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Dormancy release and germination in recalcitrant *Aesculus hippocastanum* seeds

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Abstract: The investigation of dormancy release in *Aesculus hippocastanum* seeds was aimed at estimating the proportion of coat-imposed to embryo dormancy, and studying the growth initiation providing embryo dormancy release. During winter, horse chestnut seeds exhibited 16–17 week-lasting deep dormancy, which predominantly was determined by coat-imposed dormancy. Embryo dormancy lasted for 11–12 weeks of wet cold stratification. Embryo dormancy was weak, even the embryo axes excised from deeply dormant seeds were capable of extending to the size exceeding the axis length in intact seeds at radicle protrusion. Embryo dormancy release manifested itself in gradually increasing growth capacity of both embryo axes and cotyledonary petioles. The growth initiation in horse chestnut seeds occurs only by cell elongation. During growth initiation, a more rapid fresh weight gain was observed in comparison with length increment, thus indicating that accumulation of osmotically active substances and active water uptake by embryo axis cells were ahead of their increasing longitudinal cell wall extensibility. Cell wall loosening appeared to be directly related to embryo dormancy release. The hormonal regulation of embryo dormancy release in horse chestnut seeds is discussed.

Additional key words: coat-imposed dormancy, cytokinin, embryo axis, cotyledonary petioles, growth initiation

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Introduction

Being originated from the mountains of Balkan Peninsula, horse chestnut (*Aesculus hippocastanum* L.) trees belong to few species with recalcitrant seeds spreading to the North and inhabiting beyond the Mediterranean and subtropical regions. *A. hippocastanum* was widely planted as a shade tree in Europe, first mentioned as an introduced species in 1576 (Rudolf 1974). From southern Europe it was transferred to north-eastern United States.

The recalcitrance manifests itself in the maintaining high water content in the embryo axes during dormancy continuation and perishing in the case of seed

desiccation. Great care must be taken not to dry them long because seeds lose their viability (Widmoyer and Moore 1968).

Seeds of horse chestnut trees are shed dormant in southern England and other countries of middle Europa (Tompsett and Prichard 1993; Daws et al. 2004). These seeds combine the recalcitrance with true dormancy typical of tree seeds that can be relieved by wet cold stratification (Tompsett and Prichard 1993). For this reason, the recommended regime of seed storage was stratification in moist sand or sand-peat mixtures at 5°C for about 120 days or in sealed containers at 1°C for 100 days or longer (Suszka 1966).

Under cool climatic conditions of Russia, they retain deep 4-month-long dormancy in winter, thus occupying an extreme position among recalcitrant seeds. These seeds differ from most tree seeds by their recalcitrance and from tree recalcitrant seeds by their dormancy.

Horse chestnuts are large seeds covered with a dense waxy coat. The ripe seeds have a large light-colored hilum. They contain an embryo axis consisting of radicle, hypocotyl and plumule. The axis is connected with two massive starchy cotyledons by short cotyledonary petioles. Germination occurs according to hypogeal type.

The third specific feature of horse chestnut seeds is the ability to commence germination only by cell elongation, first in hypocotyls and then in basal root cells (Obroucheva, 1999). Cell divisions begin in the root tips of 3-cm-long axes of seedlings. This peculiarity makes these seeds most suitable for studying the growth initiation as related to dormancy release and loss.

Materials and methods

The freshly-fallen seeds were collected in the Main Botanical Garden (Moscow, Russia) in early October in 2002 and 2003. They were tightly layered in wet sand-filled boxes and stored at 4°C in thermo-controlled chamber for stratification. The sand was regularly moistened throughout the entire period of cold stratification which lasted for 4 months.

For testing the germinability, the seeds were regularly transferred to enameled dishes with distilled water and incubated at 27°C in the dark. Each sample consisted of 30 seeds. Radicle emergence was recorded every day so long as germination occurred. In some samples, the seed coat was completely removed before the germination test.

In some experiments, the embryo axes (A) were excised with a scalpel and incubated in small Petri dishes on filter paper moistened with distilled water or isopentenyladenine (Aldrich Chem. Co., USA) solution. In other experiments, the embryo axes were excised together with a half of cotyledonary petiole (A+P), while in the third series the excised seed parts consisted of the embryo axis, whole petiole and a piece of cotyledon (A+P+C). In all cases, chloramphenicol (50 mg/l) was added to avoid bacterial infection.

These explants were incubated at 27°C in the dark for 7 days and regularly measured and weighed. Water uptake was evaluated by fresh weight gain in the embryo axes.

Axis growth was estimated in terms of fresh weight and length. In the case of excised axes or axes of decoated seeds, radicle emergence was taken to be at the axis length exceeding 10 mm, i.e. the axis length at radicle emergence in intact seeds.

The experiments were performed in triplicate, each replicate included 5 explants. The experiments were repeated during two years.

The data are presented as the mean \pm standart error.

Results

Proportion of coat-imposed to embryo dormancy in horse chestnut seeds

The freshly-fallen seeds were initially dormant, their dormancy extended far into post-harvest period. In order to evaluate the contribution of coat-imposed and embryo dormancy to full dormancy, we have compared the germinability of intact and decoated seeds under favorable conditions in terms of the rate of radicle emergence. The measurements were performed during the entire stratification time, starting with freshly-fallen seeds possessing the deepest dormancy and ending with rapidly germinating seeds after dormancy loss. The rate of germination gradually increased with dormancy release, i.e. with the reduction of stratification time, by the end of which seeds exhibited 100% radicle emergence during 36 h in average (Fig. 1).

The decoated seeds possessed much attenuated dormancy as compared to deeply dormant intact seeds. For example, all freshly-fallen decoated seeds germinated at most 7 days, whereas in intact seeds, germination time of 50% such seeds was as long as 25 days. The germination time of both decoated and intact seeds gradually shortened in the course of stratification up to the germination time of intact seeds after dormancy loss (Fig. 1).

The schematic representation of these data is given in Fig. 2 showing the proportion of coat-imposed to embryo dormancy in terms of radicle emergence time of 50% seeds. The upper curve shows the average time of radicle emergence in intact seeds and characterizes the full dormancy. Curve 2 indicates the time of radicle emergence in decoated seeds, whereas line 3 refers to this index in dormancy-released seeds. The area between curves 1 and 2 represents the depth of coat-imposed dormancy while the area enclosed by curves 2 and 3 characterizes the depth of embryo dormancy. Coat-imposed dormancy dominated over embryo dormancy and comprised 85–90% of full dormancy. Relatively weak embryo dormancy was completed by 11–12 weeks of stratification. During the last 2–4 weeks, the germination is delayed due only to gradually weakening coat-imposed restraint. By this time, the seeds can actively germinate even at 4°C in wet sand.

The coat-imposed prevention of water inflow is evident from Fig. 3, where the amount of water taken up by embryo axes in intact seeds and by excised axes was

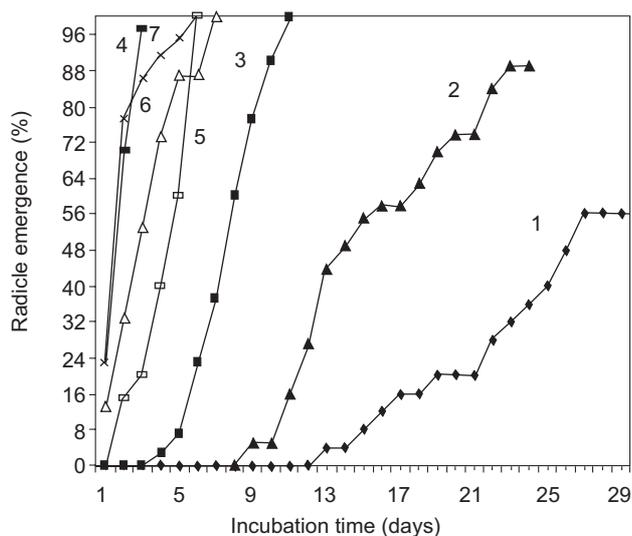


Fig. 1. Radicle emergence in intact and decoated *A. hippocastanum* seeds in the course of dormancy release. The seeds imbibed under optimum conditions

1 – Intact freshly-fallen seeds; 2 – intact seeds, 4 weeks of stratification; 3 – intact seeds, 12 weeks of stratification; 4 – intact seeds after dormancy loss; 5 – decoated freshly-fallen seeds; 6 – decoated seeds, 4 weeks of stratification; 7 – decoated seeds after dormancy loss

compared. In the embryo axes of coated dormant seeds (Fig. 3a), the initial water inflow was slow and then accelerated during second half of imbibition time. In intact seeds after dormancy loss (16 weeks), water was rapidly absorbed without delay. Fig. 3b represents the water uptake by excised axes. It shows that the embryo axes excised either from dormant or from dormancy-released seeds absorb water as rapidly as the axes inside intact seeds after dormancy loss.

Embryo dormancy and influence of petioles and cotyledons

Embryo dormancy can be imposed by an inhibitory influence of cotyledons or it represents a specific feature of the embryo axis itself. During the entire period of cold stratification, we compared the growth capacities of excised embryo axes (A), those attached to a piece of cotyledonary petiole (A+P) and of the axes attached to a piece of cotyledon through the intact cotyledonary petiole (A+P+C), during the entire period of cold stratification. The excised embryo axes (Fig. 4a) from dormant seeds were capable of elongating on their own, and their length far exceeded 10 mm, i.e. the size at radicle protrusion. Therefore, their innate growth capacity allowed the early germination to occur. However, after embryo dormancy release (Fig. 4b), they elongated more rapidly that resulted in earlier radicle emergence and accelerated embryo axis extension.

The presence of cotyledonary petioles and to a greater extent the additional presence of cotyledons promoted the elongation of the axes (Fig. 4a). No in-

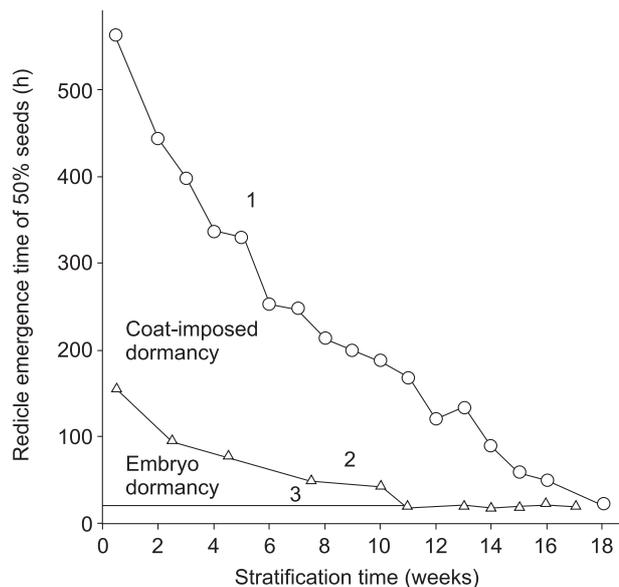


Fig. 2. Schematic presentation of the proportion of coat-imposed dormancy to embryo dormancy and dormancy absence in terms of time necessary for radicle emergence in 50% *A. hippocastanum* seeds

1 – intact seeds; 2 – decoated seeds; 3 – seeds after dormancy loss

hibitory effect of cotyledons was observed. The cotyledons actively stimulated the post-germinative growth of axes excised from dormant, and especially dormancy-released seeds, apparently due to nutrient export. In A+P+C experiments, the earlier radicle protrusion corresponded to the behaviour of decoated dormant seeds.

Special attention is to be attracted to the petioles. Figure 4a shows that the presence of a piece of petiole (5–7mm, a half of its full length in dormant seeds) in A+P system did not stimulate the axis elongation up to the radicle emergence, but significantly accelerated it later. When the seeds after dormancy loss were taken for similar experiments (Fig. 4b), the growth capacity of A+P system was greater by 30% than in dormant seeds (Fig. 4a) and similar to A. The embryo axis growth promotion by petioles occurred only in dormant seeds and may also be explained by the nutrient import, apparently as a result of reserve mobilization in growing petioles and their exhaustion by the time of dormancy release.

For radicle protrusion, it is important that the cotyledonary petioles are capable of slow elongating during early germination. When the A+P+C system was excised from dormant seeds (1–11 weeks), the petioles increased in length from 12 mm in average to 15 mm during 72-h-long incubation in water, and then to the final length of 17–18 mm by 144 h. If A+P+C were excised from dormancy-free seeds (15–17 weeks) this final petiole length was achieved more rapidly (during 72 h). The petiole elongation appeared to contribute to radicle protrusion through the coat by producing additional pressure on it from the

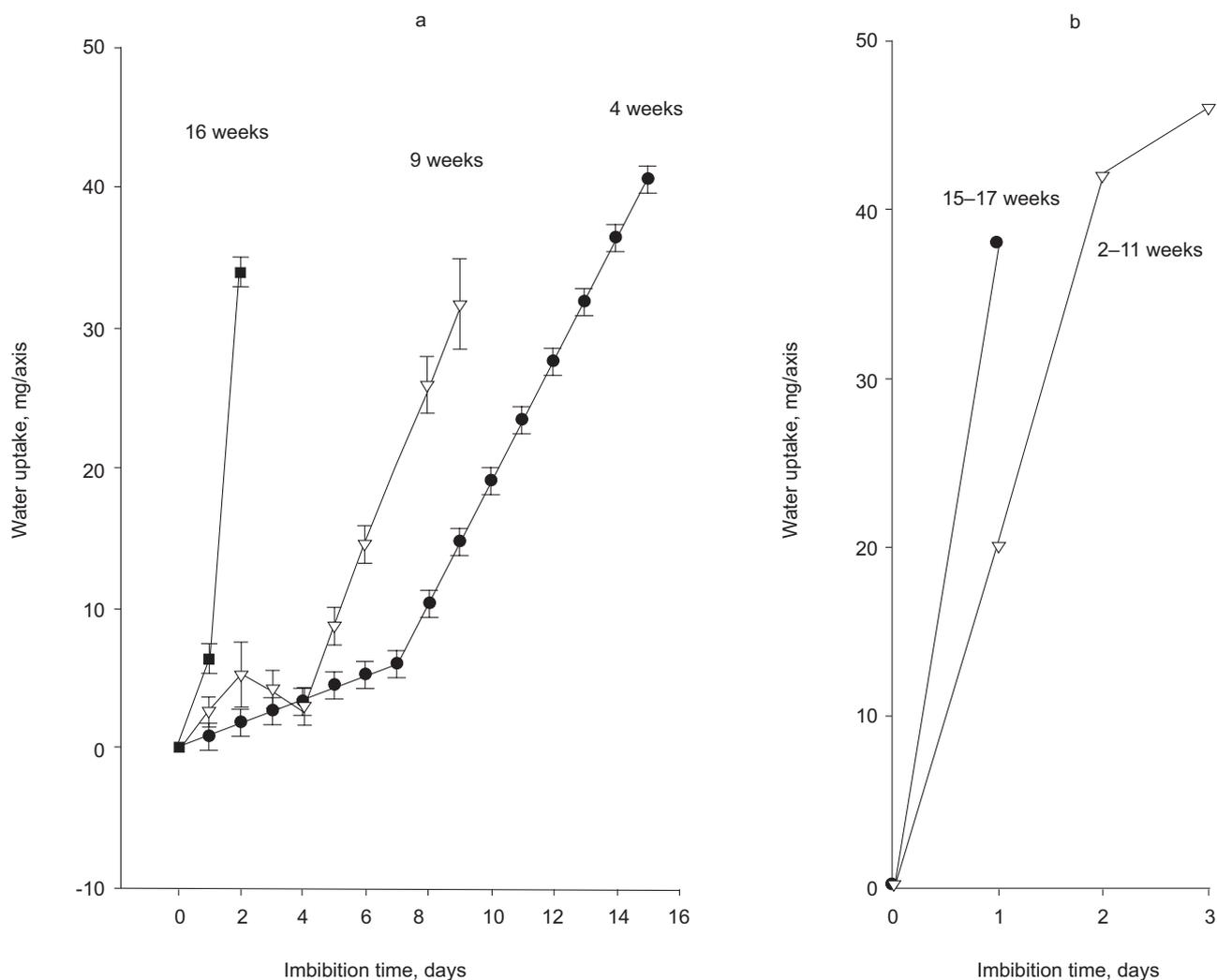


Fig. 3. Water uptake by horse chestnut embryo axes *A. hippocastanum* seeds
 a – Embryo axes within intact dormant (4 and 9 weeks of stratification) seeds and dormancy-released (16 weeks) seeds during seed imbibition under optimum conditions up to radicle emergence; b – Embryo axes excised from dormant (2–11 weeks) seeds and seeds after dormancy loss (15–17 weeks), both imbibed under optimum conditions up to the length typical of radicle emergence

inside and pushing the radicle tip, the process more efficient in seeds after dormancy loss.

The growth capacity of excised embryo axes enhanced with embryo dormancy release. It can be clearly demonstrated by changes in the fresh weight during 72 h incubation in water (Fig. 5). The embryo axes from dormant seeds (1–11 weeks) elongated slowly, whereas those from free of dormancy seeds (15–17 weeks) were growing more rapidly and achieved the fresh weight of the axes at radicle emergence, in average 104.5 ± 2.4 mg, a day earlier. In dormancy-released seeds, the weight of excised embryo axes prior to radicle protrusion amounted to 90 mg, and their growth after protrusion started in some hours. For comparison, the upper curve showed the rapid weight increase in the embryo axes grown after radicle emergence.

The growth capacity of excised axes is more readily illustrated by the gain in fresh weight at radicle emergence (248% of initial fresh weight during 72 h in

dormant seeds) as compared to the increment in length (185%). The average length of the embryo axes at the excision time was 6.5 mm at deep dormancy, but 8.5 mm after dormancy loss by seeds, while the difference in the axis weight was greater, from 50 to 90 mg. This indicates that initiation of longitudinal elongation in embryo axis cells first of all includes the processes providing water uptake and fresh weight gain, whereas the length increase depending on cell wall loosening is partially limited.

Seed dormancy release and cytokinin effects

The intact dormant horse chestnut seeds are known to respond to the treatment with isopentenyladenine, benzyladenine and its riboside by accelerated dormancy release in the middle of stratification time (Obroucheva and Antipova 2000). In partially decoated seeds, the treatment of dormant seeds with natural cytokinins also resulted in promoted dor-

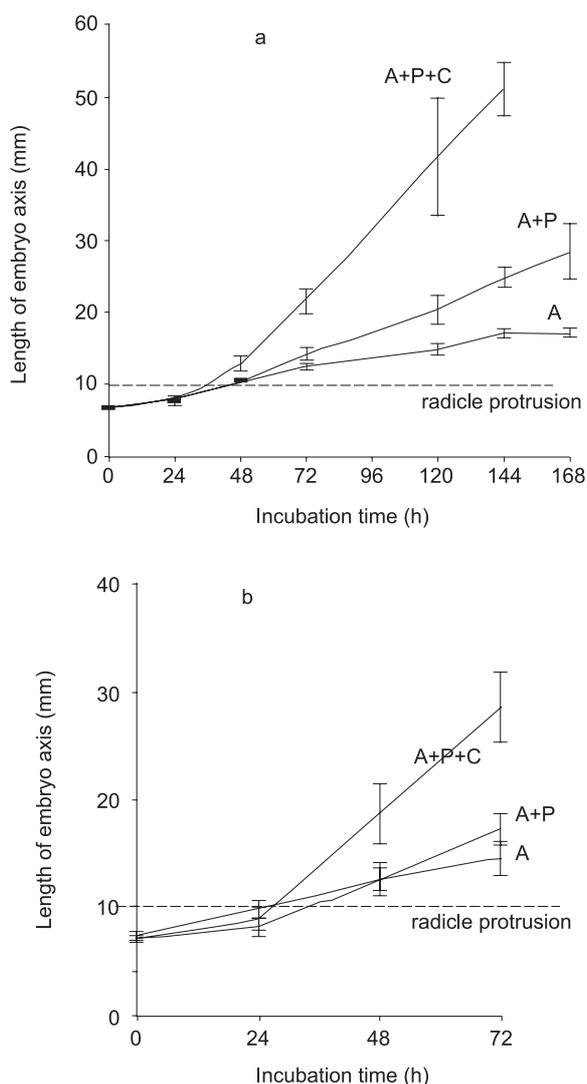


Fig. 4. Elongation of excised embryo axes (A), embryo axes with a half of cotyledonary petiole (A+P) and embryo axes with an intact petiole and a piece of cotyledon (A+P+C) in the course of *A. hippocastanum* seed dormancy release and germination. Dash line indicates the axis length at radicle emergence

a – dormant seeds, 1–11 weeks of stratification; b – seeds after dormancy loss, 15–17 weeks of stratification

mancy release, the effect being clearly evident from the onset of dormancy. Apparently, the facilitated penetration of cytokinins into partially deoated seeds has shifted the response time to the very beginning of dormancy.

In order to analyze further the cytokinin interference with dormancy release and to separate cytokinin effects on coat-imposed dormancy from embryo dormancy, we have investigated the influence of 0.01 mM isopentenyladenine, a natural cytokinin. We evaluated the growth capacity of embryo axes excised from dormant and free of dormancy seeds in the course of incubation with this hormone. These experiments showed that the increase in fresh weight was equal to that in control embryo axes incubated in water.

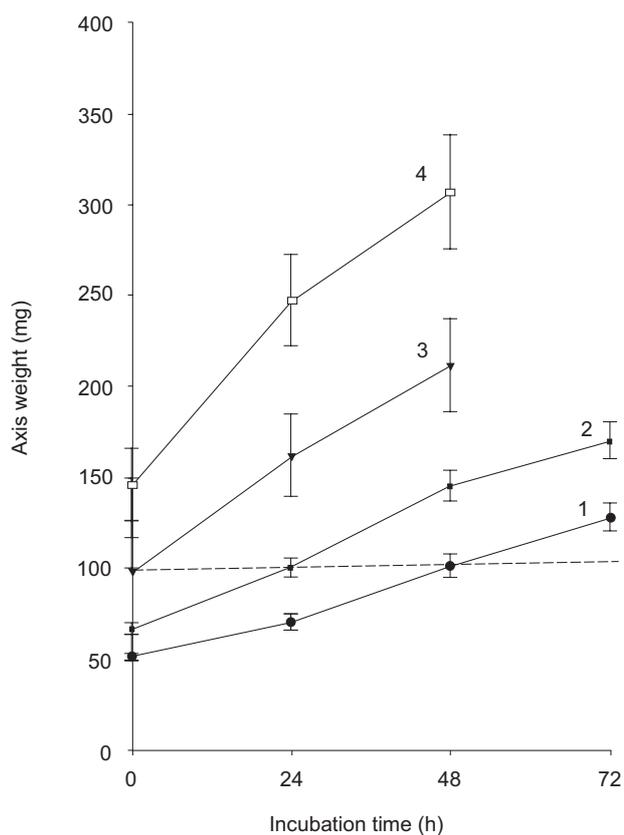


Fig. 5. Fresh weight increase after excision of embryo axes from dormant *A. hippocastanum* seeds and seeds after dormancy loss

1 – Seeds after 1–11 weeks of stratification; 2 – seeds after 15–17 weeks of stratification; 3 – nondormant seeds with nonemerged radicles; 4 – nondormant seeds with emerged radicles. Dash line indicates the embryo axis weight at radicle protrusion

When we measured the length increment, it turned out that isopentenyladenine even slightly inhibited the embryo axis growth comprising 85% of control axis size at 1–11 stratification weeks and 88% at 17 week. No stimulatory effect on pregerminative axis growth was observed. After radicle emergence, this cytokinin also hindered the extension of embryo axes, but they enlarged. The presence of cotyledonary petioles did not change the embryo axis response to isopentenyladenine.

The absence of cytokinin influence on growth capacity of horse chestnut axes indicates that the attenuation of embryo dormancy is not the cytokinin target. Therefore, cytokinins alleviate dormancy in freshly-fallen seeds and later by reducing the coat-imposed dormancy.

Discussion

The alleviation of coat-imposed dormancy manifests itself in facilitated water inflow – the process related to changes in the seed coat properties induced at least partly by cytokinins. The rate of water uptake increased during the second half of imbibition at each

stratification time tested, apparently after some preparative events that occurred in seed coat during the first half of imbibition time. Duration of imbibition period was gradually reduced with stratification and coat-imposed dormancy attenuation. After dormancy loss seeds absorbed water without delay to the level providing radicle protrusion.

The dormancy state in horse chestnut seeds results mainly from coat-imposed dormancy, the embryo dormancy did not exceed 10–15% of full dormancy. Both dormancy components gradually weakened in the course of dormancy release, coat-imposed dormancy lasted longer and attenuated steeper.

Among all phytohormones tested, only cytokinins promoted dormancy release of intact horse chestnut seeds (Obroucheva and Antipova 2000), their effect being evident earlier in partially decoated seeds. It was tempting to suppose that the target was coat-imposed dormancy. Here we confirmed this assumption indirectly as followed from the absence of any growth acceleration in excised axes by isopentenyladenine. The only effect observed was the enlargement of embryo axes. This is in accordance with well-known cytokinin effects on cell growth in elongating organs (Evans, 1984), i.e. cytokinin-induced stimulation of cell enlargement but not of cell elongation.

Embryo dormancy is weak and gradually attenuates being terminated by 11–12 weeks. The time of radicle emergence in decoated seeds, as a measure of embryo dormancy, resulted from two processes, namely the elongation of the embryo axis itself and elongation of cotyledonary petioles, which contributed to axis protrusion by pushing the radicle tip through the seed coat. In dormant seeds, the petioles elongate two times slower than in nondormant seeds.

Embryo dormancy and its release are not influenced by the petioles or cotyledons, being due to the processes occurring in the embryo axes. The excised embryo axes are capable of absorbing water at the rate similar to that exhibited by the axes located inside the nondormant seeds and of initiating cell elongation to perform the radicle protrusion in the absence of the cotyledons, petioles and the seed coat. The release of weak embryo dormancy manifested itself in the excised axes in more rapid length and weight increase up to the values characteristic of emerging axes. The influence of petioles and cotyledons became evident after radicle emergence by accelerating the post-germinative growth of seedling axes.

The capacity of excised embryo axes to commence the elongation means their competence to accumulate endogenous osmotica and increase the extensibility of cell walls, i.e. both pre-germinative processes necessary for preparing cell elongation (Obroucheva 1999). As follows from the comparison of length increment and fresh weight gain up to the radicle emergence, fresh weight of the embryo axes increased

more rapidly. It means that water uptake, as follows from fresh weight gain, occurred more readily than the longitudinal cell wall extension leading to the length increase. It is tempting to conclude that it is cell wall loosening but not osmoticum accumulation that partly retards the growth initiation in embryo axes of dormant seeds and that the embryo dormancy release is related to an increase in cell wall extensibility.

In horse chestnut embryo axes, cell wall loosening is due to activation of plasmalemma H^+ -ATPase resulting in proton excretion to wall and its acidification in embryo axes (Obroucheva 1999). This process is relieved by endogenous fusicoccin, a well-known activator of this enzyme. Fusicoccin at 0.001 mM concentration 3–4-fold stimulated the elongation of embryo axes excised from the horse chestnut seeds (Obroucheva and Antipova 2004). These data, in combination with the finding that endogenous fusicoccin appears in this axes by the 7th week of stratification (Antipova et al. 2003), permit us to suppose that activation of plasmalemma H^+ -ATPase by fusicoccin may be at least partially responsible for embryo dormancy release in horse chestnut axes.

The stimulatory effect of fusicoccin on the elongation of excised embryo axes is counteracted by 0.01 mM ABA (Obroucheva and Antipova 2004). This effect was typical of the axes prior to radicle protrusion but was not observed after the visible germination started. The content of endogenous ABA gradually decreased in the axes of intact seeds after 10–11 weeks of stratification and after radicle emergence too (Obroucheva and Antipova 2004). All these observations indicate that embryo dormancy release in horse chestnut axes may be explained in terms of hormonal regulation by ABA–fusicoccin interplay, resulting in activated acidification of cell walls and rapid cell elongation in embryo axes.

The fusicoccin capacity to overcome the ABA-induced inhibition of germination was exemplified by numerous quiescent seed species (Antipova et al 2003; Simonovich et al 2000, and references therein). However, such FC effect may be extended to dormancy loss too, as follows from the observations on dark-inhibited germination of *Paulownia tomentosa* (Grubisic et al 1988), thermo-inhibited germination of *Lactuca sativa* (Simonovich et al 2000), cold-stratified *Acer* species (Daletskaya and Nikolaeva 1987) and deep cold-induced dormancy release in *Aesculus hippocastanum* seeds (Obroucheva and Antipova 2004), although such pattern of hormonal interplay affecting dormancy release and germination seems at first glance rather unexpected.

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