Oil Red O stained

- Fig. 1. second day of germination
- Fig. 2. fifth day of germination
- Fig. 3. sixth day of germination
- Fig. 4. eighth day of germination
- Fig. 5. Carnoy fixed, inorganic polyphosphates, third day of germination
- Fig. 6. Fo-Ca Baker fixed. Sudan III and IV stained, third day of germination
- Fig. 7—8. fifth day of germination
- Fig. 9. sixth day of germination
- Fig. 10. eighth day of germination
- Fig. 11. tenth day of germination

Plate V

Changes in enzyme activity during germination of *Iris pseudoacorus* seeds; mat. unfixed. ($\times 1200$)

- Fig. 1. succinic dehydrogenase activity, first day of germination
- Fig. 2. succinic dehydrogenase activity, third day of germination
- Fig. 3. Acid phosphatase activity, third day of germination
- Fig. 4. Acid phosphatase activity, eighth day of germination
- Fig. 5. Acid phosphatase activity, tenth day of germination
- Fig. 6. Indoxyl esterase activity, third day of germination
- Fig. 7. Indoxyl esterase activity, eighth day of germination
- Fig. 8. Indoxyl esterase activity, tenth day of germination

Fig. 3. Schematic representation of changes in all protein bodies types during germination of *Iris pseudoacorus* seed

type of protein bodies, globoids may be observed after almost complete disappearance of amorphous substance (Pl. III, fig. 5, 8). However, in the third type of protein bodies, the digestion of amorphous protein and globoids is nearly simultaneous (Pl. III, fig. 9, 10). In the final period of germination of all protein bodies types only the limiting membrane remains (Pl. III, fig. 12).

The protein bodies digestion process is the most rapid in the endosperm cells adjacent to the developing embryo. However, in the case of the cells near the seed coat, time of the digestion of proteins is various. It would indicate that the proteins disappear first from the small cells.

During germination of *Iris pseudoacorus* seeds, a gradual but distinct decrease in the amount of inorganic phosphates is observed. In the first period they disappear from the amorphous protein (Pl. IV, fig. 1, 2) and later the inorganic phosphates may be seen in the cytoplasm in form of simple granules or groups (Pl. IV, fig. 1, 2, 3, 4). The size of these structures and their distribution suggests that they are globoids freed from digested protein bodies. In the final period of germination the inorganic phosphates are visible in the small spherical structures dispersed in the

cytoplasm (Pl. IV, fig. 4). On the tenth day of germination, these phosphates disappear completely.

During germination the amount of inorganic polyphosphates rapidly decreases (Pl. IV, fig. 5) and on the fifth day there are no polyphosphates in the endosperm cells.

Distinct changes take place in the quantity and distribution of lipids in the process of germination. With the decrease of protein substance, the lipid inclusions become spherical (Pl. IV, fig. 6) and replace the proteins inside the protein bodies limiting membrane (Pl. IV, fig. 7). Small lipid droplets are often observed near fragments of amorphous protein inside this membrane (Pl. IV, fig. 8, 9). When the proteins are completely digested, large lipid droplets appear sometimes inside the limiting membrane (Pl. IV, fig. 10). Moreover, the lipids are present in form of channels around the protein bodies (Pl. IV, fig. 7, 8, 9, 10). These forms of lipids are especially visible after ten days of germination (Pl. IV, fig. 11). Then, single lipid droplets connected to the net of channels around the protein bodies may be observed. On the twelfth day lipids disappear from the endosperm.

Succinic dehydrogenase activity increases during germination (Pl. V, fig. 1) and reaches its maximum on the third day (Pl. V, fig. 2). On the next few days this activity decreases until on the twelfth day when it is practically zero.

In the course of germination the localization of acid phosphatase is changed. In the first period (after three days) the acid phosphatase decreases in the globoids and a marked rise of its activity in the cytoplasm — in the spherosomes (Pl. V, fig. 3) can be observed. During germination process, this acid phosphatase activity disappears (Pl. V, fig. 4, 5) completely after twelve days.

In the first period of germination cytoenzymatical observations reveal increase of indoxyl esterase activity in comparison with dry seeds (Pl. V, fig. 6) and then a gradual decrease of this activity (Pl. V, fig. 7, 8) until it disappears completely.

During germination no changes were observed in the cell wall.

DISCUSSION

Cytochemical investigations show that endosperm cells of *Iris pseudoacorus* seeds contain storage proteins present in form of spherical bodies which are regarded as aleurone grains or protein bodies. The protein bodies are composed of amorphous protein; most of them contain globoids.

Many authors who have investigated protein bodies in the electron microscope, have observed the presence of limiting membrane (Graham et al. 1962; Horner and Arnott, 1966; Öpik, 1966; Poux, 1963) but Bagley et al. (1963), Nougarede (1963), Jones (1969)

denied its existence. Even in the light microscope, the protein bodies limiting membrane can be observed in *Iris pseudoacorus* seeds.

There are not many cytochemical research on the chemical composition of protein bodies (Poux, 1963; 1965a, 1965b).

In *Iris pseudoacorus* the protein bodies are characterized by free NH_2 groups. Sensitivity of their amorphous substance to 1 M NaCl indicates the presence of albumins and globulins. According to Konopska (1969) maturing *Iris* seeds contain globulins, albumins, glutelins and prolamins but in dry seeds there are more glutelins and albumins (80.07 mg) than globulins and prolamins (23.95 mg) per 100 mg of dry weight.

Protein bodies of *Iris* endosperm contain the inorganic phosphates. In coincide with Poux (1965b) the insoluble phosphates are inside the globoids and the soluble ones, previously precipitated during fixation by Ca^{++} and Mg^{++} , in the amorphous substance. Moreover, protein bodies also contain phytine: insoluble salts Ca and Mg phytate in the globoids and soluble K phytate in the amorphous substance (Poux, 1965b).

The cytochemical investigations show the presence of inorganic polyphosphates in globoids of protein bodies of *Iris* seeds. These polyphosphates are seldom described in the higher plants (Eschrich, 1962, 1963; Tewari and Singh, 1964). They are a high energy component and it seems that they accumulate phosphorus and energy which is used in many important processes during seeds germination.

As in the embryo cells of *Lactuca* (Paulson and Srivastava, 1968) the protein bodies of *Iris* vary in size and structure in different parts of the seeds. According to Altschul et al. (1966) the structure and number of aleurone grains may widely vary in different cells. Moreover, various forms of the aleurone grains appear in haploid, diploid or triploid storage tissue.

The protein bodies digestion in endosperm cells of *Iris* begins with the swelling of the amorphous substance and of the globoids. Next, the protein bodies amorphous substance is digested. According to Bagley et al. (1963), Horner and Arnott (1966), Öpik (1966), protein bodies first swell, coalesce and become fragmented. The volume of the protein bodies during germination may increase twofold (Smith and Flinn, 1967). These last authors consider that the next stage of protein bodies digestion is the fusion of the protein bodies into groups to form larger aggregate. Horner and Arnott (1966) distinguish two protein bodies digestion stages — fusion into larger groups which are then digested, and erosion around the edges. In the final stages of germination the protein bodies resemble a sponge structure (Bagley et al., 1963). Öpik (1966) states that the protein bodies then fuse and their membrane become part of the vacuolar one, the disappearance of storage protein leads to the formation of many small vacuoles (Srivastava and Paulson, 1968). In the case of *Iris* endosperm, neither the fusion of membranes nor

large number of vacuoles (after staining with neutral red) were observed. At the end of digestion, one central vacuole surrounded by a net formed by protein bodies limiting membranes can be seen.

Cytoenzymatical investigations of the endosperm of *Iris pseudoacorus* reveal the presence of acid phosphatase inside the globoids. This enzyme disappears from the globoids during seed germination. According to Poux (1963) acid phosphatase activity is marked around globoids, and frequently in amorphous protein. In spite of many tests, acid phosphatase was not observed in the amorphous protein of *Iris*.

The biochemical investigations show that the protein bodies apart from the acid phosphatase contain RNase, α -glucosidase, esterase, phytase, and acid protease (Matile, 1968; Ory and Henningsen, 1969).

In the endosperm cells of dry and germinating *Iris* seeds acid phosphatase also appears in the numerous spherosomes, characterized as well by the presence of indoxyl esterase. Jones (1969) suggests that the spherosomes are associated with the aleurone grains and plasmalemma in dry cells. The spherosomes which surround the aleurone vacuoles (Yatsu, 1965; Engleman, 1968) disappear during germination (Pallag and Hyde, 1964) in coincide with the results obtained from the *Iris* seed cells.

Succinic dehydrogenase activity in endosperm cells of dry *Iris* seeds is low and its activity increases during germination as was observed by Altschul et al. (1966). Biochemical data show that the synthesis of mitochondrial components occur during germination (Young et al., 1960; Cherry, 1963).

The endosperm cells of dry *Iris* seeds contain the neutral lipids. These lipids are embedded in the cytoplasm among the protein bodies. During germination the lipids appear inside the limiting membrane of protein bodies. Matile (1968) suggests that the appearance of lipids inside aleurone vacuoles (Poux, 1963, 1965b) is caused by the incorporation of this organelle into aleurone vacuoles. In this way the author identifies the lipids as spherosomes. In the light of our results, the presence of lipids inside the limiting membrane after the digestion of protein is a secondary phenomenon.

In the final stages of germination of *Iris* seeds, the lipids appear in form of channels around the protein bodies. Jennings et al. (1963) states that the protein bodies are localized within lipoprotein membranes and part of the lipoprotein structure is an integral part of the protein body. The migration of the lipid droplets to the protein bodies limiting membrane would suggest a continuity of this membrane with the membrane of the lipid. The lipids may migrate into the protein bodies limiting membrane by mechanical shifting into the spaces left by digested proteins.

SUMMARY AND CONCLUSIONS

The composition, distribution and changes of storage substances and also of some enzymes in the endosperm tissue of dry and germinating *Iris pseudoacorus* seeds were investigated.

1. It was demonstrated that storage proteins with free NH_2 groups of the albumin and globulin types constitute the main component of seeds. Besides the protein, the neutral lipids are present. The storage proteins occur in form of simple and complex protein bodies limited with a membrane. The protein bodies amorphous substance contains the soluble inorganic phosphates. Apart from insoluble inorganic phosphates, inorganic polyphosphates and acid phosphatase are found in the globoids.

2. During germination, these compounds disappear from the protein bodies at the same time as from the amorphous protein. Moreover, the lipids content in the endosperm cells also decreases.

3. Cytoenzymatical investigations demonstrate the presence of acid phosphatase and indoxyl esterase in spherosomes accumulated around the protein bodies. Indoxyl esterase is also found in the plasmodesmata. The few mitochondria show succinic dehydrogenase activity. At first the activity of these enzymes increases but during the germination it disappears.

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Badania cytochemiczne suchych i kielkujących nasion Iris pseudoacorus

Streszczenie

Badano skład i rozmieszczenie substancji zapasowych, a także niektórych enzymów w endospermie suchych i kielkujących nasion *Iris pseudoacorus*.

Wykazano, że główny składnik nasion stanowią białka zapasowe zawierające wolne grupy NH_2 , typu albumin i globulin, obok nich występują lipidy obojętne.

Białka zapasowe występują w postaci ciał prostych i złożonych, otoczonych membraną. Substancja podstawowa ciał białkowych wykazuje obecność fosforanów nieorganicznych rozpuszczalnych, zaś na terenie globoidów — obok fosforanów nieorganicznych nierozpuszczalnych występują polifosforany nieorganiczne; ponadto w globoidach obecna jest kwaśna fosfataza.

Podczas kiełkowania nasion *Iris* związki te znikają z terenu ciał białkowych równoległe z ubytkiem białka podstawowego, zmniejsza się także zawartość lipidów w komórkach endospermy.

Badania cytochemiczne wykazały obecność kwaśnej fosfatazy i esterazy indoksylowej w sferosomach zgrupowanych wokół ciał białkowych. Esteraza indoksylowa jest również obecna w plazmodesmach. Nieliczne mitochondria wykazują aktywność dehydrogenazy bursztynianowej.

W pierwszym okresie kiełkowania aktywność tych enzymów wzrasta, ale w miarę trwania procesu kiełkowania maleje aż do całkowitego zaniku.

*Zakład Cytologii i Cytochemii Roślin
Instytut Biochemii i Fizjologii
Uniwersytetu Łódzkiego*