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SEED DORMANCY AND AFTER-RIPENING REQUIREMENTS OF *FRAXINUS NIGRA* MARSH.

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SEED DORMANCY AND AFTER-RIPENING REQUIREMENTS OF *FRAXINUS NIGRA* MARSH.

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ABSTRACT

The seed of *Fraxinus nigra*, black ash, borne in a single samara, normally ripens in the autumn. At that time the seed contains an embryo which is both immature and dormant. In order to overcome this dual delay to germination the seed may be after-ripened in moist peat moss for 18 weeks at 21°C followed by 18 weeks at 4°C.

After-ripening at the warm temperature matures the embryo, whereas the subsequent cool temperature releases embryonic dormancy. During maturation the embryo length doubles and the dry weight triples. As well, the cotyledons show visual evidence of differentiation. On the other hand, for dormancy to be overcome a metabolic shift in the embryo occurs which provides the active metabolism needed by a germinating seed. This is reflected by a steady increase in the respiration capacity of the embryo throughout the cool period. At the same time a reduction in the amount of oil in the seed indicates its use as a respirable substrate.

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ABBREVIATIONS

ABA - abscisic acid

ADP - adenosine diphosphate

AMP - adenosine monophosphate

ATP - adenosine triphosphate

EMP pathway - Embden - Meyerhof - Parnas pathway

GA_3 - gibberellic acid

NAD - nicotinamide - adenine dinucleotide

NADP - nicotinamide - adenine dinucleotide phosphate

PP pathway - pentose phosphate pathway

TCA cycle - tricarboxylic acid cycle

INTRODUCTION

Macbeth once said, "If one could tell the seeds of time, which would grow this or that ---." The seed commonly defined as the fertilized ripened ovule of a flowering plant contains within the seed coats the life of a new generation. This new life may be maintained at a very low metabolic rate for many months or even years. In this restricted state the seed is equipped with a store of organic materials, minerals and growth factors; it is dependent upon the external environment only for oxygen, a suitable temperature and sometimes light.

The failure of seeds of many woody species to germinate promptly has placed widespread restrictions on attempts by horticulturists to propagate these plants. Such is the situation with black ash. In relation to Great Plains horticulture a better understanding of the dormancy of black ash is needed. Therefore, the purpose of this thesis is to furnish information leading to the arrest of dormancy in black ash seeds.

Dormancy can be defined as the failure of a viable seed to germinate when placed under favourable conditions of moisture, aeration, temperature and light. It may result from physical, mechanical or metabolic causes. Physical dormancy is often present in seeds with hard seed coats which are impermeable to water or gas exchange. Water impermeability is recognized as the cause of dormancy in Melilotus officinalis (17); oxygen impermeability seems to cause dormancy in Hydrangea (10). In a few species such as Alisma plantago (9) the seed coverings are physically strong and able

mechanically to resist the expansion of the embryo. In most cases, however, dormancy is controlled by metabolic factors. In order for this dormancy to be overcome, chemical changes must occur in the embryo. Changes may include a buildup of phosphate acceptors (31), an activation of the pentose phosphate pathway (26) or a change in the levels of hormone type regulators (7,44).

The metabolic changes which occur in the dispersed seed before germination is termed after-ripening. Since early times horticulturists have often effected the necessary changes by placing the seeds in a moist medium and storing them at the appropriate temperature for several months. Most commonly fall-ripened seeds from trees and shrubs of the north temperate zone respond best to storage at 2-7^{o}C. (17). Generally after-ripening for 1 - 3 months will be adequate to permit germination. But the length of time varies with species concerned, seed lots within a species, and seed lots from different years (41). Unlike most tree seeds the seeds of the species in question respond best to a warm (20-25^{o}C) moist treatment for 4 months followed by a chilling moist treatment for 4 months (38). This might lead one to believe that the changes occurring within the black ash embryo are more complicated and less understood than those of other seeds with a single after-ripening requirement.

After-ripening is not without ecological import. Delayed germination provides two distinct advantages to the species. Firstly, dormancy extends the period of time during which the viable seed may be carried great distances to further the distribution of the species. Secondly, a prolonged dormancy in fall-ripened seeds enables them to remain dormant so

that the young seedling will not be destroyed by the environmental extremes of winter. Both of these advantages have undoubtedly contributed to the survival of many species.

In order to provide a broader understanding of the dormancy problem and after-ripening phenomena of black ash, the results of an investigation of embryonic changes at different stages of after-ripening are presented on the following pages. Preliminary studies involve measurements of the increase in embryo length and dry weight. The central core of the thesis involves several respiration studies; namely, respiration rate, pathway and substrate. The facts presented on the following pages may unfold some secrets held mysteriously for centuries within black ash seeds.

LITERATURE REVIEW

Black ash typically grows in swampy woodlands. It is one of the major trees of the Great Lakes, St. Lawrence and Acadian Forest regions. It is the only native Fraxinus species grown in Newfoundland. Furthermore, it extends into the southeastern portion of the Boreal Forest (18). To the west it is largely restricted to the rocky woods and swampy peat soils of Eastern Manitoba (34). Just recently Ronald (32) reported vigorous stands growing along the banks of the Assiniboine River and Riviere Salle in the grassland regions west of Winnipeg. He deduced that the conditions of the habitat favourable for seed after-ripening and germination may well be much more important in determining the range of the species than are the conditions which favour subsequent tree growth. According to basic principles only where the seed germinates will the tree grow.

A dry seed contains an arrested embryonic plant. When placed under favourable external conditions of moisture, aeration, temperature and light it could well be expected to resume growth or germinate. Samish (33) defines dormancy simply as a lack of growth which continues under favourable external conditions. From this definition it becomes apparent that it may be difficult to determine whether a lack of growth should be attributed to primary dormancy or an unfavourable environment. Embryo dormancy may be regarded as a complicated system of enzyme activities with competition between enzymes for the same substrate or coenzyme. Changes brought about by after-ripening must occur before germination will result. This process is demonstrated by Villiers and Wareing (43). They say that

the excised non-after-ripened embryos of Fraxinus excelsior, European ash, will not germinate until the seed is first stored in a moist medium for several months.

Embryonic changes at the biochemical and physiological level occur during after-ripening. Although researchers have studied a great number of parameters during after-ripening, detailed accounts from any given species are fragmented. That is to say that the changes that will be discussed involve a wide range of species. It becomes scientifically inaccurate to suggest that an after-ripening phenomenom observed with one species applies universally to all species. When considering black ash, which has been given only limited study (2,38) one must accept critically the works done on seeds of other species. Based on anatomical and morphological observations (2,38,43,44,45) the embryos of black ash appear to respond to after-ripening much like those of European ash. Comparisons of changes to those of other species become highly speculative.

We have noted already that some block in embryo metabolism causes a failure to germinate. In some cases dormancy may be repression of enzyme activity. In this respect working with such widely differing seeds as Xanthium, Crataegus and Sorbus, respectively, several independent workers (11,13,15) showed an increase in catalase and peroxidase activity during the early stages of cool after-ripening. The enzymatic activity of α and β-amylase responsible for starch breakdown also showed an upward trend during cool after-ripening (28).

It is not surprising, then, that these same authors (28) showed a decrease in the total starch content in the embryo and endosperm. They

noted a corresponding increase in sucrose and reducing sugars in the embryo.

Eckerson (13) found that the fatty oil content of Crataegus gloriosa decreased during after-ripening. LaCroix and Jaswal (27) found that lipids stored in the cotyledons of Prunus cerasus were degraded to acetate and metabolized to free sugars during after-ripening. Free sugars were transported to the metabolically active embryonic axis.

Changes in nitrogen content during after-ripening show definite trends. Klein (24) reported an increase in soluble nitrogen in lettuce seeds just before germination. This caused a decrease in the protein to soluble nitrogen ratio. The results of other work (3) on Paeonia suffruticosa indicated a steady increase in protein content in embryo tissue prior to germination. Embryos after-ripened at 5°C had more protein synthesis than those left at greenhouse temperature. Similarly in the same study Barton and Bray found that aspartate and glutamate components increased markedly in after-ripened embryos. Interestingly, they noted no significant alteration in endosperm tissue. Others (5) working with Corylus avellana showed the same trend of increased aspartate and glutamate in the after-ripened embryo. However, they found no amino acid changes in the cotyledons, but did find increased nucleotide formation in the cotyledons, followed by transport to the embryonic axis. Again, in cherry seeds (30) the total nitrogen and phosphorus increased in the embryonic axis with after-ripening at 5°C. This study showed that during cool after-ripening the total nitrogen per cell remained constant; the total phosphorus per

cell increased. At this cool temperature the phosphorus accumulated as stable nucleic acids rather than inorganic phosphate.

These same workers (31) suggested that an increase in phosphate acceptors may be one of the after-ripening effects which terminates dormancy. A study of the seed of Pisum sativum (6) showed that during imbibition there was a 250% increase in ATP, a parallel fall in AMP and no change in ADP. It may well be that the amount of energy supplied to the germinating system is restricted by insufficient AMP and ADP.

Induction of changes in the levels of coenzymes associated with respiration has been suggested as one of the functions of after-ripening. Results of studies (46) of pyridine nucleotides in Vigna sesquipedalis indicated that both NAD and NADP are rate limiting factors in the metabolism of plant tissues. In studying the interrelationships between the two coenzymes it was found that NAD is fully oxidized in cotyledons and in the hypocotyl. On the other hand, NADP is partially reduced. Generally, the NADP + NADPH/NAD + NADH quotient may be low in storage organs but much higher in the active meristematic tissue. An increase in the NADP + NADPH/NAD + NADH ratio during germination appears to precede radicle emergence (6).

No doubt coenzyme levels are associated with the control of glucose catabolism. With a low NADP content in storage organs only the EMP pathway and the glyoxylate shunt are operative. With a high NADP content in the growing points functioning of the PP pathway could be significant. LaCroix and Jaswal (26) studied the relative contribution of the PP and EMP pathways in after-ripening cherry embryos. They found that near the end of the after-ripening period the relative participation of the PP pathway increased.

The time of the increase coincided closely with preparedness to germinate. The information by Gibbs and Beavers (16) that the EMP pathway is vigorous in meristematic tissue and that the PP pathway becomes more important as tissue ages seems somewhat contradictory.

All of the after-ripening changes discussed thus far are linked to the respiration rate and the availability of energy for growth. Two independent sources (26,31) showed an increase in respiration rate in after-ripening cherry seeds. The most dramatic results (31) showed that during after-ripening at $5^{o}C$ there was a 70% respiration rate increase in the whole seed and a 600% increase in the embryonic axis. On the otherhand, after-ripening at $25^{o}C$ caused a slight decrease in over-all respiration. Increased respiration was followed by growth by cell division and enlargement. The embryonic axis increased in dry weight due to translocation of stored reserves. Using NAD to determine respiration capacity it seemed that the initial effect of cool after-ripening was to increase the efficiency of respiratory enzymes, thus providing a greater supply of available energy to the embryo. However, respiration stimulation by NAD decreased as a growing condition was approached. This may mean that respiration is via the PP pathway which requires NADP. It may imply as well, that the rate limiting step shifts from the phosphorylation system to the respiratory chain itself. Possibly a lack of respiratory substrate develops. If one measures germination in terms of respiration rate it becomes difficult to separate the end of after-ripening from the beginning of germination because the first evidence for onset of germination is an increase in respiration rate (39).

In order to explain what ultimately controls respiration rates, enzymatic activity, the level of coenzymes and the breakdown of primary storage compounds, attention has been focussed on plant hormones. Sondheimer et al (35) studied the concentrations of three glucosides in Fraxinus americana. These three water soluble glucosides, found in the endosperm and embryo, accounted for 10% of the dry weight of the seed. The level of these two compounds, designated as G1-3 and G1-6, decreased as a result of germination and growth during the first 10 days. They believe that G1-3 and G1-6 perform some role in germination and growth of F. americana seed, but that GA_3 and ABA exert a regulatory effect on the metabolism of these glucosides. When germination of dormant embryos is induced by GA_3, the G1-3 and G1-6 level decreased in the same way that it did in nondormant embryos. On the other hand, in the presence of exogenous ABA no glucoside level decrease could be detected. This evidence suggests that these hormones may ultimately regulate seed dormancy in Fraxinus spp.

Two opposing theories explain hormonal activity in Fraxinus seeds. Sondheimer et al (37) explain the loss of dormancy merely as a decrease in the concentration of ABA during cool after-ripening. Support for this theory comes from the fact that leaching the excised embryo for 48 hrs. at room temperature can replace the chilling requirement for European ash. Experiments with exogenously applied ABA indicate that ABA exerts its germination inhibitory capacity in the seed rather than in the pericarp.

Contrary to this idea, Villiers and Wareing (44) report that germination results when a growth promoter increases rather than when inhibitors decrease. They extracted the growth substances from the embryo of seeds

and using a biological assay they found the production of a germination and growth promoting substance during cool temperature after-ripening. Soaking seeds which had been after-ripened for 3 months at 18-20^{o}C did not significantly reduce the water soluble inhibitor content. However, water soaked seeds germinate whereas non-soaked seeds do not germinate. They also found a growth promoter present in the embryo of chilled seeds. However, they had difficulty in explaining germination simply in terms of GA_3 and ABA because seedlings stimulated to germinate by GA_3 grew slowly compared to seedlings stimulated by chilling.

In this connection Sondheimer and Galson (36) found that leaf growth and chlorophyll synthesis are much reduced in GA_3 induced germination. This leaf growth and chlorophyll synthesis problem could be overcome by a kinetin application, but they could find no combination of kinetin and GA_3 that would overcome the effects of an application of 10 m M. ABA.

So far the explanations given for hormonal control seem too simple and fragmented to be satisfactory. However in 1971 a tean from New York (23) presented an hypothesis for dormancy and germination.

> "---This scheme clearly shows that gibberellin is the primary stimulus for germination, and the roles of cytokinin and inhibitor are secondary and essentially "permissive" and "preventive", respectively. One extremely important feature of this hypothesis is that dormancy in seeds could result, not only by the presence of inhibitors, as is generally believed, but also by absence or lack of a gibberellin or a cytokinin. Likewise for germination to take place, a gibberellin (primary stimulus) alone may not always suffice. A seed may require the assistance of a cytokinin to counteract the inhibitory effect of a germination inhibitor in order to achieve germination (permissive effect)."

In agreement with this hypothesis data from Khan (22) show a lack of interaction between GA_3 and ABA whereas kinetin and ABA do interact.

It was already indicated that gibberellins appear to be the primary stimulus for germination. Work done on the effects of gibberellin in dormant seeds (8,21) indicated quite clearly that this growth promoter operates in many seeds by breaking down starch through α-amylase synthesis. But how important are carbohydrates to a germinating black ash seed? In another study in 1970 Sondheimer et al (35) concluded on the basis of low levels of starch and polysaccharide in F. americana that these substrates may not be important for germination in this species. They found lipid concentrations high and consequently, they suggested that the primary carbon source required for germination comes from the lipid fraction. This seems to be consistent with the fact that the seed endosperm of Fraxinus spp. consists of fats and reserve proteins. Starch and soluble carbohydrates appear in the endosperm and embryo mainly in the late stages of the germinative process.

Some attention has been focussed on the significance of the pericarp in controlling dormancy in Fraxinus seeds. Both Ferenczy (14) and Steinbauer (38) stated that the pericarp has no effect in controlling dormancy, whereas Asakawa (2) observed a more rapid growth of the embryo within the seed when the pericarp was removed. In light of these conflicting results Villiers and Wareing (43) set out to find if, in fact, the pericarp did prolong dormancy. If so, how? They after-ripened naked seed, perforated fruits with 1 mm. squares cut from the top, and entire fruits. They measured the ratio of embryo length to seed length during warm temperature after-ripening.

They found that embryos within the naked seeds elongated faster than those in perforated fruits which elongated faster than those in entire fruits. They suggested that the pericarp retards the development of the embryo by restricting the oxygen supply to the seed. This could be ascribed to the deleterious effect of the film of moisture which the pericarp maintained over the seed.

The environmental conditions which best effect after-ripening are unclear. However, a medium which provides abundant aeration and much moisture must be necessary. Lindquist (29) found that Caragana seeds germinated fastest and best after they had been after-ripened in sand with 5 to 10% moisture. Another worker (15) found that moist untreated granulated peat moss at pH4 was superior to sand, muck or peak moss adjusted to more acid or alkaline pH, for after-ripening seeds of Sorbus aucuparia.

More has been reported about the temperature of after-ripening than any other factor. With black ash the need for a dual treatment has been noted (38,42). First, the immature embryo must enlarge by cell division and elongation. This is best accomplished at 18 - 20°C. After that, storage at 5°C promotes after-ripening changes. For Sorbus aucuparia seeds Flemion (15) suggested a constant temperature of 1°C or alternating temperatures between 1 and 5°C. The favourable effects of a non-constant temperature may result from creation of a balance of the intermediate materials of respiration (39). Davis (12) concluded that the best after-ripening temperature for Crataegus mollis is 5 - 6°C. Due to specific requirements of individual species these scattered pieces of work still leave the seed physiologist wondering what conditions best effect after-ripening.

MATERIALS AND METHODS

The seeds of Fraxinus nigra, borne in a single samara, usually ripen by late September in Winnipeg. At that time the flattened seed measures approximately 16 mm. x 4 mm. in size. It contains a morphologically complete embryo measuring about one half the length of the seed. The seed used for the following program was collected from Winnipeg boulevard trees on September 29th, 1970 and October 2nd, 1972. The first collection was used for determining germination percentage, embryo elongation and embryo dry weight change; the second collection was used for metabolic studies. Immediately following harvest, the seeds were sorted. Those seeds which had been bored by a Hymenoptera wasp or had been destroyed by other means were discarded. The remaining visually sound seeds were stored dry, samara intact, in a plastic bag at $4^{o}C$ until they were needed. Unless otherwise mentioned complete after-ripening implies storing seeds in moist peat (approximately 75% moisture) for 18 weeks at $21^{o}C$ followed by 18 weeks at $4^{o}C$.

Embryo Elongation Measurements

Triplicate samples each containing 15 seeds were removed from the after-ripening packages at biweekly intervals. The embryos were isolated from the seeds so that the lengths of the embryonic axes and cotyledon pairs could be measured. The accuracy of each measurement was increased with the aid of a 10x hand lens.

Embryo Dry Weight Measurements

Every 2 weeks during after-ripening, triplicate samples of 25 seeds were taken from the after-ripening packages, the pericarps were removed and the embryos were excised. With precision the cotyledons and embryonic axes were cut apart and placed in separate weighing containers, then were oven dried at 100^{o}C for 24 hours. At the end of that time they were cooled in a desiccator and then weighed accurate to 10^{-1} mg. During this complete procedure extreme care was exercised to ensure that the embryo parts were obtained free of peat moss and other foreign materials.

Germination Tests

At biweekly intervals during after-ripening 20 seeds from each of 3 replicates were taken from their respective packages. Depericarped seeds were placed between single layers of Whatman #1 filter paper in captan dusted, 9 cm., petri dishes. Each dish was moistened with 3.0 ml. of distilled water (i.e. enough water to moisten the filter paper and leave a few drops of free water). The seeds were incubated in a dark incubator at 20 - 21^{o}C and 100% R.H.

Further germination tests, carried out both in greenhouse pots and in petri dishes, were performed on seeds which had been after-ripened for 18 weeks at 21^{o}C. In addition to being partially after-ripened, they were given supplementary treatments involving water leach, 500 ppm or 250 ppm GA_3 and 20 ppm kinetin. Treatment effects and interactions were assessed using a factorial design.

Leaching was accomplished by placing the seeds in a 5 x 15 cm. screen cylinder and running cold tap water through the cylinder at a rate of approximately 8 l./min. for 24 hours. When a combination of treatments was used, leaching always preceded chemical application.

The method of chemical application in the greenhouse tests was different from the method used in the germinator tests. Those seeds intended for greenhouse planting were soaked in the chemical solution for 24 hours before they were planted. On the other hand, seeds which were plated in petri dishes were moistened directly in the dish with 3.0 ml. of chemical solution and were left in that solution for the duration of the test. The only other difference was that a GA_3 concentration of 500 ppm was applied to seeds which were to be potted in the greenhouse while only 250 ppm was applied to seeds plated in petri dishes.

Quantitative Lipid Analysis

Four replicates of 100 seeds were used to determine the total oil content. This material was dried for 24 hrs. at 110°C. On cooling it was coarsely ground in a miniature blender cup. The ground material was weighed and then placed in Swedish tubes for extraction according to the method devised by Troeng (40). The tubes were filled with 40 ml. of Skelly F (petroleum ether) and then were shaken overnight. When extraction was complete, the contents of the tubes were transferred to centrifuge tubes and centrifuged at 2000 rpm. for 10 min. After centrifugation, a 20 ml. aliquot of the oil containing solvent was pipetted from each tube to tared beakers. The solutions were evaporated carefully on a hotplate. Finally,

the oil was dried for 2½ hrs. at 110°C., cooled in a desiccator, and then weighed. In calculating the total oil content it was assumed that the solution volume occupied by the oil was not significant.

Respiration Study

Oxygen uptake at 25°C was used as a measure of respiration and was determined by a Gilson respirometer. This machine operates on the basic principle that at constant temperature and pressure any changes in gas volume resulting from biological activity are recorded directly in microliters by a volumometer (1).

Embryos to be measured for rate of oxygen uptake were removed from after-ripening packages, excised, weighed, and stored in the prepared reaction flasks at 4°C until respiration was measured. The embryos were incubated in 4.5 ml. of 0.1 M. HEPES buffer adjusted to pH 7.0. Carbon dioxide was trapped in the center well by 0.3 ml. of 1.0 N KOH. Once the flasks were secured in place, the system was allowed to equilibrate externally for 30 min. This was followed by an internal equilibration for 2 hrs. before time 0 was observed and recording began. After 3 hrs. of measuring time, oxygen uptake was recorded and the final pH of the incubation medium was checked. Each run included duplicated flasks of non-after-ripened embryos which served as a control to correct for run to run variation.

Pathway Study

The relative contribution of the pentose phosphate (PP) and Embden-Meyerhoff-Parnas (EMP) pathways to the degradation of glucose in black ash

embryos at selected intervals during after-ripening was determined using the C-6/C-1 ratio method (4). The general procedure and conditions for embryo preparation and incubation were the same as those indicated for the respiration study except for the following changes. Firstly, 5μ moles (0.3 μ C.) glucose - 1-C^{14} or glucose - 6-C^{14} was added to the incubation mixture. Secondly, 0.3 ml. of ethanolamine was used in the center well instead of KOH.

At the end of 3 hrs. both the ethanolamine and two methyl cellosolve washings were removed from the center well and transferred to a liquid scintillation vial. Then the radioactivity in each sample was counted by liquid scintillation spectrophotometry using 10 ml. of toluene-methyl cellosolve scintillation solvent prepared as follows:

Toluene---------- 667 ml.
Ethylene glycol monomethyl ether (methyl cellosolve) ---333 ml.
2:5 Diphenyloxazole (PPO)----------5.5 g./l.

Quenching was corrected by the channel ratio method.

RESULTS

Effect of After-Ripening on Germination

Seeds were after-ripened for 18 weeks at 21°C and then transferred to 4°C for cool temperature after-ripening. At biweekly intervals triplicate lots of 20 seeds were removed. They were placed on moist filter paper in petri dishes in the germinator at 20°C and 100% R.H. When the radicle protruded from the seed coat, the seed was considered non-dormant. In addition at three stages triplicate lots of 20 seeds were placed in moist soil in pots in the greenhouse and the percent germination calculated from emerged seedlings. By either germination technique the total germination after 28 days is shown in Table 1. No germination was recorded in the early stages of after-ripening. In the later stages percent germination continued to increase until the end of the after-ripening period. In some way conditions in the petri dishes were not ideal for germination. The seeds in petri dishes absorbed a film of water to their surface. This may have deleteriously restricted the oxygen supply to the embryo. As well, it could be suggested that some inhibitor must be removed before germination proceeds. Possibly in the soil it could be removed by leaching or by chemical reaction with the soil.

Effect of Gibberellin, Kinetin and Leaching on Germination

Seeds which had been partially after-ripened for 18 weeks at 21°C were treated with gibberellin, kinetin and running water (leach) in order to replace the cool after-ripening. These treatments were administered

Table 1. Germination response to after-ripening

After-ripening stage	Percent germination: Seeds on moist filter paper	Percent germination: Seeds in moist soil
non-after-ripened	0	-
2 wks. at 21°C	0	-
4 " " "	0	-
6 " " "	0	-
8 " " "	0	-
10 " " "	0	-
12 " " "	0	-
14 " " "	0	-
16 " " "	0	-
18 " " "	0	-
18 wks. at 21°C + 2 wks. at 4°C	0	-
" " " " + 4 " " "	0	-
" " " " + 6 " " "	0	-
" " " " + 8 " " "	0	-
" " " " +10 " " "	0	-
" " " " +12 " " "	5	-
" " " " +14 " " "	2	-
" " " " +16 " " "	10	-
" " " " +18 " " "	15	68
" " " " +20 " " "	37	-
" " " " +22 " " "	27	80
" " " " +24 " " "	38	87

separately and in combination. In each case even after 28 days no seeds germinated. This lack of germination probably indicates that either these seeds do not respond to these chemicals at this stage or else the improper chemical concentrations were used.

Changes in Embryo Length

At biweekly intervals during after-ripening embryos were excised; the embryonic axes and cotyledons were measured separately. The lengths recorded in Table 2 represent the mean lengths of 3 replicates. According to the analysis of variance the differences between the treatments were highly significant. It is noteworthy that the embryonic axes and cotyledons showed similar trends in their growth pattern. They both grew significantly during the warm treatment with the major increases coming near the middle of that period. Despite the similar trends observed, it is quite noticeable that the cotyledons enlarged much more than the embryonic axes with the maximum percent increases being 101.6 and 50.9% respectively. By the time the warm temperature after-ripening was complete, the embryo had attained the full size of the enclosing seed coats. Then during the following cool treatment no significant enlargement took place in either the axes or the cotyledons.

Changes in Embryo Dry Weight

The changes in embryo dry weight were followed throughout the after-ripening period. Significant changes as shown in Table 3 occurred during the warm temperature after-ripening. Although the changes in dry weight

Table 2. Changes in embryo length during after-ripening

After-ripening stage	Embryonic axis		Cotyledon	
	Length (mm.)	% of increase	Length (mm.)	% of increase
non-after-ripened	4.70	0	4.40	0
2 wks./21°C	4.77	1.5	4.50	2.3
4 " "	4.90	4.3	4.70	6.8
6 " "	5.23	11.3	5.20	18.2
8 " "	5.70	21.3	6.00	36.4
10 " "	6.37	35.5	7.37	67.5
12 " "	6.60	40.4	7.60	72.7
14 " "	6.67	41.9	8.03	82.5
16 " "	6.90	46.8	8.63	96.1
18 " "	7.03	49.6	8.50	93.2
18 wks./21° + 2 wks./4°	6.87	46.2	8.53	93.9
" " " 4 " "	6.97	48.3	8.87	101.6
" " " 6 " "	6.99	48.7	8.76	99.1
" " " 8 " "	6.84	45.5	8.64	96.4
" " " 10 " "	6.98	48.5	8.79	99.8
" " " 12 " "	7.02	49.4	8.53	93.9
" " " 14 " "	6.99	48.7	8.45	92.0
" " " 16 " "	7.09	50.9	8.78	99.6
" " " 18 " "	6.87	46.2	8.66	96.8

followed a trend similar to the changes in embryo length, the actual percent change in dry weight was much greater. Again with this measurement, the cotyledons grew much more than the embryonic axes. The embryonic axes showed a 132.6% maximum increase while the maximum cotyledon increase was 340.7%.

Quantitative Determination of Lipids

A determination of total non-polar lipids in the whole seed at 9 week stages during after-ripening was made. The results of the 4 replicates are given in Table 4. These results show that the overall oil content in the seed remains quite constant until the final weeks of after-ripening. At that time there is a significant decrease (5% level of significance) in the oil content.

The apparent high oil content after 9 weeks at 21^{o}C should have no physiological basis. Because the readings are not significantly higher than the adjacent readings they can be explained only as experimental error. Possibly the oil was not quite dry when it was weighed.

Respiration Studies

Measurements of oxygen uptake by after-ripening embryos were made using a Gilson respirometer. On each run two flasks each containing 25 non-after-ripened embryos were included as controls. Each day the duplicate values for the controls were similar, whereas from day to day the controls varied as much as 51%. This variation was noticeable as well in the after-

Table 3. Changes in embryo dry weight during after-ripening

After-ripening stage	Embryonic axes		Cotyledons	
	Weight per 25 (mg.)	% increase	Weight per 25 (mg.)	% increase
non-after-ripened	21.8	0	17.2	0
2 wks./21°C	21.9	0.5	17.3	0.6
4 wks. "	22.5	3.2	18.1	5.2
6 " "	25.0	14.7	23.2	34.9
8 " "	31.4	44.0	35.5	106.4
10 " "	41.0	88.1	49.7	189.0
12 " "	44.2	102.8	57.0	231.4
14 " "	46.0	111.0	63.1	266.9
16 " "	47.5	117.9	67.4	291.9
18 " "	50.7	132.6	69.2	302.3
18 wks./21° + 2 wks./4°	49.6	127.5	69.9	306.4
" " " 4 " "	50.4	131.2	75.8	340.7
" " " 6 " "	48.7	123.4	69.7	305.2
" " " 8 " "	48.2	121.1	68.5	298.3
" " " 10 " "	48.0	120.2	73.9	329.6
" " " 12 " "	48.9	124.3	71.8	317.4
" " " 14 " "	49.8	128.4	72.3	320.4
" " " 16 " "	50.7	132.6	75.4	338.4
" " " 18 " "	49.6	127.5	74.4	332.6

Table 4. Changes in oil content during after-ripening

After-ripening stage	Oil content (% of dry weight)				
	Replicates				
	1	2	3	4	mean
non-after-ripened	27.22	27.67	27.47	27.50	27.46[a]
9 wks./21°	28.90	28.88	28.18	28.65	28.65[a]
18 wks./21°	27.31	28.53	26.85	27.61	27.58[a]
18 wks./21° + 9 wks./4°	27.80	25.69	28.24	28.32	27.51[a]
18 wks./21° + 18 wks./4°	24.54	24.70	24.54	28.22	25.50[b]

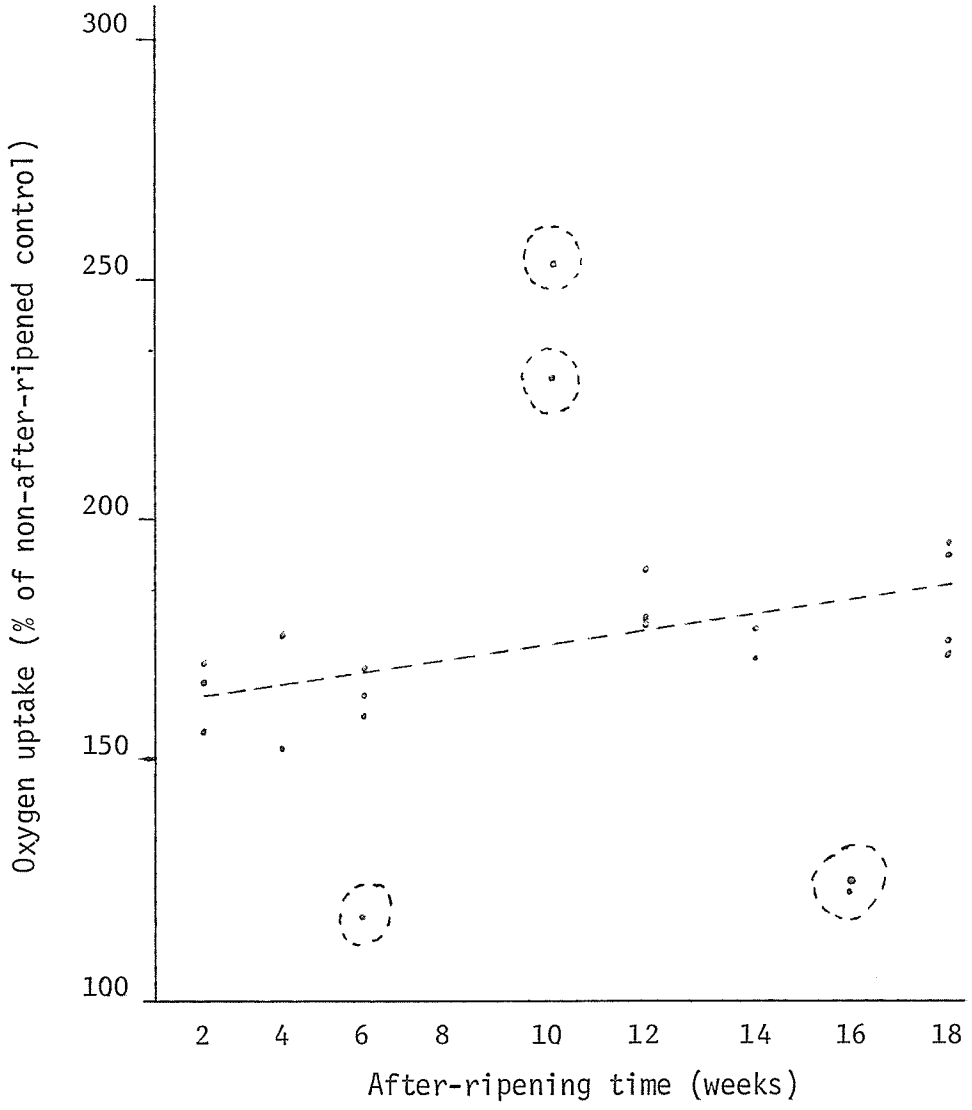
a differs from b at the 5% level of significance

ripening embryos. Consequently, the daily controls were used to remove variation from the results by converting respiration values from µl of O_2 uptake/100 mg./hr. to percent of control. Using these percent values regression lines were calculated to fit the scattered points. The results are presented in Figure 1 and Figure 2. On these graphs several points circled with broken lines fell outside the accepted limits of variability. Such points were not used for calculating the regression lines. The results show clearly a radical increase (i.e. greater than 60%) in respiration rate during the first 2 weeks of after-ripening. Thereafter throughout the warm period increases in respiration rate were only small, amounting to less than 1% per week. However with cool temperature after-ripening a different response appears to be elicited because during an 18 week interval respiration rate increased nearly 90% from non-after-ripened control. This means that during after-ripening the embryo's capacity for oxygen uptake changed from 21.6 to 56.2 µl./100 mg./hr.

Pathway Study

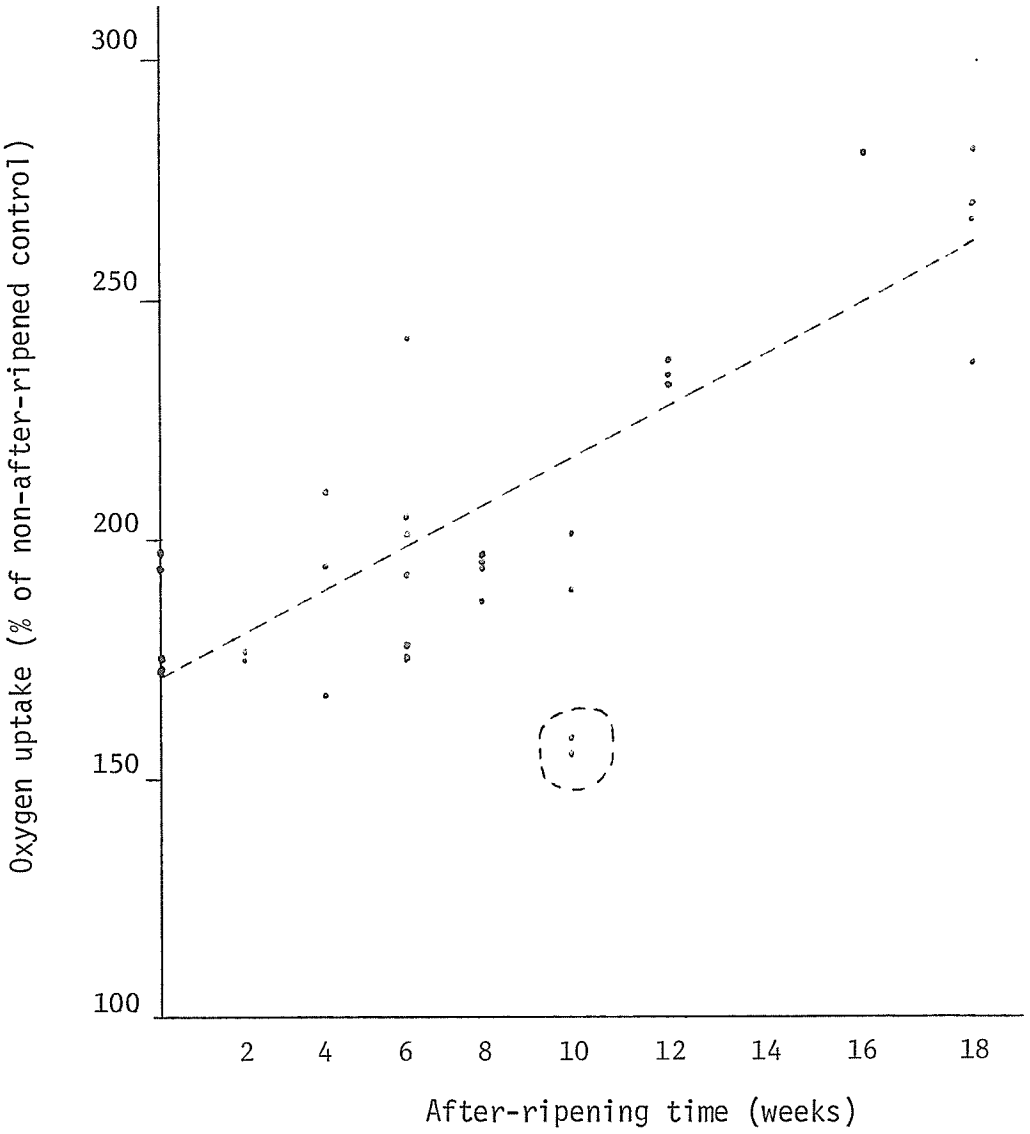
Glucose may be oxidized to carbon dioxide via the EMP pathway and the TCA cycle or alternatively through the PP pathway. If glucose is metabolized through the EMP pathway, its C-1 and C-6 carbons become distributed evenly between two - three carbon fragments which are degraded to pyruvate. From pyruvate the C-1 and C-6 carbons of the original glucose molecule enter the TCA cycle and finally are evolved at random in the form of carbon dioxide. On the other hand, degradation via the PP pathway distinguishes between C-1 and C-6. Very early in the pathway C-1 is evolved in the form of carbon

FIGURE 1. Embryonic oxygen uptake during warm temperature after-ripening.



r = .68
b = .136

FIGURE 2. Oxygen uptake during cool temperature after-ripening of embryos previously after-ripened at warm temperature for 18 weeks.



r = .85
b = .492

dioxide, whereas C-6 is retained for at least several hours in a series of intermediates.

By using paired respiration flasks containing either glucose-1-C^{14} or glucose-6-C^{14} and by collecting and counting the amount of radioactivity released after several hours, one can construct a C-6/C-1 ratio for measuring the participation of the EMP and PP pathways. C-6/C-1 ratio of unity implies complete oxidation via EMP pathway. Decreasing values for C-6/C-1 ratios imply increasing participation of the PP pathway.

The results given in Table 5 show C-6/C-1 ratios at selected intervals throughout after-ripening. They indicate that the PP pathway is important throughout after-ripening. Its relative contribution remains quite constant throughout. The results presented contain much variability. This variability can best be attributed to varying degrees of cycling in the PP pathway.

Table 5. C-6/C-1 ratios of embryos at different stages of after-ripening

After-ripening stage	Number of Observations	C-6/C-1 Ratio[a]	
		Mean	Range
non-after-ripened	6	.47	.10
18 weeks/21°C	5	.51	.39
18 wks./21°C + 18 wks./4°C	3	.62	.15
18 wks./21°C + 19 wks./4°C	4	.40	.27
18 wks./21°C + 20 wks./4°C	2	.42	.05
18 wks./21°C + 24 wks./4°C	3	.49	.28

a values given on basis of fresh weight

DISCUSSION

At the time of fruit abscision the seed of black ash has two inherent delays to germination. The after-ripening process first matures the embryo and then breaks the embryo dormancy. Maturation of the embryo is characterized by growth and differentiation of the embryonic axis and cotyledons. According to Table 3 growth consists of an increase in dry weight of more than 300%. The largest change occurred in the cotyledons. This can probably explain why the cotyledons visually showed the most differentiation. They changed from mere sheets of parenchyma tissue to differentiated organs clearly containing a vascular system. This type of growth and development also occurred in Ilex seeds (19) where the embryo was very immature when the berry fell from the tree. It developed by growth and differentiation from a spherical mass of tissue to an organism containing a hypocotyl, root axis and cotyledons. Under moist conditions at 25°C the embryo developed constantly, although slowly until the hypocotyl broke through the seed coats. Generally a temperature of 20-25°C seems suitable for embryo maturation (38,42).

Measurements of embryo weight and length seem consistent in this study. The measured percent change in embryo weight (Table 3) was nearly twice as great as that for embryo length (Table 2). This implies, confirming visual observations, that lateral and radial growth occurred.

The source of energy for embryonic development remains somewhat uncertain. The fact that embryo dry weight increased dramatically during

embryo maturation suggests that reserves must be mobilized from the endosperm and enclosing seed coats. The high oil content of the seed (greater than 25% of dry weight) may suggest that the primary reserves come from the lipid fraction. However, no significant decrease in oil content was observed while the embryo was growing rapidly during the warm temperature treatment. Possibly this was due to the variability intrinsic within the method of quantitative oil determinations. Furthermore the overall seed weight at the beginning of after-ripening was more than 20 times greater than the embryo weight. This would lead to a comparatively large lipid reserve which could be used by the embryo without significantly lowering its concentration. Most of the oil may eventually be used so that after germination the endosperm and seed coats contain a negligable quantity of the valuable energy reserve.

From the results of oxygen uptake studies it becomes apparent that two separate changes are effected during after-ripening. During the 2 to 18 week interval of warm temperature maturation, the rate of oxygen uptake per milligram of fresh weight increased very slowly. However, there was a 60% increase in oxygen uptake during the first 2 weeks which cannot be explained by present data because no readings were taken in the 0 to 2 week time interval. It seems likely that the increase would come after enough time had been allowed for lipid degradation to free sugars and subsequent sugar mobilization to the site of respiration (27).

The respiration rate of mature embryos increased 90% during cool after-ripening. Throughout this entire period the embryonic dry weight increased less than 10%. What, then, is the purpose of active respiration?

Firstly, some activity is necessary to maintain the structure and integrity of the tissue. Certainly none of the 90% increase should be needed for fundamental upkeep of the system. This leaves the possibility that this metabolism is used for redistribution of dry weight. According to previously cited literature (3,5,13) this period is characterized by buildup of nucleotides, proteins and free amino acids. In order to get this buildup there must be a shift in enzyme activity. Since each enzyme has its own temperature range for activity a cool temperature exposure may be necessary to change the relative activity of participating enzymes. In this way a supply of active intermediates needed for biosynthesis could be built-up in preparation for germination.

Often termination of dormancy results in a dramatic upsurge in respiration capacity (39). This was not noticed with black ash embryos (Figure 2). As dormancy terminated the respiration capacity increase continued steadily with no abrupt change. These results seem to suggest that a threshold respiration capacity near 25°C of non-after-ripened embryos accompanies germination. The respiration capacity measured at 25 °C gives an indication of the respiration rate in an environment favourable for germination rather than the real rate at the usual after-ripening temperature of 4°C. It should be noted that the respiration capacity would be much greater than the after-ripening rate at 4°C.

The C-6/C-1 ratio method was used to estimate the relative participation of the PP and EMP pathways to glucose breakdown. The ratioactivity of the CO_2 collected from paired flasks using glucose - 1-C^{14} and glucose - 6-C^{14} was determined. A C-6/C-1 ratio of unity implies catabolism strictly

by the EMP pathway and TCA cycle; values decreasing from one imply increasing PP pathway participation. All the values obtained from black ash embryos at different stages of after-ripening (Table 5) indicate considerable PP pathway activity. Its relative importance remained constant throughout after-ripening. These results, in addition to measurements of respiration capacity, show that in black ash a change in metabolic pathway does not accompany after-ripening and dormancy termination.

Although the paired flask method is currently used quite widely for studying metabolic pathways, it can be criticized on several points. It takes no account of selective assimilation of C-1 and C-6 into cellular constituents. Jaswal (20) found that C-6 was incorporated preferentially into amino acids, sugars and the insoluble fraction. If that were true in black ash embryos, the importance of the PP pathway would be over estimated.

A C-6/C-1 ratio measured at 25^{o}C may indicate a regime which is quite different from that functioning at 4^{o}C during after-ripening. This may be suggested because at 4^{o}C one or both of the alternate pathways may not be operating at maximum capacity. The ratio measured at 25^{o}C would indicate the metabolic pathway of the embryo attempting germination rather than the pathway active throughout after-ripening. Therefore, the results presented in Table 5 show only the pathways operating in an environment favourable for germination. In order to find to what extent each pathway operates during after-ripening the experiment would have to be run at 4^{o}C.

The effect of the pericarp on after-ripening embryos has often been considered. Some felt that it retarded after-ripening (2,43); others

stated that it had no effect in controlling dormancy (14,38). Its influence could probably be attributed mainly to the uniform film of water which it maintained around the seed. Whether this film retards, advances or does not affect after-ripening is open to speculation. It was decided to leave the pericarp intact for the duration of this study. Then, after 18 weeks at 21°C and 24 weeks at 4°C, 87% germination was finally attained. It can be deduced from this observation, that if the pericarp influences after-ripening at all, it would affect only the length of time required for after-ripening. No special pericarp treatment such as scarification is necessary to overcome its influence.

SUMMARY

The freshly harvested seed of black ash contains a dual mechanism for preventing immediate germination. Firstly, the embryo is immature, that is to say, its size must increase and its organs must differentiate. Secondly, after it has matured, its dormancy must be broken. These barriers to germination may be overcome by after-ripening in moist peat moss for 18 weeks at 21°C followed by 18 weeks at 4°C.

Maturation of the embryo produces physical changes in both the embryonic axis and cotyledons. Characteristically, the embryo length doubles and its weight more than triples. At the same time the cotyledons show visual signs of vascular development. To facilitate this growth and development the rate of oxygen uptake increases sharply at the very beginning of after-ripening and then remains almost constant throughout the warm period. After the embryo fully matures, it is still highly dormant and will not grow.

Termination of dormancy may be related to increasing metabolic activity in the embryo. During after-ripening the tissues enclosing the embryo which contain mainly lipid reserves supply the embryo with a respirable substrate. A large substrate supply becomes important to the changing embryo because respiration, proceeding by way of the EMP and PP pathways, increases steadily until dormancy is broken. In addition to maintaining the essential processes of living tissue, the active respiration produces biosynthetic intermediates which can be used later in germination. It is believed that germination will proceed once the embryo reaches a threshold level of metabolic activity.

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