Improvement of Seed Germination of *Fagus orientalis* Lipsky

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Abstract

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This thesis was seeking two main approaches for improvement of seed germination of oriental beech, a timber producing species in Hyrcanian forests in northern Iran. Germination behavior of beechnuts was enhanced either by decreasing the dormancy breakage period, or by increasing seed lot quality. A simple grading based on the weight of beechnuts, before exposing the dormant nuts to dormancy breaking conditions, significantly increased germination capacity of heavy class beechnuts, and reduced the period of dormancy breakage. Almost the same results were obtained by removing the endocarp. Applying alternative chilling temperatures, during dormancy breakage had positive effects on speed of dormancy release. These simple methods can be used with little equipment in forest nurseries and are suggested to be accompanied with more advanced techniques, like restricting moisture content during moist cold stratification period to gain maximum benefit.

Previous reports from European beech and the results from the effect of endocarp removal suggest a possible role of other agents in dormancy in oriental beechnuts. Water soluble phenolics extracts from the seed coats, significantly suppress the germination of radish seed. The endocarp may act as a barrier against exudation of these germination inhibitors.

The deep embryo dormancy presents problems when assessing the viability of oriental beech nuts. It is therefore possible to test germination performance in semi-dormant nuts to predict the nut viability in this species. A dormant seedlot was stored in sub-chilling conditions for 15 months and a series of germination tests were conducted during the dormancy breakage period of stored and fresh nuts. The results showed that mean germination times for both nut groups were almost the same, but germination capacity was statistically different only for semi-dormant nuts. Non-dormant stored and fresh nuts showed no significant differences, which indicate the complexity of dormancy release in oriental beech nuts. Abscisic acid (ABA) contents of embryonic axes of stored and fresh nuts were measured during the dormancy breakage period, and results indicated a close correlation between ABA levels and increment in germination capacity as dormancy was released.

Near infrared spectroscopy (NIRS) combined with partial least squares regression (PLS) were used as rapid and non-destructive methods for discrimination of sound and deteriorated single beechnuts. NIRS-PLS is a promising method for quality improvement of nearly all agricultural products and in this study showed 100% accuracy in separation of viable and non-viable nuts.

Key words: oriental beech, seed germination, seed dormancy, Seed storage, dormancy breakage, seed viability, phenolics, Abscisic acid, near infrared spectroscopy.

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Appendix

This thesis is based on the following papers, which are referred by roman numbers.

I. Soltani, A., Lestander, T., Tigabu, M. & Odén, P. C. 2003. Prediction of viability of oriental beechnuts, *Fagus orientalis*, using near infrared spectroscopy and partial least squares regression. *J. NIR Spectrosc.* (Submitted).

II. Soltani, A., Tigabu, M. & Odén, P. C. 2003. Alleviation of Physiological Dormancy in Oriental Beechnuts with Cold Stratification at Controlled and Unrestricted Hydration. *Seed Sci. & Technol.* (Submitted).

III. Soltani, A. & Odén, P. C. 2003. Changes in ABA levels during cold moist stratification and mid-term storage in oriental beechnuts (*Fagus orientalis* Lipsky). Manuscript.

IV. Soltani, A. 2003. Phenolics in the Seed Coat of Oriental Beech (*Fagus orientalis* Lipsky) as an Autotoxic Factor. Manuscript.

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Introduction

Oriental beech

Oriental beech (*Fagus orientalis* Lipsky) belongs to the Beech family (Fagaceae) and is closely related to its European counterpart (*F. sylvatica* L.) (Gömöry *et al.,* 1995). Some authors even consider it as a subspecies (*Fagus sylvatica* subs. *orientalis* Greuter. & Burdet.) of European beech. It is a large tree, normal mean heights in Iran are *ca.* 35 m but it can reach heights of up to 50 m, with dark grey bark and fluted bole, upswept ovoid crown with many narrow branching forks. The leaves are long-cuneate, obovate, often cupped and whole. The female inflorescence stalk is longer (8 cm), the leaves are larger (4-8 × 7-14 cm) and the number of veins in each leaf (9-14 couples) is higher than in European beech (Sabeti, 1993; Alan & Wilkinson, 1999) (Figure 1).

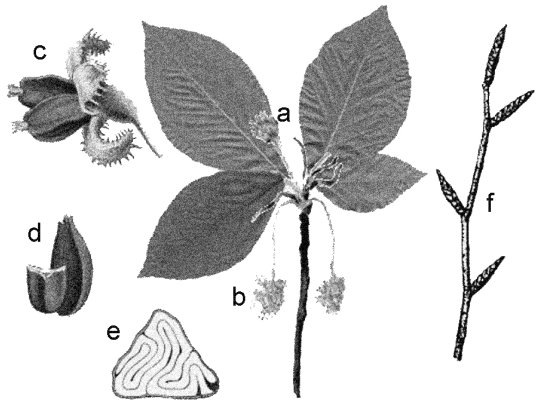

Figure 1. Oriental beech: erect female inflorescence (a), hanging male catkins (b), mature spiky cupule containing 2 nuts (fruits) (c), triangular cross section of a fruit (d), cross section of a seed with characteristic type of cotyledons folding in seed (e), long spindle-shaped buds in winter (f).

In Iran, beech flowers appear in mid to late April, about the same time leaves begin to emerge. The species is monoecious and unisexual. Male flowers occur in hanging globular catkins and female flowers in clusters of 2-4. The flowers are susceptible to late frosts in spring. Fruit is a pyramidal nut (beechnut), with a triangular section and about 1.5 cm in length, enclosed in a spiky cupule. The cupule opens by valves, which are one more than fruit number. The endocarp (inner layer of the pericarp) is a thick, smooth and shiny dark brown, hairy inside husk. Its color lightens as the beechnut dries. Seeds are non-endospermic (Figure 1).

Compared with many other forest tree species the genetic diversity of beech is high (Comps *et al.,* 1998). It is wind pollinated with an outcrossing rate varying

between 0.90 and 1.00. The gene flow is limited due to the high density within stands, favoring mating between closely spaced individuals (Merzeau *et al.*, 1994).

The extreme eastern border of oriental beech forests is Ziârat valley near Gorgân town, on the southern coast of the Caspian Sea in Iran. These forests extend westwards as separated strips towards Caucasia, Asia Minor and north of Greece, Bulgaria and Romania (Figure 2). Oriental beech is replaced by European beech (*F. sylvatica*) in central and west Balkans (Sabeti, 1993; Bektas *et al.*, 2000). In the Hyrcanian phytogeographical region in northern Iran, oriental beech forests cover the northern slopes of the Alborz Mountains, at about 680-2000 m above sea level (asl) and annual precipitation between 800 to 1800 mm, decreasing from West to East. These forests consist of three phytosociological associations: Fagetum, Fageto - Carpinetum and Carpineto -Fagetum, dominated by 2 main species *Fagus orientalis* and *Carpinus betulus* L.

Wood from oriental beech is commonly used for furniture, panelling, package boxes, tool handles, desks, fuel wood, mine props, and as timber sleepers impregnated with preservatives.

Silviculture of oriental beech in Iran

Hyrcanian commercial forests in Iran were nationalized in 1963. Since then, the area has declined significantly from 3.4 to less than 1.3 million ha in 1998. Mixed and pure stands of beech occupy less than 20% of the Hyrcanian forests and produce more than 35% of the total wood stock volume. A high proportion, *ca.* 86%, of the trees is at least 100 years old and in some areas regeneration is non-existing (Fishwick 1972, Anonymous 2000).

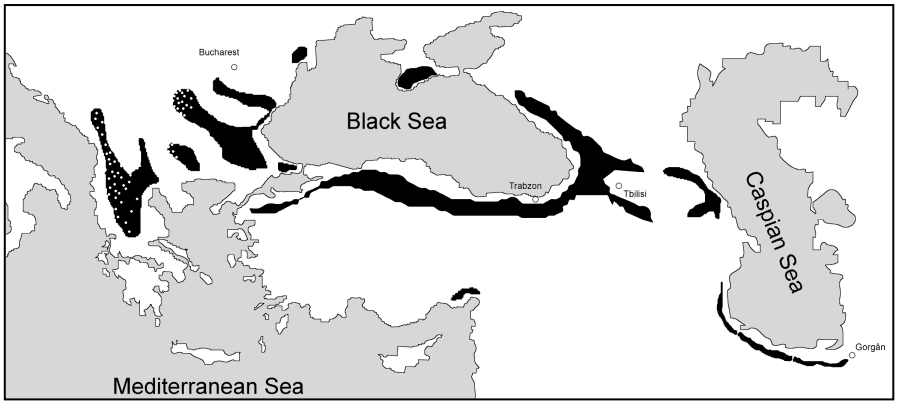

Figure 2. Ranges of oriental beech.

Beech and beech-hornbeam forests in Iran are considered as even-aged forests and managed mostly by shelterwood silvicultural system. Naturally as succession

progress the beech-hornbeam stands transform into pure beech stands since beech is shade tolerant and hornbeam is of intermediate shade tolerance, but this process seems to be inversed now. Most of the beech dominated forests suffer from human interference and are continuously decreasing in area. The rotation period for these forests is 120-125 years and complete natural regeneration is seldom achieved (Sagheb-Talebi & Schütz 2002). Unsuitable harvesting methods during last 30 years and lack of forest protection are the two main technical reasons for failure of the shelterwood system in these forests (Hosseini *et al.,* 2000). In addition, infrequent mast years occurring at intervals of five to eight years, closed canopies that rarely produce a fertile seed-crop and a high proportion of infested and predated nuts, are natural reasons that adds to the regeneration failure in beech forests (Linnard 1987, Shimano & Masuzawa, 1998). This incomplete regeneration is common also in European beech forests, which are successfully managed by shelterwood system and artificial plantation is prescribed to assist natural regeneration (Teissier du Cros, 1984).

Nearly all forest nurseries in the Hyrcanian region in Iran are located at low altitudes and few are found at levels above 1000 m asl. These nurseries were originally designed mainly for seedling production of exotic species and despite the increasing demand, currently only few oriental beech seedlings are produced due to the serious seed handling- and dormancy problems of beechnuts.

Seed dormancy

Dormancy is the state of reduced metabolic activity adopted by many organisms under conditions of environmental stress or when such stressful conditions are likely to appear. For instance, most plant species in temperate regions undergo a dormant condition before winter sets in.

Seed germination is the most critical part in the life cycle of seed bearing plants and seed dormancy is an excellent capability to increase the chance of survival by optimizing the distribution of germination in time or space (Foley & Fennimore, 1998). Seed dormancy means that the seeds are alive but they need something more than just water and permissible temperatures before they start to germinate. This inhibition of germination is caused by one or more of the following three mechanisms: 1- Chemical inhibitors that prevent growth. 2- Physical barriers that prevent the uptake or the movement of water, gases or chemicals within the seed. 3- The embryo of the seed is not fully developed and needs time after dispersal to ripen (Bewley & Black, 1994 and Baskin & Baskin, 1998).

It is not yet understood how to measure the depth of dormancy in individual seeds, but the variation can be reflected by the behavior of the seed population. For example, the degree of loss of dormancy during after-ripening of dry seedlots can be considered as a dormancy index (Murdoch & Ellis, 2000). On the other hand, the mechanisms of seed dormancy in species like oriental beech are still to be elucidated and, therefore, the classification of the different types of dormancy is

entirely based on its expression under various conditions (see Bewley & Black, 1994 and Baskin & Baskin, 1998). Thus, it is proposed to first distinguish two types of dormancy; primary and secondary (Karssen, 1982; Amen, 1986). This classification is based on timing. The primary dormancy is the state of the seed as shed from the mother plant. It helps to prevent precocious germination. The secondary dormancy is induced in a mature, imbibed seed by certain environmental conditions which are unfavorable for germination (Bewley & Black, 1989).

The level of primary dormancy in seeds is determined by several factors of genetic and non-genetic origin (Andersson & Milberg, 1998). All of these factors may cause physiological variability which is matched with differences in seed morphology (size, weight, color *etc.*) or simply heterogeneity in degree of dormancy (Bewley & Black, 1994). Therefore dormancy levels vary within the seedlot of many species among them *Fagus* spp.; a few nuts germinate without cold moist stratification or prechilling, but others in the same lot will not germinate until they are prechilled. In addition, during prechilling and dormancy release, the heterogeneity usually increases (Derkx & Joustra, 1997).

Dormancy in beechnuts

European beech nuts possess a deep embryo dormancy resulting in an enormous waste of nuts and poor seedling production in nurseries (Muller & Bonnet-Masimbert, 1989). This embryo dormancy inhibits germination of viable, mature embryos even when isolated from the seed and subjected to conditions suitable for germination (Bewley & Black, 1994; Bianco & Le-Page-Degivry, 2000).

Termination of embryo dormancy is achieved by cold moist stratification or prechilling, *i.e.* subjecting hydrated nuts to temperatures between +2 till +5°C with or without medium (El-Antably, 1976; Suszka, Muller & Bonnet-Masimbert, 1996). The time period necessary to complete dormancy release is usually quite long, ranging from 5 to 8 weeks and in some cases even up to 12 weeks (Muller & Bonnet-Masimbert, 1982).

A procedure developed by Suszka (1974) to increase efficacy and decrease heterogeneity in dormancy release involved controlled and restricted hydration of the nuts to a predetermined moisture content (m.c.). The fully imbibed nuts were subjected to +3°C and the time in weeks, X, necessary to achieve 10% germination was recorded. X represents the dormancy index of the lot. The dormancy of the whole lot is then broken by prechilling the nuts at a controlled and restricted m.c. of 30-34% without medium for X+2 weeks (X+4 weeks if dormancy is very deep) at +2 till +5°C. The restricted moisture content and low temperature prevents precocious germination. This treatment can be applied both before and after storage (Muller 1993).

Although this method has proved to be very effective in decreasing heterogeneity of stratified beechnuts, there are still controversial results from

different authors with different seedlots, moisture contents and prechilling temperatures. For a review see Gosling, (1991) and Derkx & Joustra, (1997). On the other hand the period necessary for dormancy release is not substantially decreased by the treatment and furthermore, controlled and restricted m.c. of *ca.* 30% without medium can not be achieved without the addition of fungicides (Muller *et al.,* 1999). Experience also shows that some fungicides can have harmful effects on viability of beechnuts (Suszka *et al.*, 1996).

Successful results in decreasing the time period of prechilling or cold moist stratification and increasing the efficacy of dormancy release have been obtained by using exogenous gibberellins (Nicolás *et al.*, 1996; Fernandez *et al.,* 1997) and ethephon (Falleri *et al.*, 1997). However, no results from practical handling in nurseries have yet been reported.

Abscisic acid (ABA) is of some authors considered as the plant hormone responsible for inducing and maintaining dormancy in European beechnuts (Nicolás *et al.*, 1996; Lorenzo *et al.*, 2000, 2001). It has been shown that addition of ABA reverses the effect of prechilling on dormancy release by preventing the synthesis of germination-specific RNAs and proteins (Nicolás *at al.*, 1996; 1997) and also that the ABA content in embryonic axes of European beech decreases during dormancy release (Le Page-Degivry *et al.*1997b). Other dormancy mechanisms may however be active.

For European beechnuts, Nicolás *et al.*, (1996, 1997), Thomsen (1997) and Shen & Odén (2002) showed that germination capacity and dormancy release rates were amplified by removal or mechanical scarification of the endocarp. These results are clues for a probable role of the endocarp in modifying dormancy of European beechnuts and may be explained by an effect of the hard endocarp as a mechanical barrier restricting entry of water, leakage of exudates and protrusion of the radicle. The role of seed coat/pericarp in restricting the germination of seeds is widespread in tree and shrub species and is often referred to as physical dormancy (Baskin and Baskin 1998). The seed coat or pericarp can inhibit seed germination by various mechanisms such as preventing gas exchange, water uptake, light penetration, or escape of inhibitors from the embryo (Taylorson & Hendricks, 1977). The seedcoat and to some extent the pericarp may itself contain inhibitors that block the germination process (Bewley & Black 1994). One main group of inhibitors in exudates has been characterized and referred to as oxidized phenolics (Marbach & Mayer, 1975; Werker *et al.*, 1979). Histochemical studies (Thompson *et al.*, 2001) and practical techniques (Bhattacharyya *et al.*, 1999) also revealed the presence of phenolics in the seed coat and their role in inhibition of seed germination.

Seed viability and seed deterioration

Seed viability is an indication of the capability of seeds to germinate and produce normal seedlings under suitable germination conditions (Copeland & McDonald,

2001). It has long been known that three factors; temperature, seed moisture content and oxygen pressure, are most important for viability and longevity of seeds in storage. In general, the lower the temperature and moisture content the longer the period of viability.

Based on storage behavior and the ability in maintaining viability, Roberts (1973) defined two seed classes, recalcitrant and orthodox seeds. Recalcitrant seeds must retain relatively high moisture content, usually more than 30% m.c., in order to maintain maximum viability. Species with recalcitrant seeds include oaks, sweet chestnuts, many rainforest tree species and some aquatic plants. Even when these recalcitrant seeds are stored under moist conditions their longevity is often quite short, and only occasionally exceeds more than a few months.

The majority of plants have orthodox seeds and it seems they conform to certain rules of thumb that predict well the pattern of loss of viability in relation to storage environment. A number of investigations have devised mathematical models to relate the viability of orthodox seeds to their storage environment. For example Ellis & Roberts (1981) have developed a seed viability equation, which later was improved by others (Ellis *et al.*, 1993; Stahl & Steiner, 1998). The formula has proved to be valid for a number of orthodox seeds, including seeds from forest trees (Dickie *et al.*, 1990; Zewdie & Ellis 1991), and can be used to predict the percentage viability of a seed lot in relation to storage temperature and seed moisture content.

In practice, predicting storage ability and loss of viability of orthodox seeds is however very difficult (Bewley & Black, 1989), because of different viability states in different cultivars or ecotypes, different pre- and post-harvest conditions, different oxygen pressures during storage and fluctuating environmental conditions, these monographs cannot be used as more than a rough guide to the viability of a species.

The process of seed deterioration is complex and difficult to detect in dormant seeds. It involves many biochemical and biophysical changes, including loss of enzyme activities, loss of membrane integrity and genetic aberrations. Change in seed coat coloration, reduced respiration, change in cell membrane constituents, leading to increased leaching of free fatty acids are main signs of seed deterioration (Copeland & McDonald, 2001). At the last stages of seed deterioration, necrotic and granular lesions in cotyledons can be observed. Intense drying before storage and high moisture contents and high temperatures during storage accelerate seed deterioration rate. At lower temperatures lipid peroxidation may occur either through autoxidation or enzymatically by lipoxygenase. The result is the release of free radicals which in turn create profound damage to membranes (Wilson & McDonald 1984). The loss of seed viability at high temperatures is closely related to protein modification and thermal inactivation of enzymes (Murthy and Sun, 2000). Storage fungi are a major cause of seed deterioration during storage. In situations where insects and rodents are effectively controlled, storage fungi cause

more seed deterioration than any other single agent. The fungi involved include several species of *Aspergillus* and *Penicillium* (Lisker *et al.*, 1985; Suszka *et al.*, 1996; Copeland & McDonald, 2001).

Seed viability in beechnuts

Generally, nuts of European beech are now considered as orthodox. However, they are susceptible to desiccation and Poulsen (1993) recommends a slow drying at temperatures below 20°C to m.c. of *ca.* 9%. There are, however, claims that beechnuts belong to the orthodox-recalcitrant class (Gosling 1991) or to the sub-orthodox class (Bonner 1990). European beechnuts can be stored for at least 6 years without loss of viability after drying the seeds to a moisture content of 8 to 10% at room temperature and storing them in sealed containers at temperatures from -5 to -15°C (Suszka, 1974; Muller & Bonnet-Masimbert 1982). Muller (1993) reported an increase in germination capacity after 72 months storage using a fast germination test. Gosling (1991), however, reviewed storage experiments on beechnuts and found that the germination percentage typically dropped 10% per year, at -5°C and 8-10% m.c.

In order to minimize loss of viability Poulsen (1993) suggested storage at 7% m.c. in subzero temperatures. She also calculated the viability constants for European beechnuts using two Danish seed lots and concluded that since the increase in longevity of beechnuts was related to temperature and m.c. decrease during storage, they should be classified as orthodox. Supporting this she found that her model of loss of viability was totally fitted with the data of loss of viability from storage of beechnut.

Near infrared reflectance spectroscopy and multivariate analysis

Near infrared reflectance spectroscopy (NIRS) is a rapid alternative to wet chemistry procedures for determining concentrations of major classes of chemical compounds in organic materials, such as plant tissues. No absorption bands for minerals exist in the near-infrared region; however, organic complexes and chelates may be detected (Shenk *et al.*, 2001).The method utilizes signals resulting from bending and stretching vibrations in molecular bonds between carbon, nitrogen, hydrogen, and oxygen. The principle of NIRS is based on selective absorption of electromagnetic radiation from 800 to 2500 nm in accordance with the characteristic vibration frequencies of functional groups (Osborne *at al.*, 1993).

NIR analysis relies on developing a calibration model ($Y = FX$) that relates the NIR spectra (Y matrix) of a calibration set of samples, as response block, to their known chemical property (X matrix) as predictor block, where F can be any mathematical function. This model is validated by *cross validation* method and/or a new external validation sample set and then used to predict the quality of further samples based on their NIR spectra.

Use of whole NIR-spectra as multiple variables where *e.g.* one variable comprises every second wavelength resulting in totally more than 1000 variables from each spectrum. Such huge amount of data can not easily be handled by classic statistical methods, instead by using partial least square (PLS) regression method the uncountable set of variables will be reduced to a few orthogonal principal components (Rännar, 1996).

In PLS the object variation in the predictor block is described by the X-scores, *T*, and the corresponding variation in the response block is described by the Y-scores, *U*. Basically what PLS does is to maximize the covariance between *T* and *U*. For each dimension, a weight vector, *w*, is calculated, which contains the contribution of each X-variable to the explanation of Y, in that particular dimension. The matrix of weights, *W*, contains the structure in X that maximizes the covariance between *T* and *U* in each dimension. The corresponding matrix of weights for the Y-block is designated as *C*. The matrix of X-loadings, *P*, is calculated for each dimension in order to perform the appropriate decomposition of X and Y. Hence, the decomposition of X and Y can be described as:

$$X = TP' + E \qquad \text{and} \qquad Y = TC' + F$$

The set of PLS regression coefficients, *B*, can then be calculated according to the formula:

$$B = W(P'W)^{-1} C'$$

The estimate of Y, $\tilde{Y}$ is then given by:

$$\tilde{Y} = XW(P'W)^{-1} C' = XB$$ (Antti, 1999).

Objectives

The two main objectives of the research presented in this thesis were to improve quality of oriental beech nuts and to reduce the long term dormancy period by enhancing currently available dormancy breaking methods for beechnuts. To achieve the first objective an experiment was conducted using NIR spectroscopy and multivariate analysis (Paper I), in this study the capability of NIR and multivariate analysis in discriminating viable and non-viable beechnuts was tested. Simple methods like sorting the nuts according to weight and removal of endocarp were examined in paper II to attain the second objective.

Since assessment of quality of beech nuts is lengthy due to the deep dormancy, and unreliability of the tetrazolium test for these species (Gosling, 1991), a basic study was carried out (Paper III) to compare storability of beechnuts as an index of loss of viability and abscisic acid content in embryonic axes. The result of this study was applied to examine if the germination test results of semi-dormancy released nuts were reliable to viability estimation of this species or not. This study also described the relationship between ABA and dormancy release in oriental beech.

To better explain the promoted dormancy release due to endocarp removal, the phenolic content of seed coats were analyzed (Paper IV). The germination inhibitory effect of these compounds can be potentiated in the presence of endocarp.

Material and methods

Seed sources

Three lots of oriental beechnuts were used in the experiments presented in this thesis. A seedlot with Iranian origin collected in 1999 from a natural forest stand in Hyrcanian region, north of Iran at 36°29´N and 51°08´E and an altitude of 1200 m above sea level. Another seedlot (lot # 9402) with Turkish origin was purchased from Sheffield's Seed Co. Inc. USA in 2000. Both seedlots had a moisture content (fresh weight basis) of *ca.* 9% at the time of the experiments. The third seedlot was purchased from Forest Tree Seed and Tree Breeding Research Directorate, Turkey in the autumn of 2000. The nuts were collected from a natural stand at 40°47´E and 39°29´N at an altitude of 1250 m above sea level.

Near infrared spectroscopy

A sample of beechnuts was sorted into 126 viable and 126 non-viable nuts by taking X-ray images. Near infrared diffuse reflectance spectra were recorded from 400 to 2498 nm at 2 nm intervals on single beechnuts. The raw spectra were pretreated with multiplicative scatter correction (MSC) to remove scatter effects, or orthogonal signal correction (OSC) to remove the variation in the spectra that was orthogonal to the calibrated response variable.

Multivariate calibration model was developed for each mean-centered data set with partial least squares regression (PLS) using the digitized spectra as descriptor matrix (X) and a y-vector of dummy variables as response. The number of significant PLS components to build the model was determined by a seven-segment cross-validation. The computed models were then applied to classify unknown samples in the test set.

Cold moist stratification at controlled and unrestricted hydration

Cold moist stratification at unrestricted moisture content was carried out with beechnuts with or without endocarp in semi-closed glass containers filled with sand for two, three, five and eight weeks at 5°C. In another experiment the cold moist stratification was conducted at controlled moisture content to study the effects of seed weight screening, removal of endocarp and alternating temperatures during prechilling on germination performance. Nuts were sterilized, the moisture content equilibrated to 30% and cold moist stratified at 5°C for four weeks in darkness.

Abscisic acid levels in embryonic axes

Half of a seedlot was directly used in the experiment (fresh nuts) and half was stored in sealed dark glass containers at -5°C for 15 months (stored nuts). Nuts, both fresh and stored, were cold moist stratified at maximum m.c. for 0, 2, 4, 6 and 8 weeks at 5°C using glass containers filled with moist and pre-washed sand. Samples were withdrawn after 0, 2, 4, 6 and 8 weeks for germination test and analysis of ABA levels. Another set of samples were stratified for eight weeks and then placed at 20°C for 1, 2 and 3 weeks. Each week samples were taken for analysis of ABA levels. ABA content of embryonic axis was determined according to Walker-Simmons *et al.,* (2000), using gas chromatography - mass spectrometry (GC-MS) selected ion monitoring (SIM) and deuterated ABA as internal standard.

Measurement of seed coat phenolic exudation

The rate of water uptake was measured by monitoring the fresh weight increment of 50 nuts during the first 72 h of imbibition at 5°C. Total phenolics of seed-coat and whole seed as well as water soluble phenolic content of seed coat were extracted and then quantified with half-strength Folin-Ciocalteu reagent and UV spectra (Ferraris *et al.,* 1987 with modification), based on $mg.g^{-1}$ gallic acid equivalents at 725 nm. The germination inhibitory effect of the water soluble seed coat extract was studied with a bioassay using radish seed. A sample of water soluble extract was further purified by high performance liquid chromatography (HPLC) and analyzed qualitatively with GC-MS in full scan mode, scanning from 40 to 300 amu, in order to identify phenolics with possible seed germination inhibitory characteristics.

Germination tests

Four replicates of 25 nuts or seeds from each treatment were sown on moist cotton in petri dishes and placed in a germination chamber maintained at 20°C with or without illumination. The germination test was run for 3 weeks and germinated seeds were counted and removed daily.

Data analysis

For all germination tests, germination capacity (GC) and mean germination time (MGT) were calculated as follows:

$$GC\ (\%) = (\Sigma n/N)\times 100 \qquad MGT\ (days) = \Sigma(t_i \times n_i)/\ \Sigma n_i$$

where t_i is the number of days starting from the date of sowing and n_i is the number of seeds germinated at each day and N is the total number of seeds sown (Bewley and Black 1994).

Generalized linear model (GLM) or univariate analysis was performed to determine significant differences in germination.

Results and discussion

Prediction of viability with NIRS

All PLS models developed explained more than 87% of the spectral variation and 89% of the variation between viable and non-viable nuts (R^2Y). Superior predictive ability (Q^2 = 0.959) and low prediction and calibration errors were achieved. All PLS models clearly separated viable and non-viable beechnuts in the calibration sets. The first component in one of the PLS models detected moisture content in viable nuts since the absorption bands in the 1400-1500 nm and 1880-2000 nm are mainly characterized by water molecules (Shenk *et al.,* 2001). In the same model absorption peaks at 1722 nm and 2110 nm had the largest influence in the second PLS component and may correspond to higher protein content in viable seeds which is in agreement with the general view of protein degradation and modification during seed deterioration (Cherry, 1983; Murthy & Sun, 2000). The absorption band between 750 and 950 nm is caused by methyl (CH_3) (Osborne *et al.,* 1993), and shows higher concentration of lipid (-acids) in non-viable nuts, that could be attributed to their low moisture content, which in turn enhanced the signal from other chemical compounds.

Effects of cold moist stratification on dormancy release

Cold moist stratification in sand or prechilling without medium revealed a gradual dormancy release in oriental beech similar to dormancy release in European beech (Muller & Bonnet-Masimber, 1989) and other dormant forest trees in the temperate region (Suszka *et al.,* 1996). The number of precociously germinating seeds/nuts was remarkably lower after cold pre-treatment at restricted m.c., supporting the claimed efficiency of the method in decreasing heterogeneity in dormancy release of beechnuts as suggested by Suszka and Zieta (1977).

There was a significant difference in dormancy release between nuts and seeds of oriental beech, indicating the existence of a germination delay or shallow dormancy caused by the endocarp or seedcoat. These both organs have been shown to play an important role in regulation of seed dormancy (Kelly *et al.,* 1992). They can interfere with oxygen diffusion to the embryo either by acting as a physical barrier or by consuming O_2 and limiting oxygen supply (Corbineau & Côme, 1995), restricting water uptake (Kelly *et al.,* 1992) or embryo growth (Prasad & Nautiyal, 1996). Seed coats and/or endocarps may contain germination inhibitors (Chow & Lin 1991), or prevent leaching of germination inhibitors from the embryo (Bewley & Black, 1994). These germination inhibitors may affect dormancy levels

through hormonal regulation *e.g*. by catabolism of ABA (Bewley & Black 1994; Barthe *et al.*, 2000).

Interestingly a highly significant difference was observed among seed weight classes in terms of speed of dormancy release. Heavy seeds germinated faster (lower mean germination time) and with higher germination capacity. Positive correlation between seed weight and germination capacity is common nearly in all seeds and is related to higher nutrient supply and thereby stronger and faster radicle emergence. The relationship between seed weight and dormancy release is reported only in a few non-woody plants (Mumford, 1990; Springer, *et al.,* 2001). In beechnuts, this correlation can be explained with the high genetic diversity in these species (Comps *et al.,* 1998). The cotyledons are, unlike the megagametophyte in angiosperms, from both maternal and paternal origin, adding to the diversity in seed morphology of this wind pollinated species with high outcrossing rates.

The results from the experiments also showed that alternating temperatures during cold moist pre-treatment is preferable. El-Antably (1976) showed that the amount of the plant hormones indoleacetic acid, gibberellins and cytokinins increased more substantially in nuts incubated at alternating temperatures than at constant temperature, during cold moist stratification of European beechnuts. It has been reported that gibberellins are promoting the release of dormancy in European beechnuts (Nicolás *et al.,* 1996, 1997, Fernandez *et al.,* 1997, Lorenzo *et al.*, 2000, 2001).

ABA content and seed viability

There were significant differences in germination capacity of fresh and stored beechnuts after six weeks of cold moist stratification. The germination capacity of stored nuts was *ca.* 10% lower than that of fresh nuts, which is in agreement with previously reported results for European beechnuts (Gosling, 1991; Poulsen, 1993; Derkx & Joustra, 1997). However, the difference was not statistically significant after eight weeks of cold moist stratification, which indicates a delay in dormancy release in stored nuts. The lower germination capacity after storage can either be the result of loss of viability or deepening of the dormancy, *i.e.* nuts unable to germinate after storage might be dormant or dead.

There were no significant differences in mean germination time of fresh and stored nuts during cold moist stratification indicating that the vigor was changed neither during storage nor during dormancy release. Since loss of vigor usually precedes loss of viability (Bonner, 1998) it can be speculated that the delay in germination was caused by a difference in dormancy degree and not viability loss. However, it can be concluded that the minimal decrease in germination capacity over the 15 month storage time indicates a good storability of oriental beechnuts under these conditions which agrees well with similar reports for European beech

(Muller & Bonnet-Masimbert, 1982, 1989; Muller, 1993; Suszka *et al.,* 1996; Muller *et al.,* 1999).

As dormancy release progressed, the ABA level in the embryonic axes rapidly decreased in fresh nuts. Although there was an overall decreasing tendency of ABA levels also in the embryonic axes of stored seeds, the concentrations of ABA were relatively higher than in fresh nuts, indicating a deepening of the dormancy degree. Interestingly, the ABA level dropped sharply, in embryonic axes of both stored and fresh non-dormant nuts after one week incubation at +20°C. This might indicate an increasing metabolism of ABA in the embryo as germination begins. Previous results from different species, among them European beech, showed that the ABA levels increased as dormant seeds were incubated at higher temperatures (Berrie *et al.*, 1979; Dunwell, 1981; Walker-Simmons, 1987; Le Page-Degivrye *et al.*, 1990). Although there are no records of ABA levels during incubation of non-dormant beechnuts at higher temperatures, our results are in agreement with similar results reported for sunflower (Bianco *et al.*, 1994) and chick-pea (Iglesias & Babiano, 1995).

From our results it can be concluded that ABA can potentially be considered as a dormancy releasing indicator in oriental beech during cold moist stratification treatment. Similar results previously been shown for European beech (El-Antably, 1972; Le Page-Degivry *et al.*, 1997a; Le Page-Degivry *et al.*, 1997b).

Seed coat phenolic content and toxicity

This study showed that the endocarp in oriental beech seed does not prevent the seed from imbibing water. The estimations of water soluble phenolics in seed coats of oriental beech showed that the absolute dry weight of water soluble phenolics in the endocarp cavity can easily reach up to 5×10^{-4} GAE per mm^3 imbibed water. A concentration ten times lower than this value inhibited germination of radish seeds by more than 50%. Significant differences were found between amounts of total phenolics in exudates from nuts with endocarp and seeds without endocarp implicating that the phenolics are trapped in the endocarp cavity, after imbibition and during stratification. These results can explain the advantage of endocarp removal for the release of dormancy in oriental beech. High levels of phenolic substances leach out when the seed coats are damaged or removed (Qi *et al.*, 1993).

Phenolic compounds are widely distributed in plants, and several functions have been attributed to them. Plant phenolics have antipathogen, antiherbivore and allelopathic properties (Harborne, 1989). Free or conjugated phenolic substances, present in seeds of a number of species, have been implicated in the regulation of seed germination (Williams, & Hoagland, 1982; Hamilton, & Carpenter, 1976; Enu-Kwesi & Dumbroff, 1980; Sreeramulu, 1983). The simple phenolics that were identified in this study are common in oilseed (Ribéreau-Gayon, 1972) and well known inhibitors of germination (Bhattacharyya et al., 1999) particularly inhibiting

cell division in the growing roots (Vaughan & Ord, 1990). For instance *p*-hydroxybenzoic acid is a germination inhibitor in the seed coat of papaya seed (Chow & Lin 1991) and 2-hydroxycinnamic acid and coumarin are potent inhibitors of both germination and subsequent root growth of radish seed (Aliotta et al., 1993).

Conclusion

This study showed that non-viable beechnuts (Fagus orientalis) with granular lesions in the cotyledons and the same weight as sound nuts can efficiently be discriminated from sound nuts by near infrared spectrometry and multivariate classification.

Simple techniques like grading the nuts according to their weight, mild nut scarification and pre-chilling in alternating temperature can not only improve the germination capacity and shorten the seedling production time, but also decrease the period of dormancy release in this species.

Oriental beechnuts showed a good mid-term storability without any significant loss of viability after complete dormancy release, applying eight weeks of cold moist stratification. Difference in germination capacity of semi-dormant fresh and stored lots is more dormancy dependent, and shows a complex relationship between primary dormancy degree and viability assessment in this species. Storage may however induce secondary dormancy too. Abscisic acid levels in the embryonic axes are inversely related to germination capacity indicating a possible role of this hormone in the maintenance of dormancy in oriental beechnut.

Simple phenolics in the seed coat are also involved in regulation of germination or dormancy in this species, potentially as autotoxins.

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