Characterization and prediction by NIR in lupins seeds

Bachelor project
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Preface

This project has been supervised by Christian Andreasen, Associate Professor and Head of Crop Science, employed at the Department of Agriculture and Ecology/Crop Science.

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Prefacio

Los altramuces son una fuente rica en proteína y es un cultivo agronómicamente muy atractivo debido a su capacidad de fijar nitrógeno. Gracias a estas propiedades, este cultivo es muy interesante, ya que puede ser utilizado como fuente de proteína para mejorar otros productos alimenticios, además de cumplir un papel importante en la rotación de cultivos. El objetivo de este proyecto de investigación es la caracterización de semillas de altramuz según variedad, localización y tiempo de cosecha y la predicción del potencial de germinación, usando espectros NIR (espectroscopia de infrarrojo cercano) para el análisis de datos. Se observó correlación entre la variedad y ubicación, que es muy útil en las industrias de semillas y cultivo de plantas como un método de detección rápida para determinar qué variedades en el mercado se diferencian más y cuales son más estables en diferentes condiciones de desarrollo. De acuerdo a la predicción del potencial de germinación, sería un avance tecnológico importante, ahorrando tiempo y dinero en la investigación y/o procesos de producción en industrias de semillas y alimentos.

Lo que hemos realizado podemos decir que es una huella dactilar química en semillas. La espectroscopia NIR correlaciona con diferentes compuestos químicos presentes en la semilla, por lo que para ver en los resultados diferencias entre variedad, localización y cosecha, las muestras deben ser diferentes entre sí. Se utilizaron semillas de tres variedades diferentes, desarrolladas en tres lugares distintos y cosechadas en cuatro momentos distintos. Se encontró correlación entre variedad y localización.

En cuanto a la predicción del potencial de germinación, no se consiguió debido al modelo planteado y similitud de las muestras, pero se plantearon soluciones para mejorar los resultados y poder hacerlo viable en futuros proyectos.

Para el manejo de datos, se utilizaron técnicas de PCA (clasificación), PLS (predicción) y MSC (corrección). Para la obtención de datos se han realizado ensayos de germinación y crecimiento, medidas NIR con espectrómetro de ultima generación y test químicos Kjledahl.

Los resultados fueron positivos, pero no tanto como lo esperado debido a la similitud de las muestras. Este proyecto abre puertas a futuros proyectos en la investigación de ésta técnica que en un futuro será fundamental en mejora vegetal, industrias de semillas y alimentos.

1. Abstract

Lupine seeds are a rich source of proteins and agronomically attractive because of their ability to fix nitrogen, but they have been in a second place as legume crop. Thanks to these properties, this crop is very interesting, because it can be used as a protein source to improve other food products, besides making a significant role in crop rotation. The aim of this research project is the characterization of lupin seeds according to variety, location and harvest and prediction of germination potential, using NIR spectra for the data analysis.
We observed correlation between variety and location, which could be very useful in seed industries and plant breeding as a fast screening method to determine which varieties at the market would differentiate most and which would be most stable in different growing locations. According to prediction of germination potential, it would be an important technological advancement, saving time and money on research and/or production processes in seed and food industries.

2. Introduction

Lupins have never been a popular crop as food source, despite their ability to fix nitrogen as well as being a great source of protein, becoming in an important option in crop rotation. Its high protein content makes this crop an untapped source which, when added to other food products, can enrich their nutritional value and improve their technological properties.

The objective of this project is to study the possibility of using NIR method as an aid for certain processes, which would facilitate future research in plant breeding and the food industry.

This project also carries out a test of dormancy in lupins, because it will be necessary to perform the germinations *(Seed Dormancy Experiment).*

Following this research we will be able to answer the following issues:

- **Characterization of lupin samples according to variety, location and harvest time.**

- **Measure the germination potential of different seed lots**

The NIR analysis will be carried out by non-destructive methods on a number of lupin seed lots for both objectives. It will be used as plant material seeds from three different lupin varieties, grown on two different locations and harvested at separate dates. Afterwards Kjeldahl method will used to measure the variability of samples to correlate the NIR data.

This project does not seek to validate or reject the method to investigate as it is something innovative, and will require further work to develop it. We want to find a logical conclusion to serve as a starting point for future researches.

3. Theory

3.1. Lupin

3.1.1 Description

The term lupin is widely used to describe the seeds of different domesticated Lupinus species. Lupins or lupines, are members of the genus *Lupinus L*. in the legume family (Fabaceae). Concern to division *Magnoliophyta (Angiospermae)*, subclass *Magnoliatae (Dicotyledoneae)* and order *Fabales* (Kurlovich, 2002).

Is a large genus of herbs and shrubs that are distributed all over the world. Mostly within the species are herbaceous perennial plants, but some are annual and a few are shrubs. They can measure between 0.5 and 1.5 meters, and sometimes up to 3 meters.
The leaf shape is very easy to recognize because it's characteristic to only this genus. They are green to grey-green and in many species with bare silvery hairs. The leaves are palmate and divided into either 5–28 leaflets, or reduced to a single leaflet in a few species with long petioles connected to the stem by a cushion with elongated stipules. Primary true leaves are opposite while other leaves that are unseen before germination are alternate (Kurlovich, 2002).

The inflorescence is polyanthous apical truss with alternate, semi-verticillate or verticillate arrangements of flowers. The flower of lupin is monoecious, zygomorphous and hermaphroditic. It has concrescent stamens, superior ovary and apocarpous gynaeceum. Flowers are bisexual, irregular, blue, purple, and yellow in racemes. They're produced in dense or open whorls on an erect spike. Each flower is 1-2 cm long with a typical pea flower shape with an upper 'standard' or 'banner', two lateral 'wings' and two lower petals fused as a 'keel'. The flower corolla is zygomorphus and papilionaceaous, with an ovary with pistil and 10 stamens. The ovary has four or more seedbuds, and the cotyledons are large with a short caulicles (Kurlovich, 2002).

Photograph 1: *Lupinus L*

The fruit is a pod containing several seeds. Each plant can produce primary, secondary, and tertiary pods with each pod containing 3 -7 seeds. These seeds are very diverse in size, shape and color. The size is approximately 1cm and with oval morphology. The seed coat is smooth or fine-meshed, with white, beige or black color and is composed of 83% dietary fibre. The cotyledons are yellow and composed of 13% of fat, 35% of proteins and 38% of carbohydrates (Kurlovich, 2002).

The pod surface is rough, with white, black color, and in most cases with brown speckles. Sometimes the seeds are separate from each other (Kurlovich, 2002).

The seeds have a hard seed coat that require pretreatment to induce a faster germination. For example *L. Angustifolius* became impermeable to water when their moisture content was reduced to 10 to 12%.
We can use mechanical scarification, a hot water soak and cold stratification at 9ºC to induce a prompt germination. We can improve the germination significantly by also applying a treatment with concentrated sulfuric acid (Riemenschneider et al. 2008). We will speak more about this in the point *4.2.1 Seed Dormancy Experiment*

Photograph 2: Lupin seeds

3.1.2 Distribution and ecology

The genus comprises between 200 and 600 species ***(Appendix 1)***, with greater diversity in South America and western North America, in the Mediterranean region along with Africa. Lupins have low climate and soil condition requirements. In their natural environment lupins grows on poor, sandy soils and rarely on calcareous or alkaline soils. Lupins also require pH conditions under 6.8 and can't grow in soils with medium-high content of lime (Gladstones et al. 1998).

They are a cool season crop so are cold tolerant and survive frosts up to -9ºC. Lupins varieties which are not selected don't show a great cold resistance, but there are varieties selected which have had positive responses to frost with mortality rates below 15% in extreme temperatures up to -14 ºC. These improved varieties are valid for autumn sowing. The different species grow well in temperatures between 10 and 14 ºC, with optimum temperatures between 20 and 25 ºC, and with negative effects in their growth with temperatures above 29 ºC. High temperatures can reduce growth, increase flower or pod abortion and reduce yield, so an early planting is recommended. Cold sensitivity depends on the state of the plant, supporting better low temperatures in rosette state - being much more susceptible in a state of flowering (Kurlovich, 2002).

Lupins are sensitive to heat and water stress during flowering and pod fill, so the planting should occur as early in the spring as possible to encourage flowering and branching. However if we make the plantation very early when cold temperatures can affect the seed or seedling, plant growth, plant height, and days to maturity can be reduced therefore reducing the yield (Carvalho et al. 2005).

Production is limited by the variation in the amount and distribution of rainfall. A lack of water during vegetative and/or reproductive growth stages is one of the most limiting factors for seed growth. Depending on the sensitivity to stress level of the plant, this will be more or less tolerable to water deficiency (Carvalho et al. 2005).

In Australia some of these crops (L. angustifolius, L. albus and L. luteus) are grown in regions of winter dominant rainfall and are sown in autumn. In Northern Europe the same lupin species are spring sown and grown over summer with the season start dictated by soil temperature for germination (Carvalho et al. 2005).

Grain quality depends by the rate and duration of grain filling, which is a process that happens after the period during a flower is fully open and functional (post anthesys). The availability of assimilates and photosynthates are crucial for proper development process (Carvalho et al. 2005).

The flowers attract insects with their colour, presence of pollen and secretion of a scented liquid from the vexillum. Lupin flowers are self-fertile, self-pollinated, but some of them are predisposed to cross-pollination. There are species which are almost exclusively self-pollinated, such as *L. Angustifolius,* and self-pollinated with facultative cross-pollination (Kurlovich, 2002).

3.1.2.1 Agronomic suggestion [3,4]

1. Appropriate land for growing lupins:

    - Lupins perform best on coarse-textured soils (sandy and loam type soils).
    - Finer soils with greater water holding capacity may increase root rot potential.
    - Medium to high drainage soil type is required.
    - Low to medium levels of organic matter is preferred.
    - High organic matter can increase disease potential.
    - Lupins are sensitive to soluble salts.
    - Optimum pH between 5.0-7.0 pH.
    - Fertility requirements are moderate.
    - Adequate moisture for germination, flowering, and pod set is required.

2. Early planting is recommended to avoid high temperatures at flowering, which can cause abortion of flowers, cold and frost tolerance.
3. Inoculation with *Rhizobium lupini* or *Bradyrhizobium sp.* is required.
4. Well drained soils are essential in minimizing root disease problems.
5. A continuous legume rotation can cause growing problems.
6. A well-tilled, smooth seed bed is essential to ensure consistent planting depth and germination.
7. To date, insects have not posed an economic concern.
8. Full season weed control is necessary for optimum yields.
9. Seeding rate: 140 - 150 lbs./acre
10. Seeding depth: 0.5 - 1.5", depending on soil type and moisture.
11. Roll the field before emergence to ensure good soil contact around the seed to insure quick germination.
12. Lupins should be harvested as soon as they are ripe. Delays can result in significant yield losses due to lodging, pod shattering and pod drop. Lupins must be harvested within three weeks of maturity.

    A moisture meter should be used to determine when the lupin crop is ready, and harvest should start as soon as the moisture content reaches 14%.
13. Lupins should be dried down to 13% moisture.

3.1.3 History and actuality

Lupin is well established among the world`s prehistoric crops. Seeds were found in Egyptian graves from more than 3000 years ago. Greek and Roman authors wrote about the use of lupins for green manure, forage for stock and as a pulse for human consumption when alternative foods were scarce (Black et al. 2006).

This crop has been cultivated for at least 2000 years, and probably was first raised in Egypt. In fact, lupin seeds have been used for human consumption and as a medicinal plant in Egypt and other countries for thousands of years (Gladstones et al. 1998).

The seeds of this legume were popular with the Romans, who spread the plant's cultivation throughout the Roman Empire. The name 'Lupin' is from the Latin (lupines) that means *wolf*. It's also called *bean of the wolves* because at that time, seeds or beans that were rich in protein and bitter taste were seen as a poor food. [2]

In spite of lupin have a long history, its cultivation as a mechanized grain crop is of very recent origin. Serious breeding efforts and developing management procedures started in Europe and Australia no longer than about 30 years ago (Black et al. 2006). Breeders from Poland, Holland and Germany recognized the importance of collection and evaluation of wild and landraces lupins. Identification of local phenotypes, that are adapted to specific climate condition and soil types, may have provided large improvements in grain yield (Jørnsgård et al. 2005).

Taming the undesirable characteristics of the wild forms was the subject of selection by farmers. This yielded populations with various degrees of non-dehiscent fruits, permeable seed coats to obviate seed dormancy and diminishing content of toxic substances. Recent mutant breeding and selection paved the way for the development of reasonably stable cultivars. [1]

Varieties have been improved to have better agronomical characteristics, higher yield and disease resistance. The production of these varieties is continued today by breeding programs in Australia, Chile, Denmark, Germany, Poland, Russia, Ukraine and USA. *L. Angustifolius* breeding has been the major focus in Australia, otherwise *L. Albus* and *L. Luteus* have received more attention in Europe. They are harvested for grain, green manure or made into silage. Often the grain produced is fed on-farm to livestock. [1]

Healthy food is gaining more interest in developed countries and pulses have encountered a revival of interest from European consumers, in spite of being known in the past as the poor man's meat. A diet consisting Lupin is recommended because it is recognized that frequent consumption may reduce cholesterol levels and reduce risk of coronary heart disease and diabetes. This legume is presented as a staple food for vegetarians.

In the European Union, pulse consumption has increased by 34% during the past 10 years to 3.9 kg/capita per year, although differences between countries are still large (Torres et al. 2005). The most common places where you can find Lupini dishes are Mediterranean countries, especially in Portugal, Egypt, Italy, Brazil and Spain.

Lupins also have a great ornamental value. Some species, such as *L. Polyphyllus* and hybrids like *L. Regali* are common garden flowers, because they have a high range of colours and lots of flowers. Others, such as *L. Arboreus* are considered invasive weeds when they appear outside their native range. [2]

3.1.3.1 Industry Background

Southern Australia´s narrow-leafed lupin industry is by far the largest and most technically sophisticated agricultural industry based on a species of the genus Lupinus. In the 19th century botanists Richard Schomburghk and Ferdinand Von Mueller introduced many species of lupins into Australia, which later were promoted as fodder and green manure crop by the State Department of Agriculture. From no commercial production at the time of the release of the first cultivar on 1967, the area sown has increased to 1.3 million ´ha` and a production of about 1.4Mt in 1996. The industry began in south-western Australia and this region has dominated the lupins industry throughout its history.[1]

3.1.4 Chemistry and nutrition

Although lupin grains have been used for human consumption for thousands of years, their utilization in modern food production is still limited. Nevertheless, lupin is attracting interest worldwide as a potential high protein food ingredient suitable for human consumption (Torres et al. 2005).

Legumes are very satisfactory sources of proteins and play a very important role in human and animal feeding. They contain 20-25% protein, which is 2 or 3 times higher than the content in cereals, so they have been considered as leading candidates for protein supply to malnourished areas of the world. (Sujak et al. 2006)

The protein content varies widely between species of the same genre, for example between *L. Angustifolius, L. Albus, L. Luteus and L. Mutabilis. L. Angustifolius* has the lowest quantity of protein with 31.1%, followed by *L. Albus*, *L. Luteus* and *L.Mutabilis* with 42.4%, occupying a privileged place in the vegetable kingdom. We can consider *L. Albus* and *L. Mutabilis* as potential oil sources, with a content of 11.5% and 18.7%. The crude fibres content exceeds as much as 10% in *L. Angustifolius* and *L. Luteus.* It's recommended that these seeds are used for animal consumption to reduce the high fibre

content by breeding, to facilitate digestion. However in human consumption, the seed hull can be considered a valuable fibre source (Birk et al. 1989).

Regarding to microelements, Lupins are a great source of iron (Fe), zinc (Zn) and copper (Cu) compared with other legumes. Also lupin has a very high level of manganese (Mn). Up to 350mg/100g in comparison to other species that contain less than 4mg/100g. However, they have low levels of phosphorous (P) and calcium (Ca) (De Carvalho, 2005).

Lupine seeds are used as a source of protein in feed and food in various parts of the world, not only for its nutritional value (high in protein, fat and fiber) but also for its easy adaptation to poor soils and different climates. Human consumption has increased in recent years. Lupine flour is added for its nutritional value and also provides functional properties in baked goods, protein concentrates and other industrial products. They are also being used to make lactose free milk and yogurt analogues. Besides this, it is also used to produce alternative flours for people with gluten allergy. For example, lupin flour can be added to pastas and bread to make them more nutritious. In the Middle East and Asia, which traditionally use soybean in processed food, lupins have been accepted by taste panels. This research tries to open potential new markets for lupins (Sipsa, 2008).

One positive characteristic of lupins compared with soybean and other legume crops, is that lupins have lower levels of undesirable elements (phytic acid and saponins) and lower quantities of lectins and protease inhibitors, improving the protein digestion (Sujak et al. 2006).

Lupin seeds are a rich in and an important source of dietary proteins, oil and compounds of potential use in nutrition, medicine and agriculture. Ultimately, they could replace well accepted seeds, such as soybean (Sujak et al. 2006).

3.1.4.1 Antinutritional compounds and Toxicity

Utilization of legume protein has had a lower potential, partly due to deficiency of some of the essential amino acids in these proteins, and also due to the presence of antinutritional compounds. The nutritional quality of lupin grains is not only defined in terms of their nutrients, also by their anti-nutritive substances. The most important antinutritional compounds or antinutritional factors (ANFs) present in legume seeds, are *protease inhibitors, lectins, tannins, saponins and phytates.* ANFs can cause negative effects in man and animals by influencing digestive and metabolic processes (Muzquiz et al. 2009).

Legume seeds contain substances which considerably detract from their value. In lupins, the downside of their widespread utilization in human and animal nutrition is the quinolizidine alkaloids, which are toxic and bitter. Quinolizidine alkaloids, occurring in lupins, are the largest single group of legume alkaloids with clear ecological functions in

the defense of the plant. Lupin is toxic because of its alkaloid content, sparteine and lupanine in particular (Muzquiz et al. 2009).

In order to improve the nutritive value and digestibility, and to reduce antinutritional factors, methods such as soaking, dehulling, germination, fermentation, cooking, heat treatment and irradiation can be applied.

The levels of alkaloids in seeds or meal can be reduced through a de-bittering process involving soaking or washing with water. After this process, the alkaloid content in seeds is about 500mg/kg. But in the 1920s, German plant breeders produced the first selections of *alkaloid-free or sweet* lupin, which can be directly consumed by humans or livestock. The mean alkaloid content of marketable sweet lupin seed is on average 130-150 mg/kg (De Carvalho, 2005).

With the appearance of this variety, the most important constraint for the consumption is the high level of *α-galactoside*, which can cause negative effects such as flatulence, osmotic effects and a reduction in protein utilization and net dietary energy.

Different investigations have been carried out to develop treatments to reduce or remove the content of *α-galactosides* and to enhance the nutritional quality of lupins. Germination, fermentation and genetic manipulation have been used to obtain nutritively improved legumes, decreasing level of antinutritional factors and at the same time improving the concentration and bioavailability of their nutrients (Khalil et al. 2006). Furthermore, some other technological procedures, such as enzyme addition to hydrolyze specific antinutritional factors and non-starch polysaccharides or even to improve the digestibility of proteins (proteases), have also been investigated. (Torres et al. 2005).

Lupins are becoming increasingly recognized as alternative crop to soy, because they have the full range of essential amino acids and can grow in different climates. Newly varieties of sweet lupin, that lack any bitter taste and require no soaking in salt solution, are grown extensively in Germany.

"Schmidlin Mezarps (1973) reports manifestatisons of intoxication in human beings after consuming the bitter seeds and estimated that what the toxins do in human beings runs at about 11 to 46 mg/kg of weight".

"Hudson et al. (1976) considers that a daily consumption of lupin alkaloids amounting to 500 mg/day involves no risk".

"Culvenor and Petterson (1986) recommended that lupin seed should not exceed an alkaloid content of 0.02% for human consumption".

The protein content and protein quality as well as antinutritional compounds in the lupin seeds have been investigated, but relatively little is known concerning their carbohydrate composition.

3.1.5 Responses to inoculation: Nitrogen fixation

Bacterial inoculation is a technology to improve crop productivity and soil fertility. It should be performed in soils which contain a low and inefficient indigenous rhizobial population (Tahir et al. 2009).

Lupin can perform symbiosis with bacteria of the genus *Rhizobium lupini* and *Bradyrhizobium sp.,* which are found as microsymbionts in other leguminous crop. Due to its symbiosis with nodule bacteria, lupin possesses a high ability to fix nitrogen, with the means to obtain molecular nitrogen from the atmosphere, which is used to produce protein and other nitrogen substances. Result of this symbiosis is that the lupin plant fixed significant amounts of nitrogen, reaching to 67 - 78% of the total nitrogen requirement in lupin plants inoculated with *Bradyrhizobium sp.* being unnecessary nitrogen fertilization for high performance (León et al).

Lupins-rhizobia association may make available nitrogen compounds in soil through symbiotic fixation. One hectare of lupins can fix in a year between 150 and 316 kg of nitrogen, with an average of 227 kg N/ha, producing a significant saving of nitrogen fertilizer and an important contribution to the sustainability of productive ecosystems in time (León et al).

The rate of accumulation of nitrogen is not the same during all stages of growth, with the greatest intensity during flowering, formation and growth of the sheath, to decrease during grain filling. An efficient inoculant must have high efficiency strains, in nitrogen fixation and root infection besides being free from other bacteria, fungi or other contaminants that may be detrimental to the Rhizobium or plant. Also the soil type represented an important factor on the nodulation, affecting significantly the dry weight of nodules per plant. Opinion is divided on the question of the propensity of Bradyrhizobial inoculant to survive on seed. It has been described as a poor survivor when applied to lupins (León et al).

Mineral nutrients are also very important in nitrogen fixation process. Nodulation and nitrogen fixation appear to require more phosphorus and greater levels of iron and cobalt than does the host plant growth (J. S. Gladstone, 1998). Without limiting factors of symbiotic, nitrogen fixation allows the plants lupine ensures optimal growth, grain yield and protein, being certainly necessary to inoculate the seed to ensure good nodulation and subsequent fixation nitrogen (Tahir et al. 2009).

Studies in Australia show that the soil's mineral nitrogen had an increase of 41 kg/ha after planting lupine, also shown that cultivated wheat after lupins was practically free of

disease. It has also found that that seed's inoculation improves 50% the yield and 28 to 48% the protein concentration. Yield increases up to 100% in *L. Albus* and 200% in L. *Angustifolius* were obtained when inoculated with *Bradyrhizobium sp* (León et al).

Seed inoculation and subsequent nodulation induce a greater total dry matter production. Lupins being a nitrogen fixing legume have developed a special role as a rotation crop with wheat and other cereals. They can also benefit the following crop by reducing disease burdens (León et al)

Bacterial crops were made available to farmers once scientists discovered that successful nodulation wasn't generally possible without inoculation.

3.1.6 Importance

There is not a reason why lupin should retain a minor position among other legumes. The lupine has gained importance due to their agronomic characteristics in rotation with cereals such as their capacity to fix nitrogen, low nutritional requirements and an ability to release the phosphorus from the soil. It is also a good alternative for animal feed because of its high nutritional value, especially given the high protein which, added to other food products, can enrich their nutritional values and improve their technological properties, thus giving higher quality foods. It's an interesting option in the rotation of winter annual crops such as lupine-wheat (Gulewicz et al. 2008).
It has to be noted that lupin is relatively a new crop compared with soybean and other grain legumes, which have many years of intensive research behind them (Birk et al. 1989).

3.2 Seed Quality

The nutritional quality of lupin grains is not only defined in terms of their nutrients. Parameters of seed quality for lupins include permeability, physical damage, seed size and weight, germinability, nutrient content, seed origin and freedom from fungal or viral infection (Gladstone et al. 1998).

3.2.1 Physical damage

Lupin seed is susceptible to damage during harvesting and in handling and transport. It is necessary to practice good harvest techniques for seed crops, and perform the harvest in the correct time. Transfer of seed using mechanical augers may also cause physical damage and should be minimized (Gladstone et al. 1998).

3.2.2 Size and weight

These are important factor of lupin seed because of its effect on stand density, which is determinant of seed yield.
The relation between seed weight and germination is not constant, but a positive correlation unites them, the heavier seeds show a higher germination percentage.

On the other hand, the effect of seed weight on growth is very constant. A large seed size induces the production of more vigorous seedlings with heavier fresh and dry weight. The larger seeds are also more tolerant to unfavorable conditions during early growth (Huyghe, 1993).

At constant plant density, smaller seeds produced smaller plants, and were consistently yielding lower than larger seeds. There are other factors, such as deep sowing, where large seed may be advantageous (Gladstone et al. 1998).

3.2.3 Germination

Commercial lupin seeds have high permeability to water, and doing a hand-harvest in the correct time (physiological maturity), 99-100% germination can be expected. Despite the high germinability, germination testing is still essential to ensure that a poor quality or damaged seed doesn´t result in low stand density and thus limit yield (Gladstone et al. 1998).

3.2.4 Nutrient content

Most species of Lupinus seed have a good quantity of protein (43%), a good proportion of fibers (25.5%), adequate amounts of sugars (13.5%) and minerals such as cobalt, phosphorus and potassium (5.5%). The nutritional quality of lupin grains is not only defined in terms of their nutrients, also by their anti-nutritive substances.

Low levels of manganese and phosphorus reduce emergence and growth of lupin seeds and seedlings, respectively, even in the presence of manganese fertilizer (Gladstone et al 1998).

3.2.5 Seed origin

Such as seed size, there are undoubted effects of seed source which may affect the growth, affecting the yield. This is due to differences in the environmnt such as which seed matures, presence of different diseases, etc. Until that time, selection of a healthy seed crop, careful harvesting and routine testing for germination and CMV are the agronomic requisites for selecting seed (Gladstone et al. 1998).

3.2.6 Seed borne diseases

Infected seeds are usually non-viable, but in *L. Albus*, infected seeds may give infected seedlings. The viral diseases cucumber mosaic cucumovirus (CMV) and bean yellow mosaic potyvirus (BYMV) may be seed transmitted in narrow-leaved lupin and *L. Albus* lupin. Anthracnose, potentially a serious disease, is also seed borne.

The importance of seed transmission for the persistence of the virus also means that detection and use of virus free seeds stocks can usually eliminate the diseases in commercial productions (Gladstone et al. 1998).

3.2.7 Seed inoculation

Seed inoculation has been very successful for lupins. As I have explained before, lupins can perform symbiosis with bacteria of the genus *Rhizobium lupini* and *Bradyrhizobium sp*. Due to its symbiosis lupin possesses a high ability to fix nitrogen, making nitrogen fertilization unnecessary and improving soil conditions for future crops.

Commercial peat inoculants is mixed with a methyl cellulose adhesive and applied to the seed, which is then dried and sown. The sown has to be as soon as possible and avoid seed treatment with fungicides and insecticides (Gladstone et al. 1998).

3.3 Spectroscopy

Analytic technique concerned with the measurement of the interaction of radiant energy with matter as a function of wavelength (λ), which provides useful information about components containing covalent binding, such as carbohydrates, protein and lipids. Its used in physical and analytical chemistry for the identification of substances through the spectrum emitted from or absorbed by them (Sherman).

3.3.1 Instrument

There are different instruments to measure the spectrum. In our case, the measurements have been performed with a NIR instrument.

We wanted a last generation instrument to measure the samples, so we had to move to the *Research Centre of Flakkebjerg* for the most innovative, which is in one of their labs.

3.3.2 Method

3.3.2.1 Near Infrared Reflectance (NIR)

NIR is a chemical fingerprint where different wavelengths correlate to different chemical compounds.

It's a non-destructive method which offers the advantage of fast sample processing measuring several broth constituents simultaneously. On the other hand, this method also has disadvantages, such as interference caused by unexpected features, which can introduce errors in the analysis (Macedo et al. 2002).

The near infrared area extends from the upper wavelength end of the visible region at about 780 nm to 2,500 nm (Ozaki et al. 2007).

Is especially important for practical applications, due to greater depth penetration of NIR radiation into the sample than in case of mid IR spectroscopy range. It is used in agriculture and food industry for quality and safety process (Wang et al. 2007). This analytical method is also used for quantitative determination of protein, moisture, starch, oil, lipids and cellulose in agriculture products (Osborne).

3.3.3 Data processing

Spectroscopic fingerprints are interpreted by the method of multivariate data analysis (Chemometrics): Principal component analysis (PCA) for classification, Partial least squares regression (PLS) for correlation and multiple scatter correction (MSC) as a method to remove possible noise (Esbensen, 2000).

3.3.3.1 Principal Component Analysis (PCA)

Expressing the data in such a way as to highlight their similarities and differences. Transform a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The other main advantage of PCA is that once you have found these patterns in the data, and you compress the data reducing the number of dimensions, without much loss of information. If a multivariate dataset is visualized as a set of coordinates in a high dimensional data space (1 axis per variable), PCA supplies the user with a lower dimensional picture (Smith, 2002).

In our case, PCA method has helped us to make a classification of our data with those variables; seed varieties, harvest time and location.

3.3.3.2 Partial Least Squares Regression (PLS)

Partial least squares regression is an extension of the multiple linear regression model. It is used to find the fundamental relations between two matrices, X and Y (Abdi, 2007).

PLS regression is a recent technique that generalizes and combines features from principal component analysis and multiple regressions. Its object is to predict or analyze a large set of dependent variables from a set of independent variables or predictors (Abdi, 2007).

It's probably the least restrictive of the various multivariate extensions of the multiple linear regression model. This flexibility allows it to be used in situations where the use of traditional multivariate methods is severely limited, such as when there are fewer observations than predictor variables (Esbensen, 2000).

PLS has been used in many disciplines, but in chemometric has become an important method for modelling linear relations between multivariate measurements (Esbensen, 2000).

In our case, PLS has been used as an exploratory analysis tool to select suitable predictor variables. In a PLS the algorithm tries to find correlation between the spectra (X-matrix) and the germination variable (y).

3.3.3.3 Multiple scatter correction (MSC) [2]

Several transformation methods are known for multivariate data analysis. The reason for transforming data is to remove noise from X matrix.

Multiple scatter correction (MSC) is a pre-treatment proposed for spectra data in which dispersion effects of radiation are estimated, or changes in optical step in relation of an ideal sample which is introduced as a reference (m).

In principle, this estimate should be made in areas of spectrum that does not contains chemical information. However, in practice the full spectrum is corrected.

MSC assumes that the totality of chemical information contained in a spectrum is contained in reference spectrum (m) and in another term denominated (αi) which represents the set of variation of unknown and irrelevant spectrum.

This correction, in some cases, can lead to some mistakes and the elimination of interesting information of spectrum.

Pearson correlation coefficient

The Pearson correlation coefficient is a statistical index that measures the linear relationship between two quantitative variables. Unlike the covariance, the Pearson correlation is independent of the scale of measurement of variables.

The calculation of linear correlation coefficient is accomplished by dividing the covariance by the product of the standard deviations of two variables:

$$r = \frac{\sigma_{XY}}{\sigma_X \cdot \sigma_Y}$$

The correlation index value varies in the interval (-1, +1)

If r = 0; There is no linear relationship. But this does not necessarily imply total independence between the two variables. Maybe are non-linear relationships between the two variables.

If r = 1; There is a perfect positive correlation. The index indicates a total dependency between the two variables called direct relationship: when one increases, the other does in the same proportion.

If 0 < r < 1; There is a positive correlation.

If r = -1; There is a perfect negative correlation. The index indicates a total dependency between the two variables called inverse relationship, when one increases the other decreases in the same proportion.

If -1 < r <0, There is a negative correlation.

3.3.4 NIR application to Agricultural and Food Engineering

NIR has been used as quality testing in wheat since the late 1970s.

NIR as an analytical technique provides a quantitative and qualitative characterization of the spatial distribution in heterogeneous samples (biological materials), which is very used because of its fast and low cost per test and requires a relatively small quantity of sample. It is used to replace some of the chemical tests necessary as part of a quality testing program (Cen et al. 2007).

The many advantages of NIR spectroscopy don't appear to have attracted the same attention from crop breeders. For example, the application to determine the chemical composition in soybean is more developed if we compare with other crops such as lupins. In soybean NIR has been applied for protein, moisture, oil, crude fiber, P, Na and Ca amongs others, and in lupins only for protein and aminoacids.
It is also used in other aspects. For example in Australia it's used to predict fertilizer requirements of cereal crops or to help farm managers to segregate or blend on its protein content prior to delivery (Osborne).

NIR method is not only used in cereals and cereal products, also in milk and dairy products, meat, fish, fruit and vegetables, confectionery, beverages, food authenticity, etc. This method is also making headway in the field of genetic engineering. It is possible to detect some chromosomal translocations (mutations) by NIR spectroscopy (Ozaki, 2006).

Its moving slowly to the Kjeldahl method (destructive chemical test), as it is much more environmentally safe, since it avoids chemical compounds such as sulfuric acid.

3.4 Kjeldahl method

Analytical method developed by Johan Kjeldahl, which is used the estimate the protein content in foods. The amount of protein present is calculated from the nitrogen concentration of the food. However, the protein content measured is not perfect because this method also measures non-protein nitrogen in addition to the nitrogen in proteins.

Briefly, the method consists of warming the substance we want to measure with sulphuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulphate. Then is distilled to convert the ammonium salt to ammonia, whose quantity is the amount of nitrogen in the sample. After, the ammonia reacts with boric acid and the remainder of the acid is then titrated with a sodium carbonate solution with a methyl orange pH indicator. This actually makes use of specific catalysts to yield a faster decomposition. [2]

3.5 Applied Lupin Varieties

Seed Dormancy Experiment: Line *Violet* has been used as seed material in this experiment. We don't need to number the seeds as in "NIR analysis" because we only want to know the effect of cold treatment in any lupin seed.

NIR analysis and Kjeldahl method: Three lupin varieties have been used in this experiment; *Bora*, *Prima* and *PD*. These are from two different farms in Højbakke and Ytteborg (PD only from Ytteborg) and from four different harvests in Bora and Prima, and five different harvests in PD. The harvests occurred at different times: Højbakke 2009, Ytteborg 2008 and 2009. We have four replications per harvest and 23 seeds per replication (23 seeds/sample). The seeds have to be numbered and sorted correctly.

4. Materials and methodology

4.1 Materials

4.1.1 Materials for Break Latency Experiment

Photograph 3: Germination trays

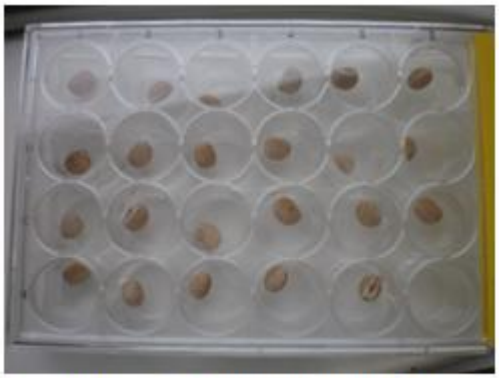
Cold treatment: To apply the cold treatment, we have used: dry sand, trays, markers, clean lupins and fridge (9°C).

Germination: To germinate the seeds, we have used: sand, trays, markers, clean lupins, climate chamber (~20°C) and water.

The trays are without holes because the sand is very fine, and would come out by the same. In the cold treatment we don't have to water the seeds because we don't want to germinate them, only apply the cold treatment. The markers are to differentiate between different trays.

4.1.2 Materials for NIR analysis

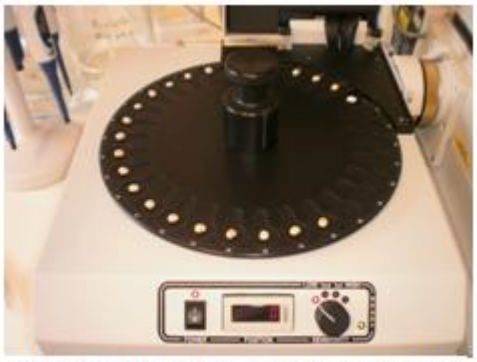
Seed processing (cleaning and numbering): Before start with the researches, we have cleaned and numbered all the samples. We have used a specific air machine for the cleaning, and to number we have used special **boxes of 24 holes** (23seeds/box).



Photograph 4: Seed numbering box

Photograph 5: Seed identification

NIR analysis: For the NIR analysis, we have used the NIR instrument and the software of NIR. The measures we have realized in a specific lab in the *Research Centre of Flakkebjerg*, with a latest generation NIR instrument.

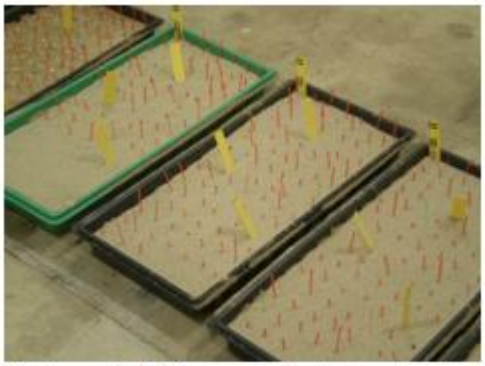


Photograph 6: NIR instrument (Research Centre of Flakkebjerg)

Photograph 7: NIR instrument (Research Centre of Flakkebjerg)

Germination: To germinate seeds, we have used: sand, trays, identification marks, clean and numbered lupins, climate chamber (~20°C) and water.

Photograph 8: Germination Trays in Climate Chamber



Photograph 9: Process prior to germination (sowing and identification)

4.1.3 Materials for Kjeldahl method

The content is shown in ***Appendix 2***.

4.2 Methodolgy

4.2.1 Seed Dormancy Experiment

In many cases the seeds germinate immediately, but many plants, especially species of trees and shrubs have developed the ability to control the initiation of germination to coincide with periods of the year in which natural conditions favorable for survival of seedlings. To this internal mechanism is called latency, lethargy or dormancy. For example, seeds that spread in early summer as cherry, *Prunus avium*, necessarily need to go through periods of heat (summer) followed by periods of cold to germinate, which means that until spring the following year the spread would be unable to germinate. Others need to deteriorate the surrounding decks and in nature it occurs in many cases after passing through the stomachs of birds that feed on fruit (Bonner, 2008).

Everyone who wants to germinate a latent seed must try to approach as much as possible to the natural germination process through what we call pre-sowing treatments.

There are two basic types of seed dormancy. The first is called exogenous dormancy, which is caused by the presence of a hard shell that protects the seed and prevents the ingress of water or oxygen to the embryo so it cannot be activated. The second type is called endogenous dormancy (embryo dormancy or internal dormancy) that is caused by the condition of the embryo that does not allow germination.

The objective of this test is to show whether using cold treatments, we can break latency to improve the germination of lupin seeds.

The line *Viol* has been used as seed material. We have used 400 seeds for the cold treatment and another 400 as "control" to compare the results. We have used 4 trays for each treatment (cold/control), and 100 seeds per tray. The seeds were placed in a green plant tray without holes. The process was performed during 1 week at 9 °C in dry sand, to avoid germination.

Photograph 10: Sowing

After the cold treatment, the germination took place in Climate Chamber at 20ºC with 12 hours of light and 12 hours of darkness (Climate Chamber number 18 in the Climate Chamber Hall of Taastrup). For the germination process, we have filled 3 cm of sand on the tray, which is equivalent to 5 litres of sand. To put the seeds evenly in the trays, we used a timber with holes as a template. Once the seeds are evenly placed, we pressed the seeds down until they flushed with the sand and filled another 2 cm of sand on the top, only 2 litres of sand is used or else it would be too heavy. Finally, 2 ½ litres of water were added to each tray. We used room temperature for the sowing.

The count was conducted for normal and abnormal plants. We counted as abnormal those whose development was slower; unopened cotyledons, plants underdeveloped respect to normal.

Normal

Photograph 11: Normal seedlings

Abnormal

Photograph 12: Abnormal seedlings

First counting was 5 days after sowing and the second 10 days after.

Sowing day	20-11-2009
Days in chilling(9ºC)	20/11/2009 – 27/11/2009
Days in Climate chamber(20ºC)	27/11/2009 – 7/11/2009
Irrigation(2,5L/tray)	1st- 27/11/2009
	2nd- 1/12/2009
Counting	1st- 2/12/2009
	2nd- 8/12/2009

Table 1: Planning test

4.2.2 NIR analysis

Three different varieties of narrow leafed lupin were analyzed by NIR. The analysis has been carried out by non-destructive methods on a number of different lupin seed lots. The seeds are of three different varieties grown on two different locations, with contrasting environmental conditions, and harvested at different times, to create enough variability.

First of all, we have cleaned all the samples with a specific machine by air. Then, all the seeds have been numbered to correlate the data obtained by NIR and germination. To number the samples we have used special boxes of 24 holes, which we used 23 (1seed/hole), and we have written in each box the following information: ***sample number, year, location, variety and harvest time.***

The samples were organized like this:

Year	Location	Variety	Harvest num.	Replication	Sample ID
2009	Hojbakke	Bora	1. Harvest	1	1
				2	2
				3	3
				4	4
			2. Harvest	1	5
				2	6
				3	7
				4	8
			3.Harvest	1	9
				2	10
				3	11
				4	12
			4.Harvest	1	13
				2	14
				3	15
				4	16
		Prima	1. Harvest	1	17
				2	18
				3	19
				4	20
			2. Harvest	1	21
				2	22
				3	23
				4	24
			3.Harvest	1	25
				2	26

				3	27
				4	28
			4.Harvest	1	29
				2	30
				3	31
				4	32
	Ytteborg	Bora	1. Harvest	**1**	**33**
				2	34
				3	35
				4	36
			2. Harvest	1	37
				2	38
				3	39
				4	40
			3.Harvest	1	41
				2	42
				3	43
				4	44
			4.Harvest	1	45
				2	46
				3	47
				4	48
		Prima	1. Harvest	1	49
				2	**50**
				3	51
				4	52
			2. Harvest	1	53
				2	54
				3	55
				4	56
			3.Harvest	1	57
				2	58
				3	59
				4	60
			4.Harvest	1	61
				2	62
				3	63
				4	64
2008	Ytteborg	PD	1. Harvest	1	65
				2	66
				3	67

				4	68
			2. Harvest	1	69
				2	70
				3	71
				4	72
			3.Harvest	1	73
				2	74
				3	75
				4	76
			4.Harvest	1	77
				2	78
				3	79
				4	80
			5.Harvest	1	81
				2	82
				3	83
				4	84

Table 2: Sample numbering

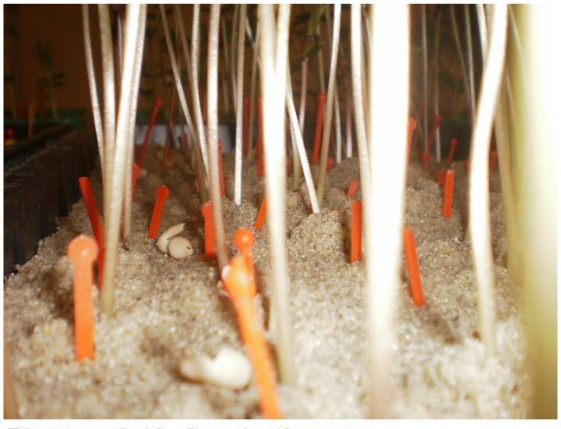
The sample number **33**(Ytteborg-Bora-1.Harvest-1.Replication) has 22 seeds, one broke. Sample number **50** is missing (Ytteborg-Prima-1.Harvest-2.Replication). The numbers in red are the samples which have been used in Kjeldahl method, in point 4.3.

After ordering the samples, they were packaged and sent to ***Research Centre of Flakkebjerg***, where we measured them in a specific lab with a latest generation NIR instrument.

After NIR analysis, all the data was saved to compare with the germination data.

Before the germination, it was necessary to employ cold method to break the dormancy, as we have demonstrated after the previous test *(Seed Dormancy Experiment)*. The samples were 1 week at 9 °C in dry sand.

The germination was performed with same materials and under the same conditions as in Break Latency Experiment: in Climate Chamber at 20ºC with 12 hours of light and 12 hours of darkness.



Photograph 13: Germination process

The counting was programmed 5 and 10 days after the sowing. After 5 days, any seed was germinated, so all the measurements were realized after 10 days.

The counting was based on: no germinated seeds, normal and abnormal seedlings.

No-germinated: The radicle does not break the seed coat.

Photograph 14: No germinated seeds

Abnormal: Under-developed seedlings; closed cotyledons, reduced size, root necrosis, rolled leafs, etc.

Photograph 15: Under-developed seedlings

Photograph 16: Root necrosis

Photograph 17: Abnormal root

Photograph 18: Abnormal root

Photograph 19: Rolled leafs and abnormal root

Photograph 20: Under-developed seedlings

Normal: Well-developed seedlings. Vigorous and disease-free.

Photograph 21: Normal and abnormal seedlings

Photograph 22: Normal seedlings

Photograph 23: Normal seedlings

4.2.3 Kjeldahl method

In this experiment, destructive test Kjeldahl have been carried out to measure the variability of the samples (by the protein content), to correlate the NIR data. It has followed the *American Analytical Cereal Chemists (AACC)* standard method.

1. Weigh and accurately 1g finely ground sample. Place in digestion flask and add polyethylene packet of catalyst and 25ml concentrated H_2SO_4 (reagent 1) to flask. Digest till solution is clear and then 30 min longer, remove and cool but do not allow to crystalize.

2. Place 300ml bottle or flask containing 50ml boric acid-methyl red-methylene blue indicator solution (reagent 7) under condenser tube with tip of condenser tube immersed under surface of solution. Add to original flask that is cooling 250-300ml tap water and antibumping agent, if not previously added.Gently add 50ml concentrated H_2SO_4 (reagent 4), connect to condenser with tight-fitting rubber stopper, and swirl. Boil until all ammonia has distilled and then set receiving bottle down so that condenser tube is completely drained.

3. Titrate distillate to neutrality with stdandar 0.1*N* H_2SO_4, using buret graduated in 0.1ml. Read mililiters of acid used, directly from buret.

4. Run blank determination periodically, using all ingredients except sample. Correct buret reading for nitrogen in reagents as shown by blank.

5. Results and discussion

5.1 Seed Dormancy Experiment

		1st count: 2/12/2009		2nd count: 8/12/2009			
	Samples	**Normal**	**Abnormal**	**Normal**	**Abnormal**	**No-germinated**	**Total seeds**
Control	1	1	0	59	39	2	100
	2	0	0	41	53	6	100
	3	5	0	49	40	11	100
	4	0	0	45	48	7	100
Chilling 9ºC	1	0	0	68	29	3	100
	2	3	0	69	27	4	100
	3	0	0	63	28	9	100
	4	4	0	45	45	10	100

Table 3: Germination results

1st count: The germination is higher after the cold treatment. All are normal with both treatments.

Control		9°C	
Normal	Abnormal	Normal	Abnormal
6	0	7	0

Table 4: Germination results in the 1st count

2nd count:

Control			**9ºC**		
Normal	**Abnormal**	**No-germin.**	**Normal**	**Abnormal**	**No-germin.**
194	180	26	245	129	26

Table 5: Germination results in the 2nd count

Germination percentage:

Control			9ºC		
Normal	Abnormal	Total	Normal	Abnormal	Total
48,50%	45%	93,50%	61,25%	32,25%	93,50%

Table 6: Germination percentage

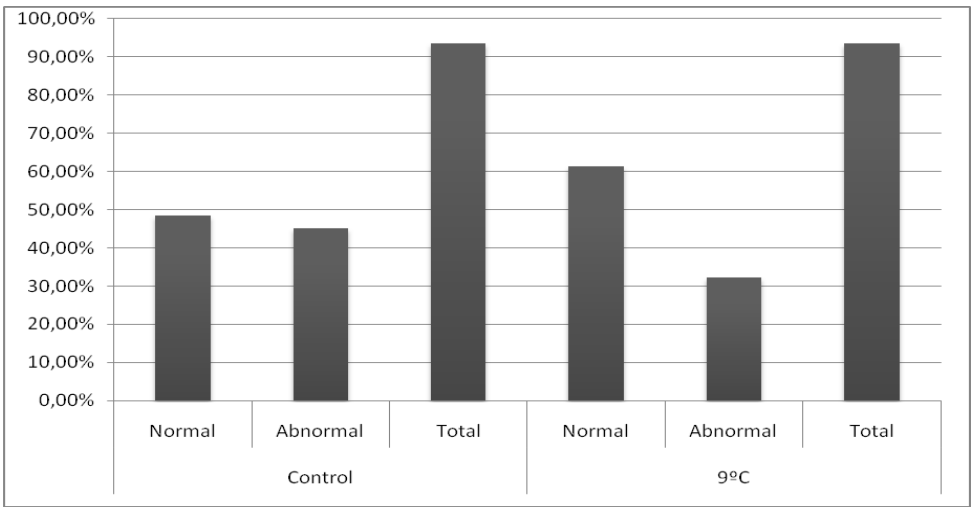


Figure 1: Germination percentage histogram

With or without treatment, we obtain the same germination percentage (93.5%). But as we can see, we get larger number of normal plants after cold treatment (9 ° C). After observing the data obtained in the experiment, we concluded that a cold treatment prior to germination improves the quality of seedlings, but not the percentage of germination.

In terms of germination percentage, the results are not entirely representative. Theorically the percentage should be higher after cold treatment.

5.2 NIR analysis

Results obtained by NIR analysis in laboratory have been processed by PCA, PLS and MSC, explained in previous sections, to see and compare them in a more simple way and take conclusions easier.

The objective of Principal Component Analysis (PCA) is to transform into a more relevant coordinate system and a dimensionality reduction. In PCA we make a principal component model of the essential correlation structure of X. The most commonly used plot in multivariate data analysis is the score vector for PC1 (X-axis) versus the score vector for PC2 (Y-axis) (Esbensen, 2000). Our variables in this study are: seed variety, harvest time and location in axis X, and the samples in Y.

Partial Least Squares Regression (PLS) has been used to predict and analyze dependent variables from independent variables or predictors. We took the values 0, 1 and 2 to predict the results, in different states. The value 0 is for the lupin seeds which are not germinated, the value 1 for abnormal seedlings *(see point 4.2.2)* and number 2 for the seeds that have been developed correctly.

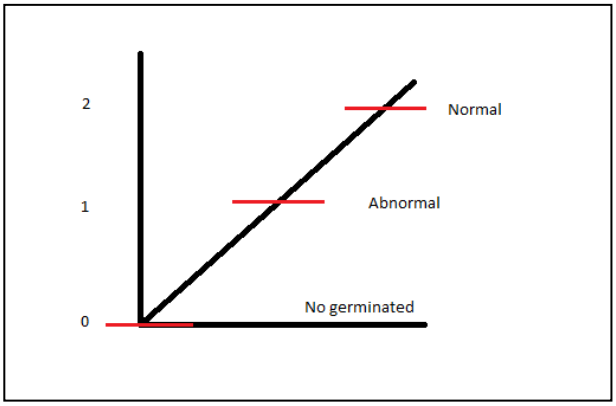


Figure 2: Values of prediction

PLS models are interpreted in the same way as PCA models.

Multiple scatter correction (MSC) has been used to remove noise from X matrix.

5.2.1 NIR results

NIR measurements on a total of 1908 seeds were carried out. The raw data from the IR measurements are plotted in a line plot in order to look at the structure of the data ***(Figure3)***.

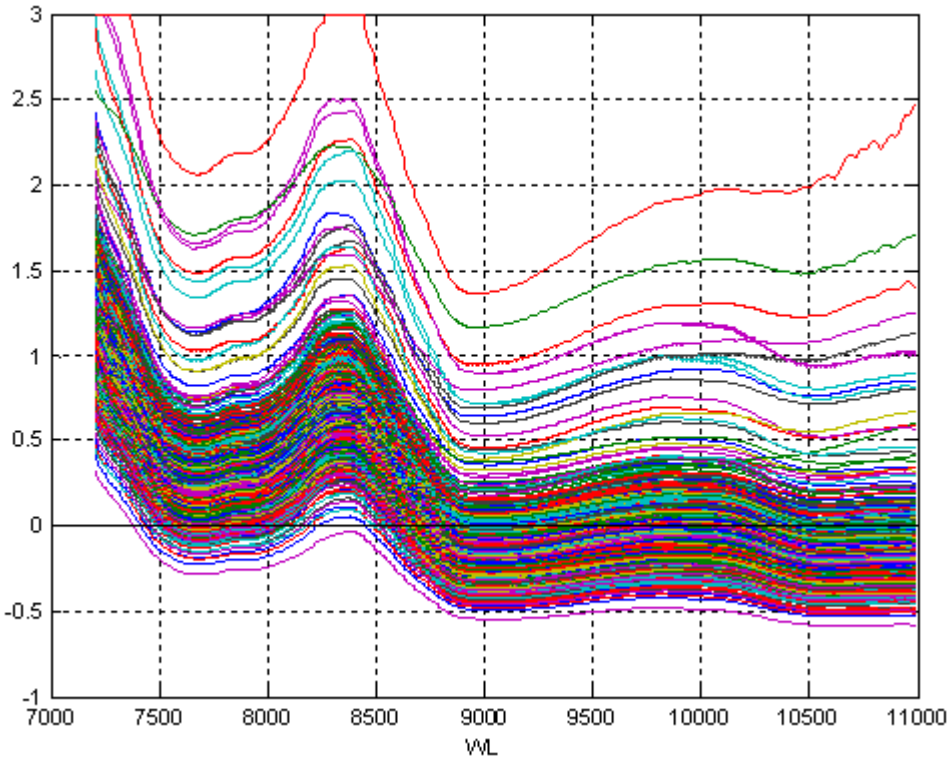


Figure 3: Raw NIR spectra for all samples (7000-11000 cm-1)

As we can see in ***Figure 3,*** NIR spectra of all the samples are similar in curve form, is a good sign that the measures were made correctly. But as we can observe, there are some of the samples which have a different progress than the rest.

In ***Figure 4***, PD samples are shown with green colour, to see if we can see something interesting.

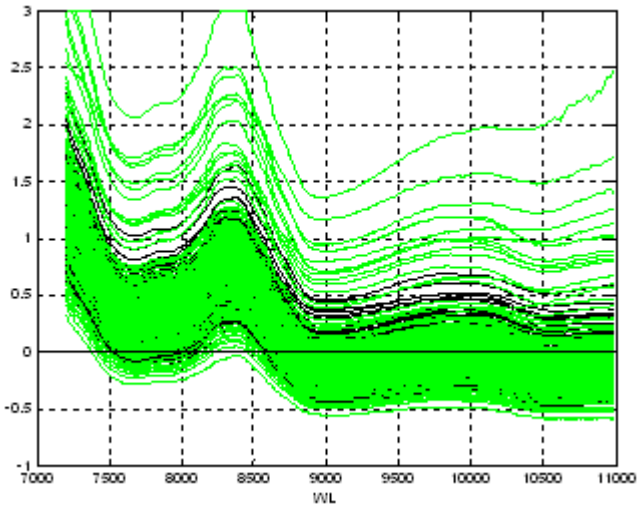


Figure 4: Raw NIR spectra for all samples (7000-11000 cm-1). PD samples are shown with green colour

As we can see in ***Figure 4***, samples that look different comparing with the others are of **PD** variety, which probably are outliers.

We have transform data to remove noise from X matrix, using Multiple Scatter Correction (MSC) as we can observe in ***Figure 5***.

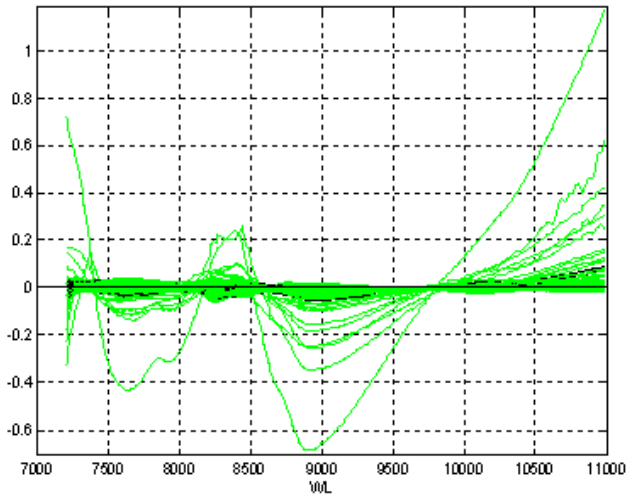


Figure 5: MSC (multiple scatter corrected) NIR spectra for all samples (7000-11000 cm-1). PD samples are shown with green colour.

We can see very clearly the sample (PD variety) which is very different from the others. We need more information about this sample to take conclusions. For this, we have coloured the PD samples in two colors depending on the harvest moment, harvest 2 with pink and harvest 5 with blue.

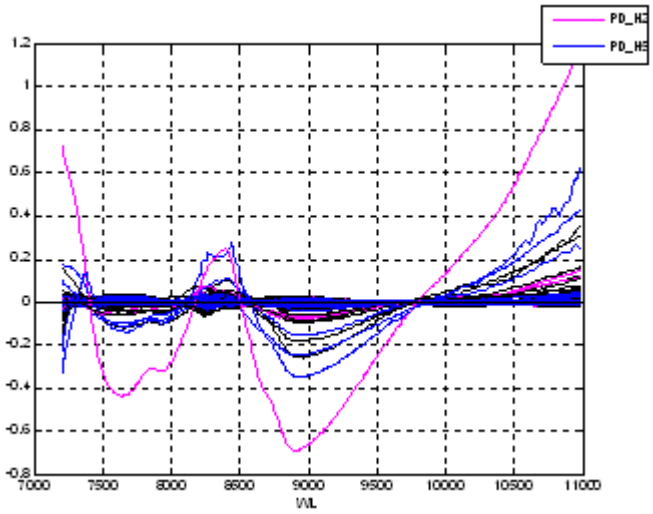


Figure 6: MSC NIR spectra for PD samples (7000-11000 cm-1). PD samples from harvest 2 are shown with pink colour and harvest 5 with blue

As we can observe in ***Figure 6***, the possible outlier belongs to a sample of harvest 2 **(PD_H2).** To detect the outliers we have used PCA method as we can see in ***Figure 7***.

Principal Component Analysis (PCA)

A PCA of all measurements is conducted to illustrate data and detect the possible outliers. In the ***Figure 9*** we can see the samples of PD variety coloured according to harvest time.

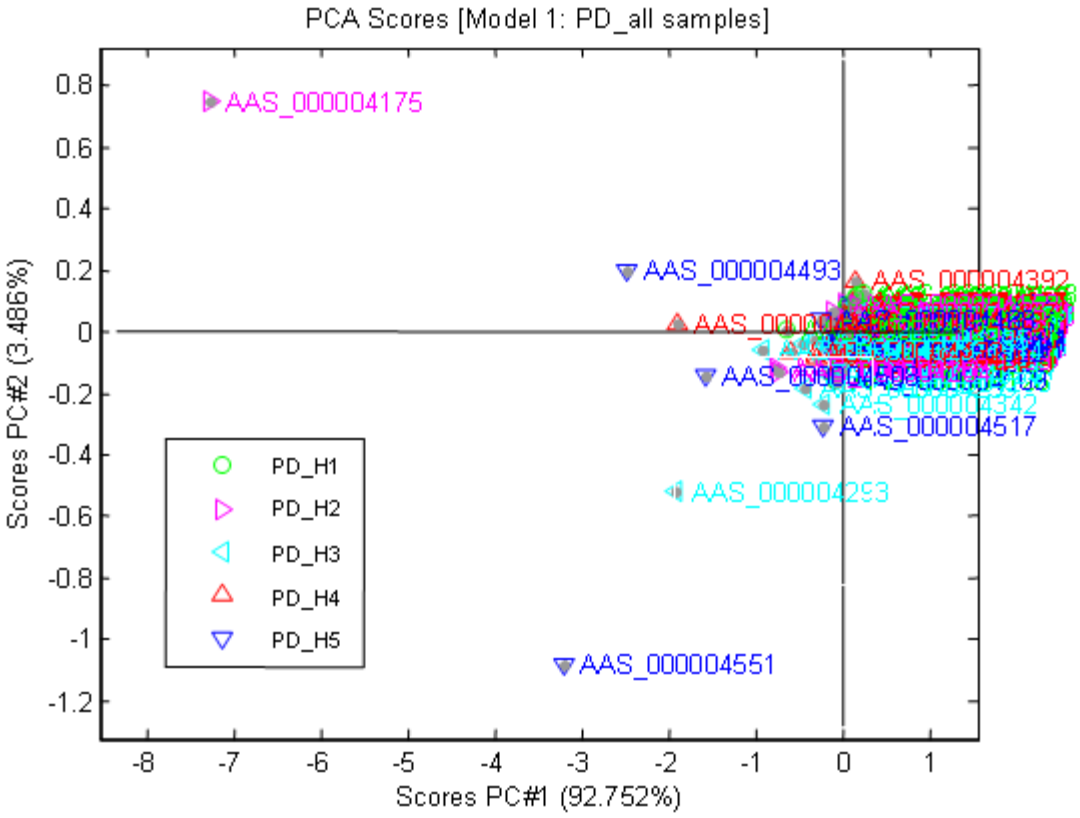


Figure 7: PCA (principal component analysis) of PD samples coloured according to harvest time

There is a singular object in the upper left corner ***PD_H2*** (AAS_000004175) (PD variety/ Harvest 2), which probably is an outlier, as well as the line plot showed before. It has the most negative score for PC1, approx. -7.5, and a very large positive score for PC2, approx. 0.8.

Despite this, we can't see any correlation (in groups of harvest: the data of the same harvest, more or less grouped together) between different harvests.

We have removed sample ***PD_H2*** (AAS_000004175) as an outlier, because when this samples is very different from the others it can hide the variation and patterns there might be within the other samples. Maybe we can observe correlation between harvest data in PD variety.

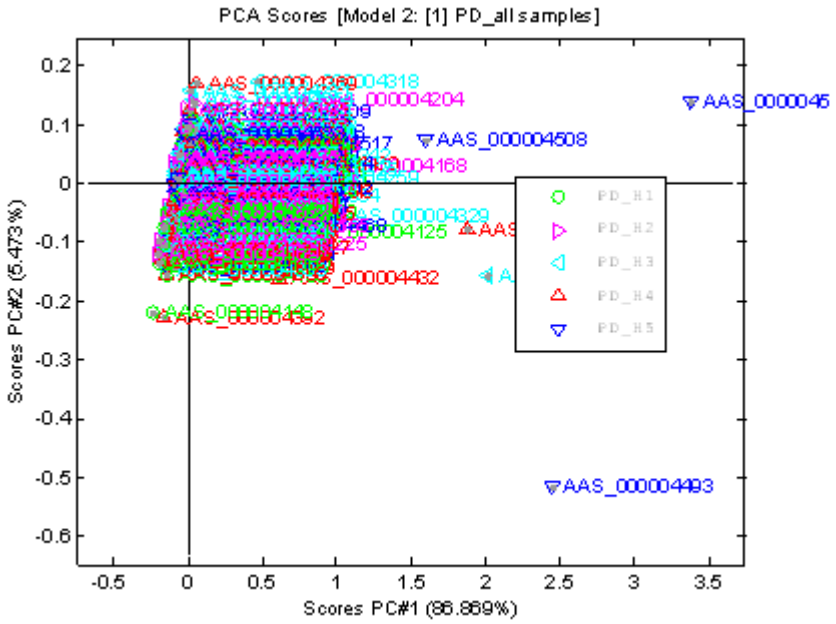


Figure 8: PCA (principal component analysis) of PD samples coloured according to harvest time without sample 4175

As we can see in ***Figure 8*** after remove the sample ***PD_H2***, there are two samples (PD_H5) that differ from the other samples and from each other. This is not very logical, we should see a group of seeds from the same harvest time differing from the other harvests, not only one seed. This means that they continue a different trend comparing with the rest of the samples.

We have continued the same process as in Figure 9 and 10, to see if we can get any correlation. We have removed the following samples: 4175, 4293, 4410, 4493, 4508, 4551, 4125, 4168, 4204, 4318, 4329, and 4432.

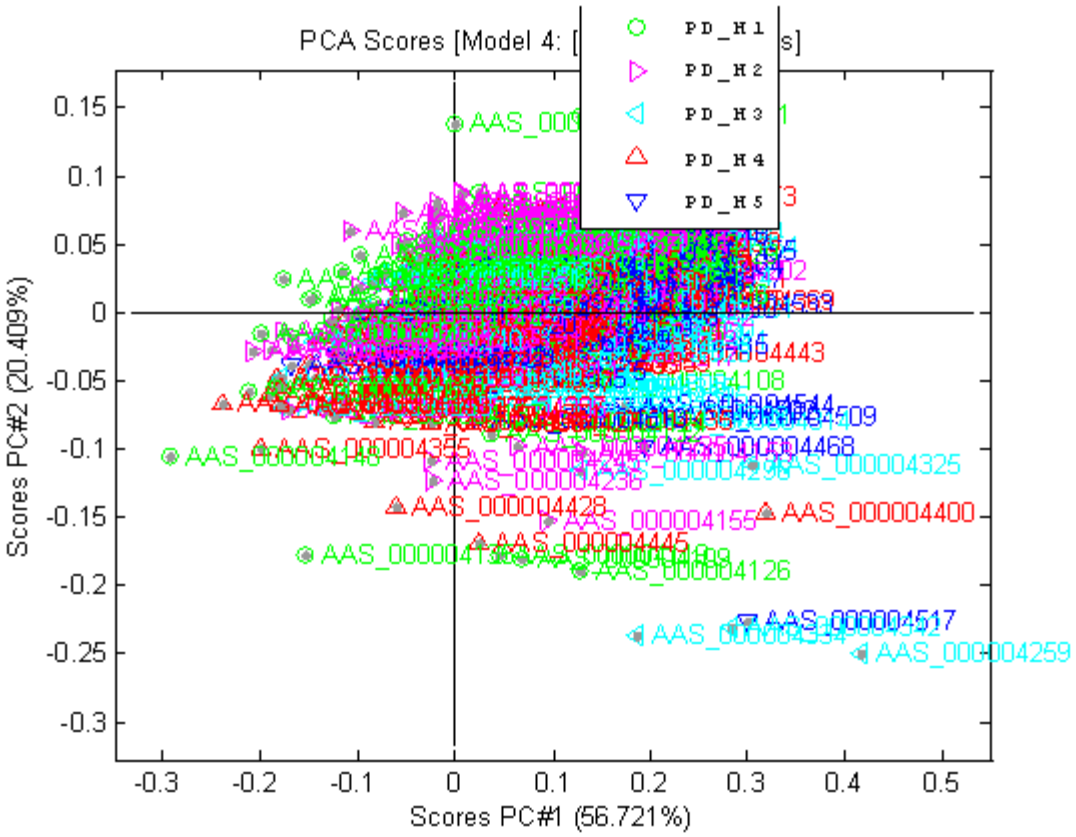


Figure 9: PCA (principal component analysis) of PD samples coloured according to harvest time without sample 4175, 4293, 4410, 4493, 4508, 4551, 4125, 4168, 4204, 4318, 4329, 4432

As we can see in ***Figure 9***, definitely, we can't see any correlation between different harvest time (H1, H2, H3, H4 and H5) with NIR method. This may be due to seeds of PD variety are very similar (similar harvest, we can't differentiate between them) or that NIR method is not the correct one for this process. It is too soon to take conclusions, we have to continue studying the data. Also may be due to a failure in the measure process, but we rule out this possibility.

.

The same procedure is carried out for Bora and Prima varieties, because it may be that samples of PD variety are similar between them, and not of Bora or Prima. Score plots are shown after we remove the possible outliers for abbreviation reasons, ***Figure 10 and 11***.

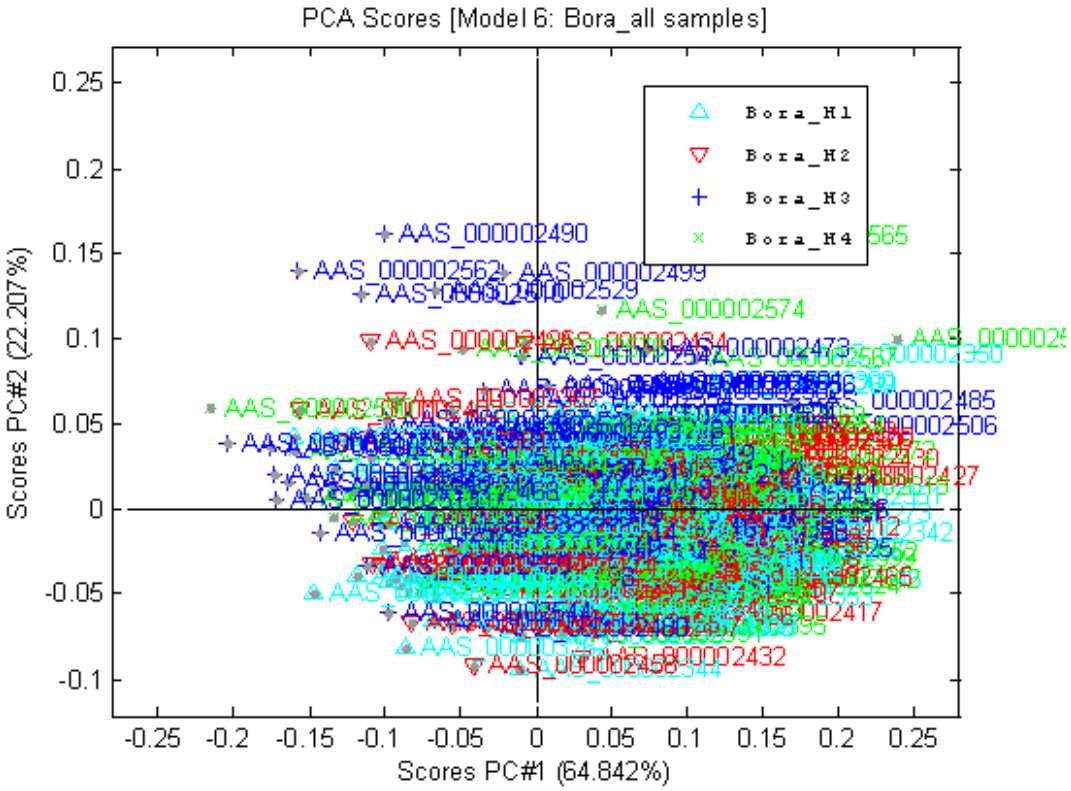


Figure 10: PCA of BORA samples coloured according to harvest time

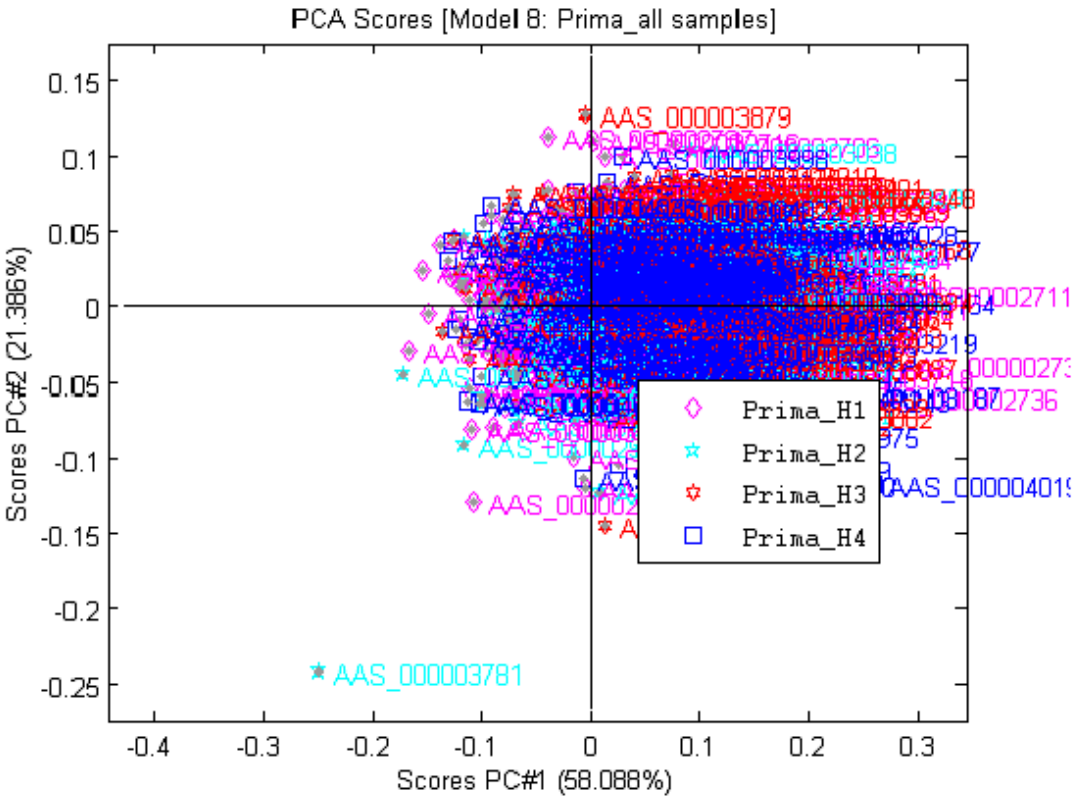


Figure 11: PCA of PRIMA samples coloured according to harvest time

We have obtained the same result with Prima and Bora. We can't see any correlation in the data. For example, according to the results in ***Figure 10***, we come to the conclusion that the samples are very similar because all are completely mixed. For a difference, we should see the data broken down by color. This may be because the harvest was not sufficiently different, or because of the failure of the method.

In the PCA of ***Figure 11***, we tried to remove the sample AAS_000003781 (Prima_H2) as an outlier without any changes, so therefore we included in the model.

We have also study the relation between locations, basing on Bora and Prima samples. We couldn't perform with PD variety because it was only grown at Ytteborg. ***Figure 12*** and **15** shows that there aren't any differences between seeds that have been grown in Taastrup or Ytteborg. This may be due to the fact climate conditions are very similar in both places. If one thing is certain, it's that weather affects seed development significantly. Can also be due to the inefficiency of the method.

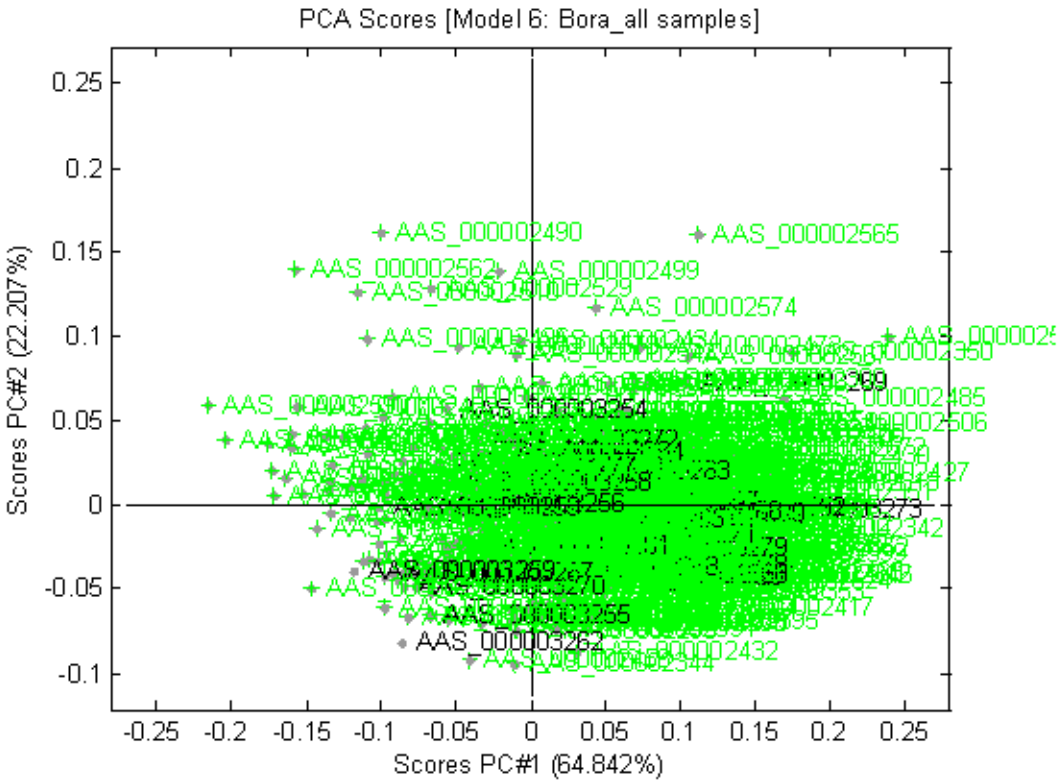


Figure 12: PCA of BORA samples coloured according to location (Taastrup green, Ytteborg black)

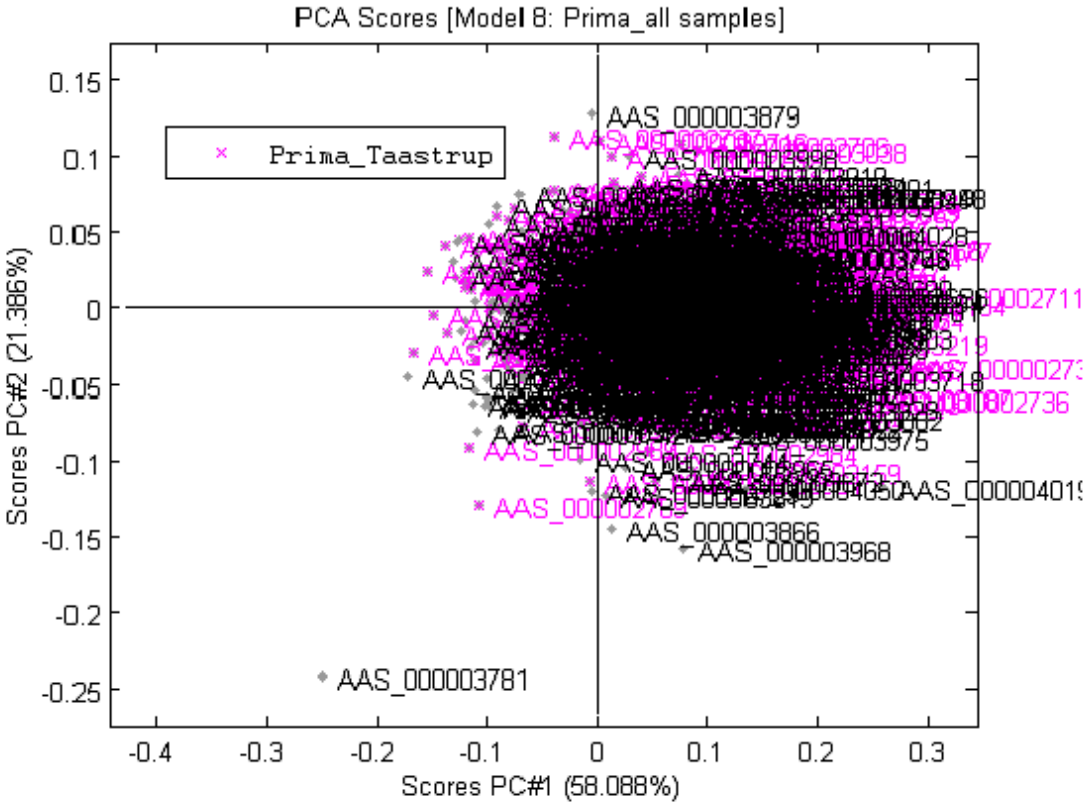


Figure 13: PCA of PRIMA samples coloured according to location (Taastrup pink, Ytteborg black)

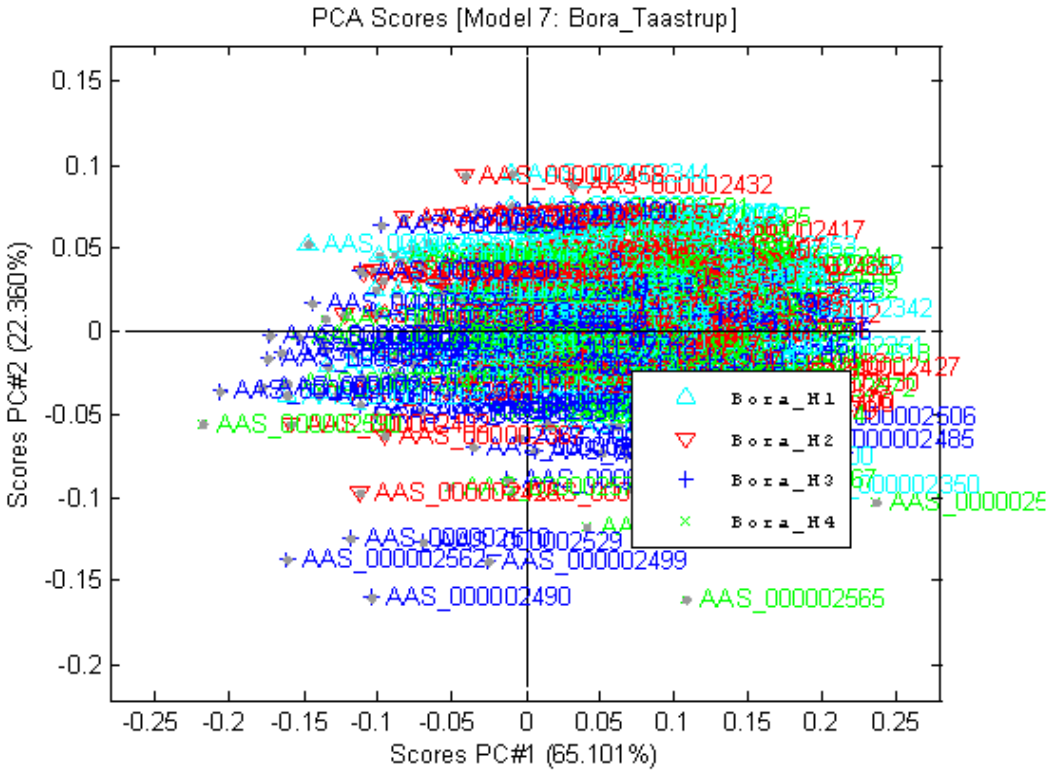


Figure 14: PCA of BORA samples coloured according to harvest time for samples grown at Taastrup

In ***Figure 10 and 12***, we have tried to correlate harvest and location respectively, with Bora variety seeds. ***Figure 16*** shows the different harvests at Taastrup of Bora variety, to see if harvest and location is correlated. But we haven't obtained any positive result.

Now, we are going to study the relation between varieties using PCA. The seeds of PD variety are shown with green colour, Prima with blue and Bora with red.

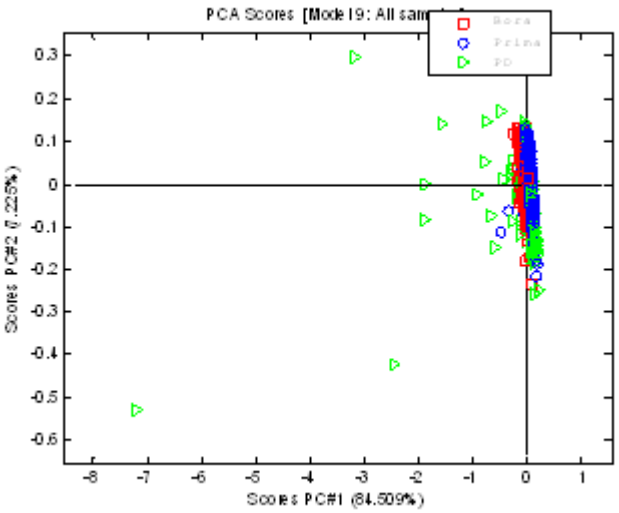


Figure 15: PCA of all samples coloured according to variety

We see a small difference between varieties in ***Figure 15***. PD data are very distributed, so we have removed them, to see better the distribution of Bora and Prima data, as we can see in ***Figure 16***.

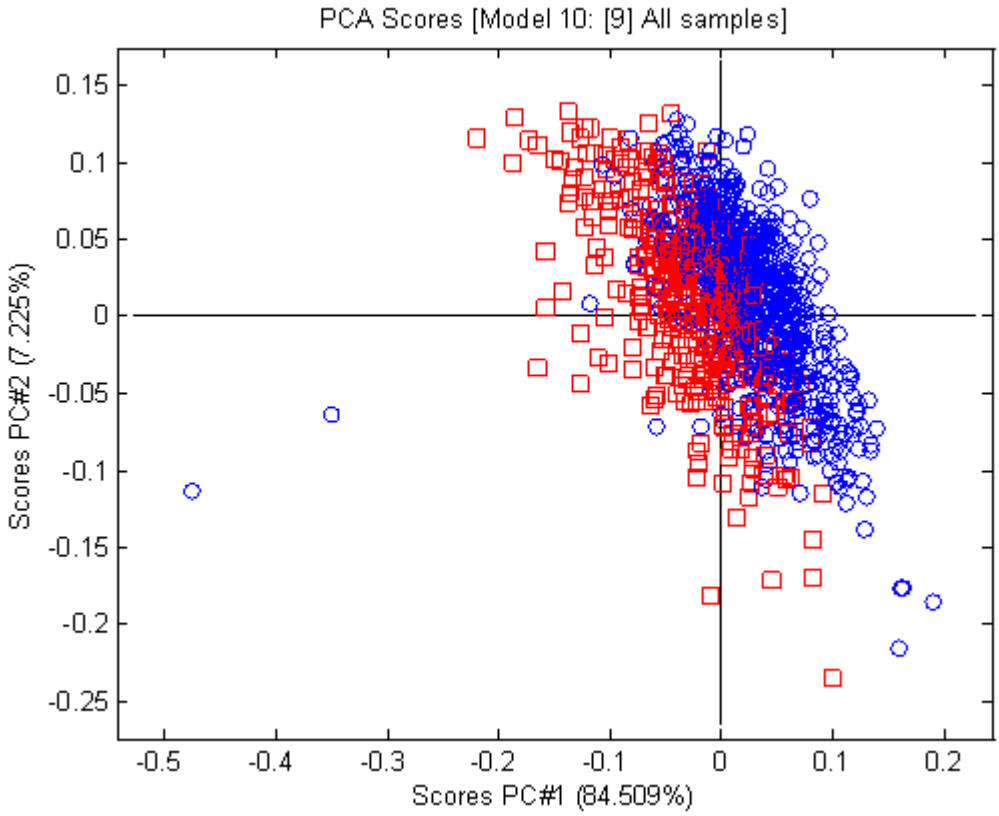


Figure 16: PCA of all samples coloured according to variety where only BORA (red) and PRIMA (blue) samples are shown

There is a trend that Bora and Prima differentiate from each other. We see the difference of the data according to variety using NIR method. The separation is not perfect, but it could be a positive conclusion.

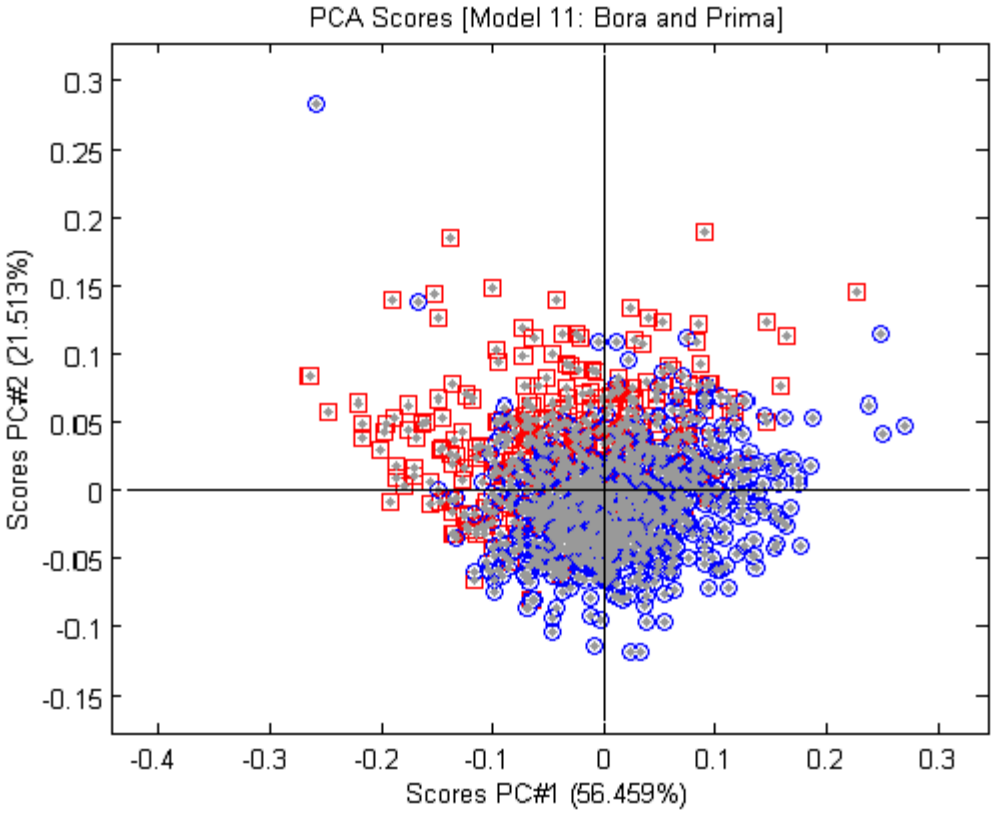


Figure 17: PCA of BORA and PRIMA samples coloured according to variety

Figure 17 shows the two varieties, which Bora and Prima are marked with red and blue respectively.

As we can see in ***Figure 18***, we have marked the samples of harvest 1 and 4 in ***Figure 17***, to conclude that we can't correlate the data according to harvest, due to the similarity of the samples in terms of harvest or inefficiency of the method.

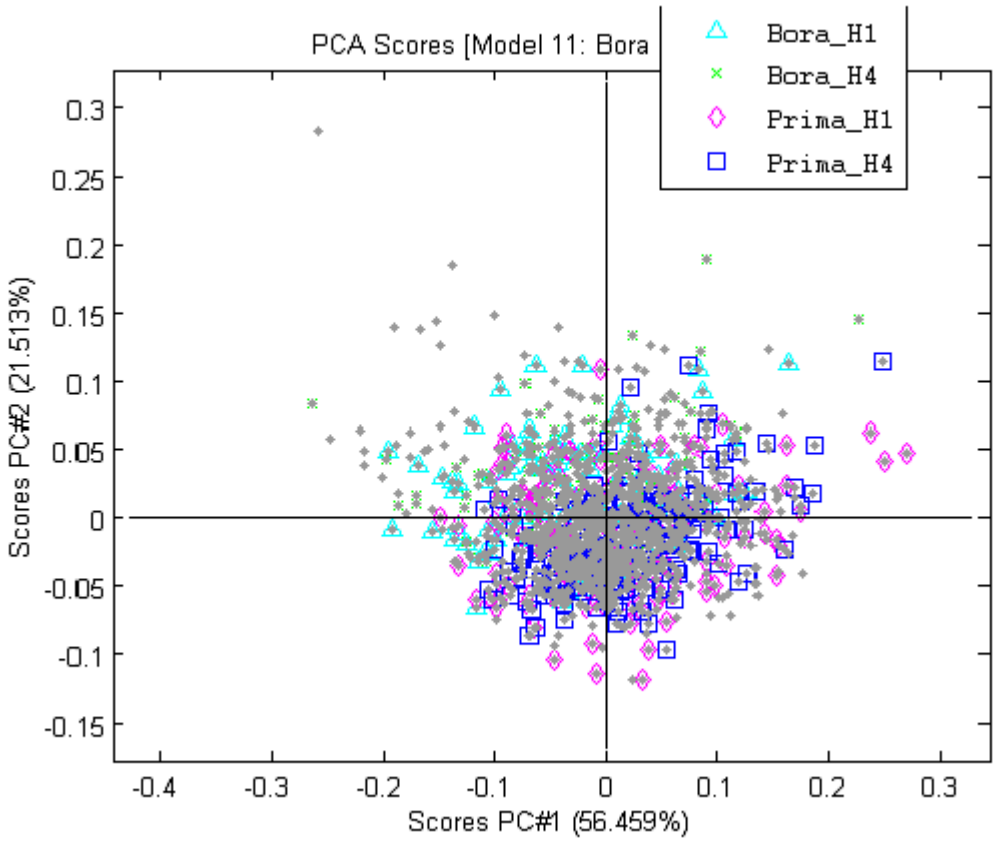


Figure 18: PCA of BORA and PRIMA samples coloured according to harvest time 1 and 4 for the two varieties

Partial Least Squares Regression (PLS)

PLS is carried out with the three varieties (Bora, Prima and PD), to predict the germination. We have chosen the values 0, 1 and 2 to predict the results, in different states. The value 0 is for the lupin seeds which are not germinated, the value 1 for abnormal seedlings and number 2 for the seeds that have been developed correctly.

Next we performed a PLS with germination data obtained after NIR measurements. The data are coloured by variety.

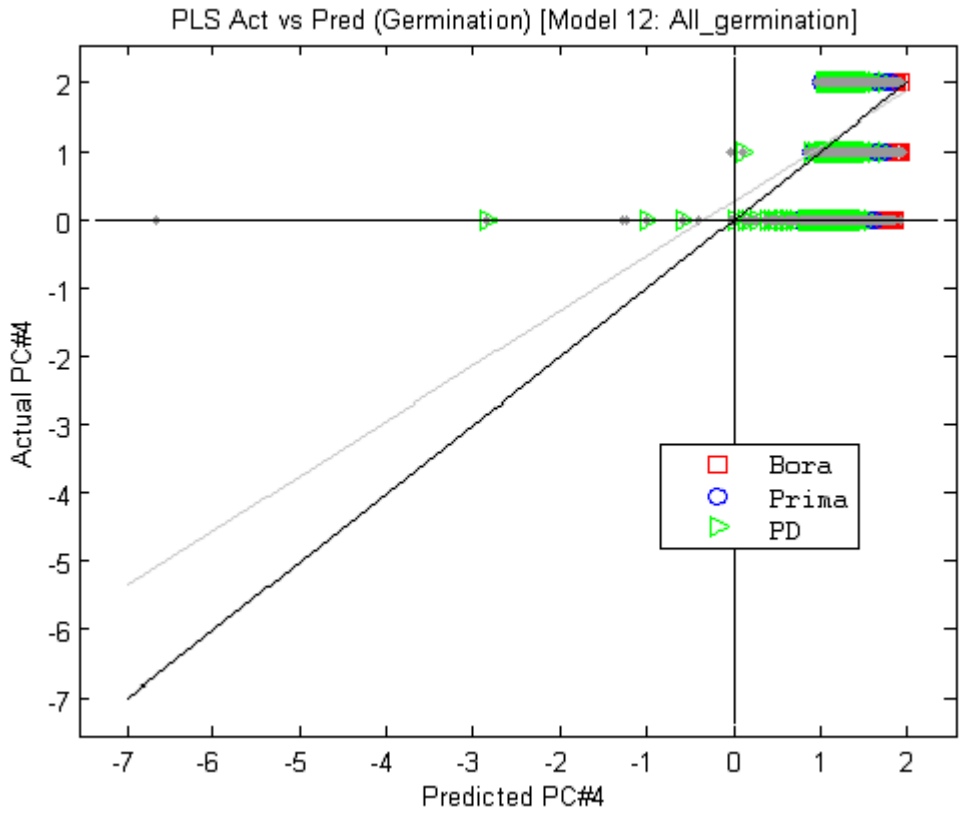


Figure 19: PLS (partial least squares) regression of all samples colored according to variety. MSC NIR spectra are used to predict germination. r = 0.27, rmsep = 0.73

As we can see in ***Figure 19***, the PLS is not consistent with model planned in point 4.2. Despite this, we must point out, as we can see in ***Figure 19***, that seeds that are not germinated (PD variety) are located to the left of zero, as shown in the theoretical model proposed before. However, the correlation index value r = 0.27 shows that the datas are not representative, being in principle impossible the prediction.

After we continued with the same procedure, doing PLS regression of Bora, Prima and PD samples coloured according to location and harvest time (studied individually).

Next we show some of the PLS as demonstration:

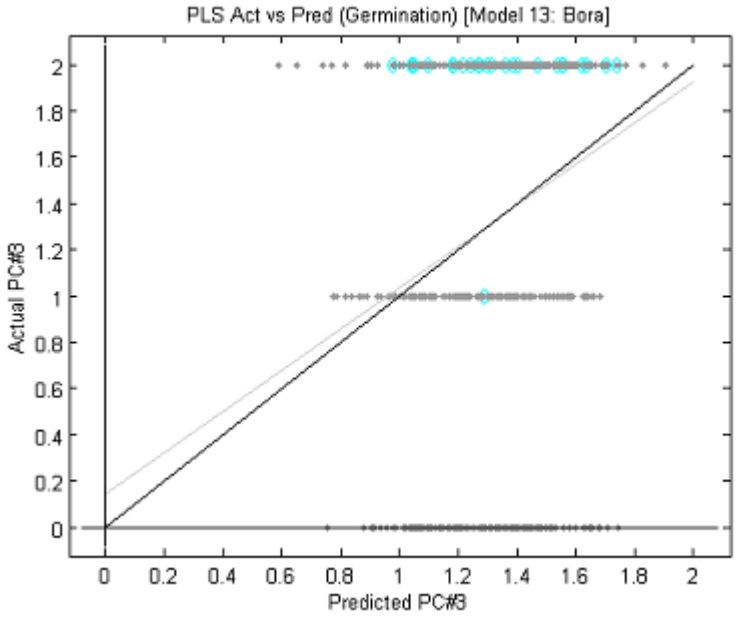


Figure 20: PLS (partial least squares) regression of BORA samples colored according to location (light blue = Ytteborg). MSC NIR spectra are used to predict germination. r = 0.19, rmsep = 0.81.

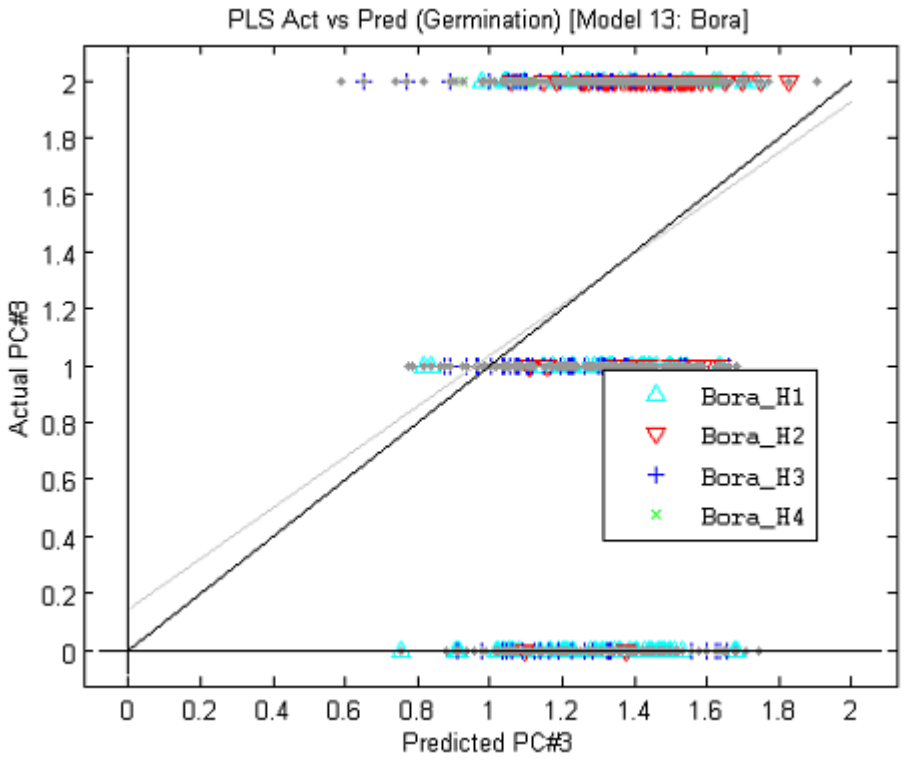


Figure 21: PLS (partial least squares) regression of BORA samples colored according to harvest. MSC NIR spectra are used to predict germination . r = 0.19, rmsep = 0.81.

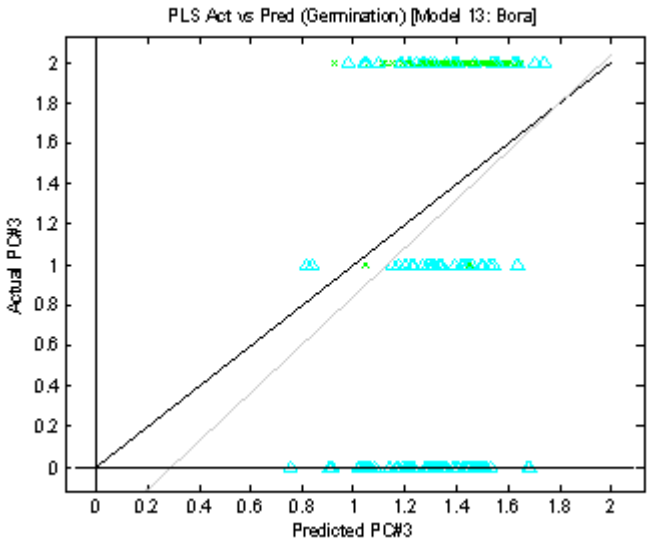


Figure 22: PLS (partial least squares) regression of BORA samples colored according to H1 and H4

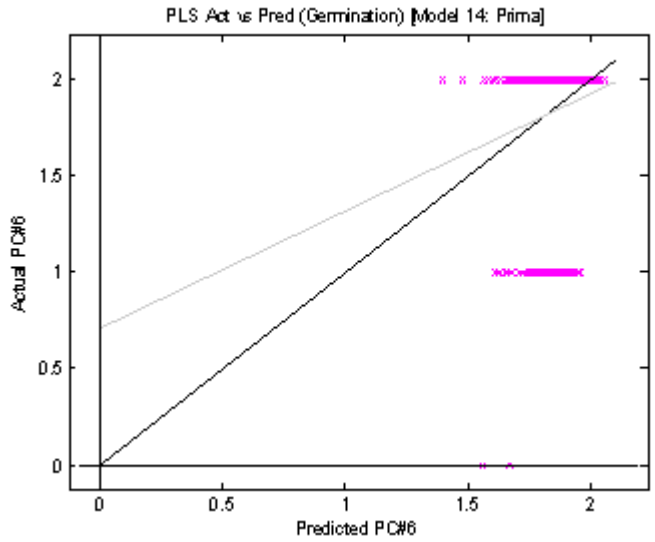


Figure 23: PLS (partial least squares) regression of PRIMA samples colored according to Taastrup

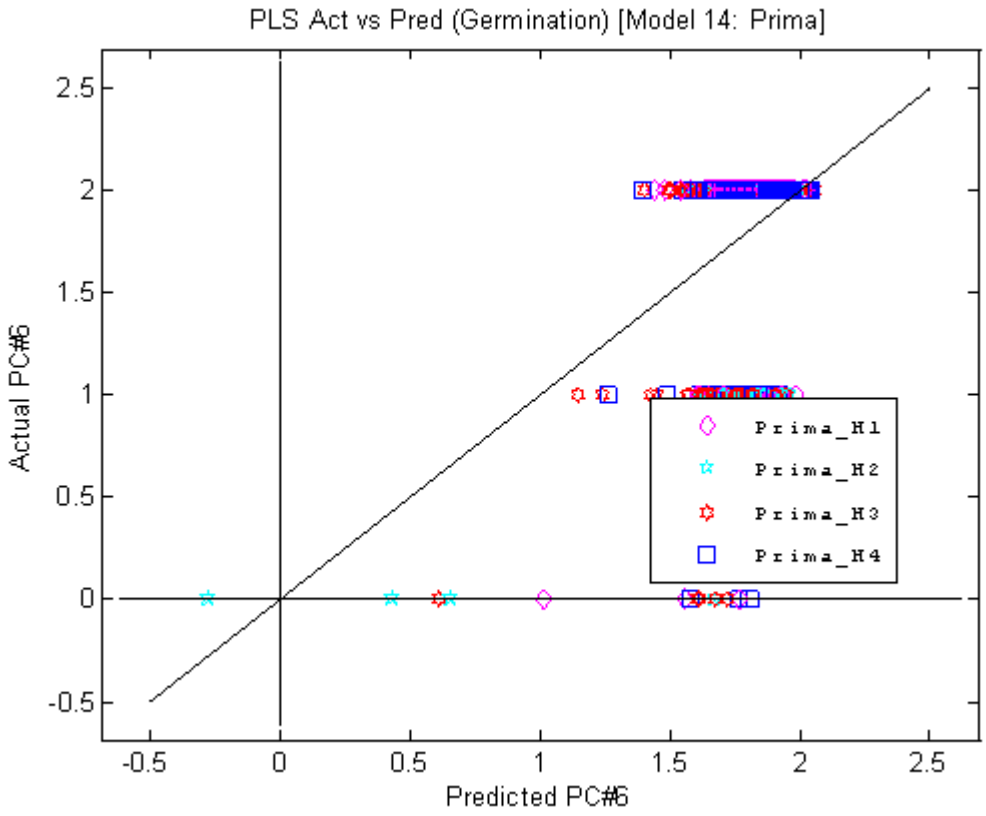


Figure 24: PLS (partial least squares) regression of PRIMA samples colored according to harvest time. MSC NIR spectra are used to predict germination. r = 0.35, rmsep = 0.44.

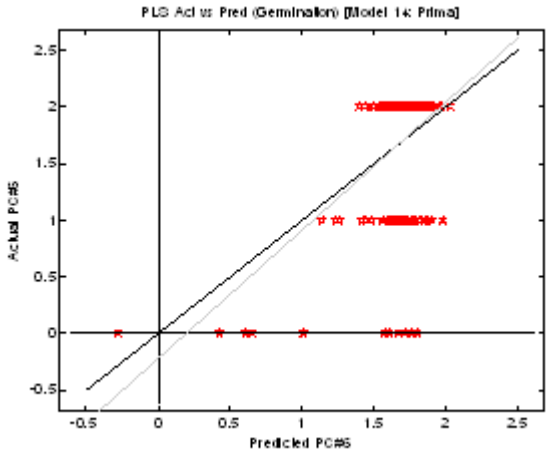


Figure 25: PLS (partial least squares) regression of PRIMA samples coloured according to Ytteborg

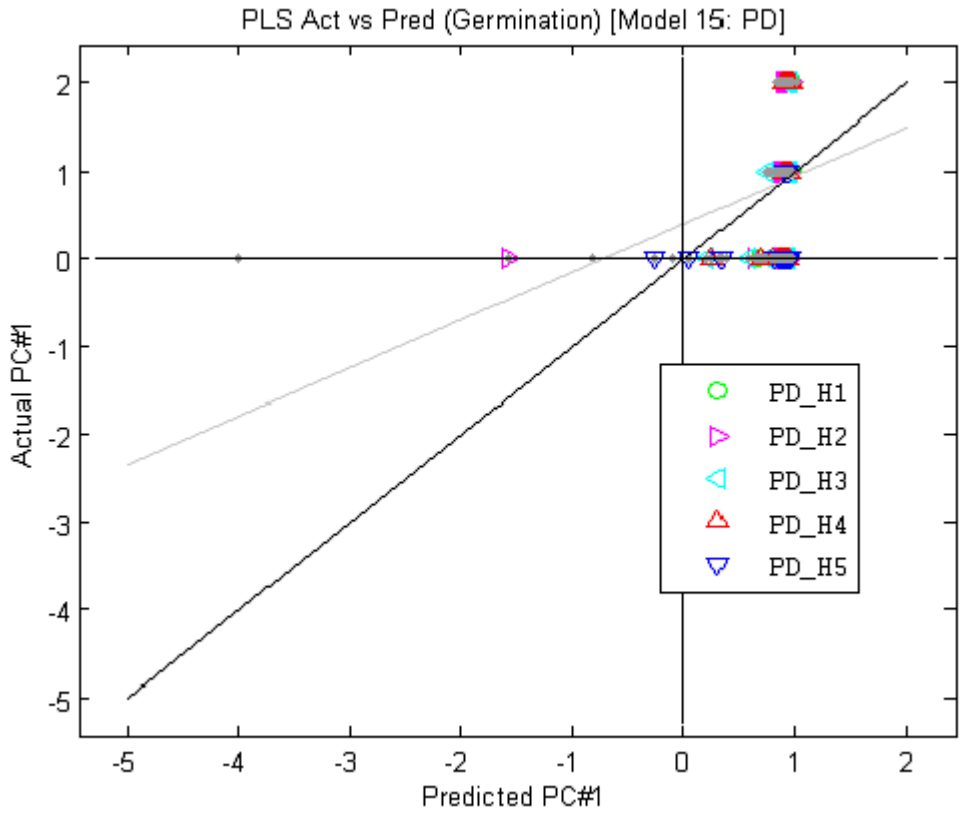


Figure 26: PLS (partial least squares) regression of PD samples coloured according to harvest time. MSC NIR spectra are used to predict germination. r = 0.12, rmsep = 0.84.

As we can see in all the PLS, in none of the cases we have obtained significant results, due to the low correlation index value (r), being impossible to draw meaningful conclusions. PLS is not consistent with the model planned before *(see point 5.2).*

After seeing those results, we reject the method for such data. We discard the PLS as effective method.

The reason why we chose only to predict germination with PLS is that we cannot use the PCA algorithm to predict something. In a PLS the algorithm tries to find correlation between the spectra (X-matrix) and the germination variable (y). It is correct that if there was a nice correlation between non-germinated, normal and abnormal seeds it would also have been possible to see in a PCA. PCA is a classification where the only data is the spectra. If we instead of names gave the germination characteristics (0, 1, 2) there would be a pattern if it was correlated. We chose to use PLS instead because we really do not see many patterns in the data

5.2.2 Germination results

The germination results are shown in ***Appendix 3***.

5.3 Kjeldalh method

ID.	Afvejet g	Vol. HCL (ml)	HCL (M)	N i prøve (g)	% N / g prøve	% prot. as is	% prot. as is
24 --1	1,0098	33,8	0,1	0,0473	4,6883	29,3020	29,42400966
24 --2	1,0222	34,5	0,1	0,0483	4,7274	29,5460	
45 --1	1,0692	37,3	0,1	0,0522	4,8864	30,5398	30,85402911
45 --2	1,035	36,85	0,1	0,0516	4,9869	31,1683	
52 --1	1,0419	34,9	0,1	0,0489	4,6918	29,3235	29,92052363
52 --2	1,004	35	0,1	0,0490	4,8828	30,5176	
61 --1	1,0218	35,7	0,1	0,0500	4,8937	30,5857	30,50184843
61 --2	1,0447	36,3	0,1	0,0508	4,8669	30,4180	
64 --1	1,0118	36,6	0,1	0,0513	5,0667	31,6667	30,89909923
64 --2	1,0096	34,75	0,1	0,0487	4,8210	30,1315	
51-1	1,0242	34,95	0,1	0,0490	4,7797	29,8730	29,80988796
51-2	1,005	34,15	0,1	0,0478	4,7595	29,7468	
49-1	1,002	35,6	0,1	0,0499	4,9764	31,1027	30,96513994
49-2	1,0365	36,5	0,1	0,0511	4,9324	30,8276	
62-1	1,0197	34,9	0,1	0,0489	4,7939	29,9619	29,99171541
62-2	1,041	35,7	0,1	0,0500	4,8035	30,0216	
33-2	1,0065	35,9	0,1	0,0503	4,9959	31,2246	
63-1	1,0435	36,2	0,1	0,0507	4,8591	30,3691	30,87344199
63-2	1,0253	36,75	0,1	0,0515	5,0204	31,3778	

Table 7: Kjeldalh test result

as is = not corrected for moisture content

As we can see in ***Table 7***, the samples have more or less the same amount in protein, although belonging to different crops. This means that the samples are very similar. Therefore we can conclude that due to too little variations in the samples, it is impossible to make predictions with NIR.

6. Final conclusions

In this study we haven't obtained any clear conclusion in either of the main objectives, so investigations should be continued for more stable conclusions.

However, there has been a focus on variety and location, which serves as a starting point for following projects, and lads us to believe that NIR method could be very useful in some investigation fields and industries amongst many others.

NIR is a chemical fingerprint where different wavelengths correlate to different chemical compounds, so to distinguish samples of different variety, location and harvest time. This should be possible through NIR if the samples differ in their chemical composition. In our case we have had difficulties with the characterization. It can either be because the NIR made failing measurements or because the samples do not differentiate from each other. Kjeldahl method was used to measure this variability of the samples, in terms of protein content. Samples which should have had a maximum variability, such as harvesting 1 and 4, showed almost the same protein content. This indicates that the samples are too homogeneous to be described by NIR. NIR cannot classify any groups if there are no chemical differences between the samples. This little variation among the seeds is maybe because the crops were not properly spaced in time of harvest and/or location.

Regarding to prediction of germination by NIR, using Partial Least Squares Regression (PLS), the results have been poor, unable to get any positive conclusions. It has been on one hand what we expected and on the other not. As we have discussed above, NIR obtains the chemical fingerprint of the sample that it is measuring. Nevertheless germination is not only chemical but is also a physiological process. Due to this, it was not expected that NIR could be used as a **direct** method for prediction of germination.
If there had been correlation between germination ability and one or more chemical compounds (e.g. fat or protein content) that could also be related to germ size (> fat , > germ size) it would be possible to use NIR for an **indirect** method to predict germination. We say indirectly, because germination is also influenced by other factors.
It was not possible to predict germination with NIR and therefore it can be concluded that there do not seem to be any correlations between germination and the chemical compounds. This might also be due to the model proposed, being difficult to make a prediction where the y-variable is only three numbers; 0: No-germinated/ 1: Abnormal/ 2: Normal. A possible solution would be to increase the information level in y-variable of the model. When we refer to the size of the seedling (good developed or underdeveloped), we could specify more, for example adding the length in centimeters; 0: No-germinated/ 1: Abnormal [0-12cm]/ 2: Normal [>12cm].

In this project the whole NIR spectra has been used for data analysis to characterize and predict germination. It might be possible to obtain better results selecting only a part of a spectra or wavelength. In other words primarily where the wavelength related to the fat or protein contents that were used in the prediction of germination. However, again this work is not worth doing if the samples do not differentiate.

7. Perspective

If it would be possible to do the characterization of lupin samples according to variety, location and harvest time, the seed industry and plant breeders could use NIR as a fast screening method to determine which varieties at the market would differentiate most and which variety that would be most stable in different growing locations. For example, if two varieties were grown at two or three different locations, and the PCA showed that all samples from one variety was in one cluster, and the other variety samples spread out according to location, you could conclude that variety one was more stable than the other, since its not influenced be different environments (growing locations) as the other is.

If it would be possible to predict germination characteristics, the seed industry could use NIR as a screening method. This would really make a difference for the industry as they did not have to make a lot of time consuming germination tests, thus reducing time and space, besides the economic savings that would be assumed.

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9. Appendix

Appendix 1: Most important species of Lupinus genre

- *Lupinus adsurgens*
- *Lupinus affinis*
- *Lupinus albicaulis*
- *Lupinus albifrons*
- *Lupinus albus*
- *Lupinus* × *alpestris*
- *Lupinus andersonii*
- *Lupinus angustiflorus*
- *Lupinus angustifolius*
- *Lupinus antoninus*
- *Lupinus arboreus*
- *Lupinus arbustus*
- *Lupinus arcticus*
- *Lupinus argenteus*
- *Lupinus aridorum*
- *Lupinus arizonicus*
- *Lupinus benthamii*
- *Lupinus bicolor*
- *Lupinus bingenensis*
- *Lupinus brevicaulis*
- *Lupinus breweri*
- *Lupinus burkei*
- *Lupinus caespitosus*
- *Lupinus caudatus*
- *Lupinus cervinus*
- *Lupinus chamissonis*
- *Lupinus citrinus*
- *Lupinus concinnus*
- *Lupinus constancei*
- *Lupinus cosentinii*
- *Lupinus covillei*
- *Lupinus croceus*
- *Lupinus dalesiae*
- *Lupinus duranii*
- *Lupinus diffusus*
- *Lupinus elatus*
- *Lupinus elmeri*
- *Lupinus luteolus*
- *Lupinus luteus*
- *Lupinus lyallii*
- *Lupinus macbrideanus*
- *Lupinus michelianus*
- *Lupinus micranthus*
- *Lupinus microcarpus*
- *Lupinus minimus*
- *Lupinus mutabilis*
- *Lupinus nanus*
- *Lupinus nevadensis*
- *Lupinus nipomensis*
- *Lupinus niveus*
- *Lupinus nootkatensis*
- *Lupinus nubigenus*
- *Lupinus obtusilobus*
- *Lupinus odoratus*
- *Lupinus onustus*
- *Lupinus oreganus*
- *Lupinus padre-crowleyi*
- *Lupinus palaestinus*
- *Lupinus parviflorus*
- *Lupinus peirsonii*
- *Lupinus perennis*
- *Lupinus pilosus*
- *Lupinus plattensis*
- *Lupinus polycarpus*
- *Lupinus polyphyllus*
- *Lupinus pratensis*
- *Lupinus prunophilus*
- *Lupinus pusillus*
- *Lupinus regalis*
- *Lupinus rivularis*
- *Lupinus rupestris*
- *Lupinus saxosus*
- *Lupinus sericatus*
- *Lupinus sericeus*

- *Lupinus excubitus*
- *Lupinus flavoculatus*
- *Lupinus foliolosus*
- *Lupinus formosus*
- *Lupinus grayi*
- *Lupinus guadalupensis*
- *Lupinus havardii*
- *Lupinus hirsutus*
- *Lupinus hirsutissimus*
- *Lupinus holmgrenianus*
- *Lupinus hyacinthinus*
- *Lupinus jaimehintoniana*
- *Lupinus kuntii*
- *Lupinus kuschei*
- *Lupinus lapidicola*
- *Lupinus latifolius*
- *Lupinus lepidus*
- *Lupinus leucophyllus*
- *Lupinus littoralis*
- *Lupinus longifolius*
- *Lupinus shockleyi*
- *Lupinus smithianus*
- *Lupinus sparsiflorus*
- *Lupinus spectabilis*
- *Lupinus stiversii*
- *Lupinus subcarnosus*
- *Lupinus succulentus*
- *Lupinus sulphureus*
- *Lupinus texensis*
- *Lupinus tidestromii*
- *Lupinus toratensis*
- *Lupinus tracyi*
- *Lupinus truncatus*
- *Lupinus vallicola*
- *Lupinus variicolor*
- *Lupinus villosus*
- *Lupinus wyethii*

Appendix 2: Kjeldahl method. Materials

It has followed the standard method of *American Analytical Cereal Chemists (AACC).*

Seeds: We have chosen those samples; 24, 33, 45, 49, 51, 52, 61, 62, 63 and 64 to test whether the cultivars differ and if harvest time affects the protein content.

Apparatus:

- Kjeldahl flasks, Pyrex, 800ml capacity. Used for difestion and destillation.
- Digestion heaters, 600W. Heater unit should boil 250ml water starting at 25° in 5 minutes with hot burners.
- Digestion unit. Consist of electric heaters, large lead tube, fume stack and suction fan capable of exhausting toxic fumes to outside air.
- Distillation unit. Consist of Iowa State-type connecting bulbs 36x100mm, Pyrex glass condenser tubes, pure gum-rubber stoppers and tubing, electric heating units (600W), condenser tubes capable of being kept cool with adequate amounts of cool water during distillation and with thermo-water control on stills. Upper ends of bulbs or traps connect with high quality rubber tubing to condenser tubes and lower ends with rubber stoppers to 800ml distillation flask. Lower ends of condenser tubes have rubber-connected glass or polyethylene tubes.
- Receiving bottles or flasks, 300ml capacity.
- Proper burets for dispensing: H_2SO_4, NaOH, boric acid indicator solution.

Reagents:

1. H_2SO_4, concentrated (93-98%), nitrogen-free.
2. Catalyst. Polyethylene packets containing 15g potassium sulfate, 0.7g mercuric oxide, and approximately 0.1g pumice stone
3. Antibumping agent. Either zinc metal, 20-mesh, or pumice stone.
4. NaOH, pellets or solution, nitrate-free. For soluiton, dissolve 450g solid NaOH in 1L water.
5. Methyl red-methylene blue indicator. Mix 2 parts 0.2% alcohol methyl red solution with 1 part 0.2% alcohol methylene blue solution.
6. Standard H_2SO_4, 0.1*N*
7. Boric acid-methyl red-methylene blue receiver solutio

Appendix 3: Germination results

Sample ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1																					AB		AB
2		AB		AB				AB							AB			AB	AB				
3				AB							AB		AB	AB				AB	AB		AB	AB	
4	AB	AB	AB		AB		AB	AB		AB	AB		AB								AB		AB
5	N	N		N	N	N	N	AB	AB	N	N	N	N	N	N	N	AB	N	N	N	N	N	N
6	AB	N	N	AB	N	N	N	AB	AB	N	N	N	N	AB	N	N	N	N	N		N	N	N
7	N	N	AB	N	N	N	N	AB	N	N	N	N	AB	N	N	N	N	N	N	N	N	AB	N
8	N	N	N	N	N	N	AB	N	AB	N	N	N	N	N	N	AB	N	N	N	N	AB	N	N
9	N	AB	N	N	N	N	N		N	AB	AB		N	N	AB	N	AB	AB	N		N		
10	AB	N	AB	AB		AB			N	AB	AB	AB		N		AB	AB	N			N		N
11			N	AB	AB	AB	N	AB	AB	AB	AB	AB	AB	AB	AB			AB	N	N		AB	AB
12	N	AB	AB		AB	N			N	AB	AB			AB	AB	AB	AB	AB	N	N	AB	N	N
13	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
14	N	N	AB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
15	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	AB	N	N	N
16	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
17	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
18	N	N	N	N	N	N	N	N	AB	N	N	N	AB	N	N	N	N	N	N	AB	N	N	N
19	N	N	N	N	N	N	N	N	N	N	N	AB	N	AB	N	N	N	N	N	N	N	AB	N
20	AB	N	N	N		N	AB	AB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
21	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	AB	N	N	N	N	AB	AB
22	N	N	N	N	N	AB	N	N	N	N	N	N	N	AB	AB	N	AB	AB	N	N	N	N	N
23	N	N	N	AB	N	AB	N	AB	N	N	N	N	N	N	N	N	AB	N	AB	N	N	N	N
24	N	N	AB	AB	N	AB	N	N	N	AB	N	AB	N	N	AB	N	N	N	N	N	N	AB	AB
25	N	N	N	N	N	AB	N	AB	AB	N	N	N	N	N	N	N	N	AB	N	N	AB	N	N
26	N	N	N	N	N	N	N	N	N	N	N	AB	N	N	N	N	N	N	N	AB	N	N	N
27	N	N	N	N	N	N	N	AB	AB	N	N	N	AB	AB	N	N	N	N	AB	N	N	N	N
28	N	N	N	N	N	AB	N	N	AB	N	AB	N	N	AB	AB	N	N	AB	AB	AB	AB	N	N
29	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
30	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	AB	N	N
31	N	N	N	N	AB	N	N	N	AB	N	N	N	N	AB	N	N	N	N	N	AB	N	N	AB
32	N	N	AB	N	N	N	N	AB	N	N	N	N	N	N	N	N	N	N	N	AB	N	N	N
33	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	AB	N	N	N	N	
34	N	N	N	N	N	N	N	N	N	N	AB	N	N	N	N	N	N	N	N	N	N	N	N
35	N	N	AB	N	N	N	N	AB	AB	N	N	N	N	AB	N	N	N	N	N	N	N	N	N

36	N	N	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N	N	N	AB
37	N	N	N	AB	N	N	N	N	N	AB	N	N	N		N	N	N	N	N	N	N	N	N
38	N	N		N	AB	AB	N	N	N		N	N	N	N	N	N	AB	N	N	N	N		N
39	N	N	N	N	N	N	N	N	N	N		N	N	N	N	AB		AB	N	AB	AB	N	N
40	N	N	N	N	N	N	N	N	N	N	N	N	N	N		N	N	N	N	N	N	N	N
41	AB	N	N	N	AB	N	N	N	N	N	N	N	N		N	N	N	AB	N	N	N	N	AB
42	N	N	N	N	AB	AB	AB	N	N	N	AB	AB	AB	AB	AB	AB	N	N	AB	AB	AB	N	AB
43		N	N	N	N	N	N	N	N	AB	AB	AB	AB	AB	AB	AB		N	AB		AB	AB	
44	N	AB	N	N	N	N	AB	N	N	N	N	N	N	N	N	N	AB	N	N	N	N	N	N
45	N	N	N	N	N	N	N	N	N	N	N		N	N	N	N	N		N	N	N	N	N
46	N	N	N	N		N	N	AB	N	N	N	AB		N	N	N	N	N	N	N	N	AB	N
47	N	N	N	N	N	N	N	AB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
48	N	N	AB	N	N	N	N	N	N	AB	N	N	N	AB	N	N	N	N	N	N	AB	N	N
49	N	AB	N		N	N	N	N	N	N	N	N	AB	N	N	N	N	N	N	N	N	AB	AB
50																							
51	N	N	N	N	N	N	N	N	N	N	N		N	N	N	N	N	AB	AB	N	N	N	N
52	N	N	N	N	N	AB	AB	N	N	N	N	N	AB	AB	N	N	N	N	AB	N	N	N	N
53	N	N	AB	N	N		N	N	N	N	N	N	N	N	N	N	N	AB	N	N		N	N
54	N	N	N	N	N	AB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	AB	N	N
55	N	N	N	N	N	AB	N	N	N	AB	N	N	N	N	N	AB	N	N	N	N	N	AB	
56	AB	N	N	N	N	N	AB		AB	AB	N	N	N	N	N	N	N	N	N	N	N	N	N
57	N	AB	AB	N	N	N	N	N	N	N	N	N	N	N	N	AB	AB	AB	AB	N	AB	N	N
58	N	N	N	AB	AB	N	N	AB	N	AB	N	N	N		AB	N	AB	N	N	N	N	N	N
59	N	N	AB		AB	AB	N	N	AB	N	N	N	AB		N	N	AB	AB	N	N	AB	N	N
60	AB	N	N	N	AB	AB	N	N	N		AB	AB	N	N	N	N	AB		N	N	AB	AB	N
61	N	N	N	N	N	N	N	AB	N	N	N	N	N	N		N	N	N		N		N	N
62	N	AB	N	N	N	N	AB	N	N	N	N	N	N	N	AB	N	N	N	N	AB	N	AB	AB
63	N	N	N	N	N	N	AB	N	N	N	N	N	N	AB	AB	N	N	N	N	N	N	AB	AB
64	N	AB	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	AB	N	N	N
65	AB		AB	AB	N	AB		AB	AB	N	AB	AB	AB	AB	AB	N	AB	N	AB			N	N
66		AB		AB	AB		N		AB	N	N	N	N	N	N	N	AB	AB			N	N	N
67			AB					AB		N	N	N	AB		N		AB			N			
68	AB	AB				AB		N	AB					N			AB	AB		AB	N	AB	N
69		N		AB	N		N	N	N	N	AB	AB	N	N	N		N	N	N	N	N	N	
70	N	AB	N	N	N		N	N	N	N	AB	N	N	N	N	N	N	N	N	AB	N		N
71	N	N	N		N	AB		N	N	N	AB	N	AB		N			AB	N	N	N	N	AB
72	AB	N	N	N	N	N	N		N	N	N		N	N	N	N	N	N		AB		AB	N
73		AB	AB	N	AB		N	N			N	AB	N	N	N	AB	N	AB			N	N	
74	N	AB	AB		AB	AB	AB	N	AB		AB	N	AB	AB	AB	AB	AB	N	AB			AB	AB
75				AB	N		N	N	N	AB	AB	AB	AB		N	AB			AB	N	AB	AB	
76				N			N	N	N	AB			N	N	N		N				AB		AB

77	AB	N	AB	AB	N	AB	AB	N	N	AB	N		N		N	N	N	AB	AB		AB	N	AB
78	N	AB	AB	AB	N	AB	AB			N		N	N	N	AB	AB	N	AB	AB	AB	AB		AB
79	N	AB		AB		AB	AB		AB			N		AB			AB	N		AB	AB		AB
80		AB	AB	AB	AB		AB	AB		AB	N	N		AB		AB		AB			AB	AB	
81									AB		AB		AB					AB					
82																							
83											AB					AB	AB						AB
84			AB				AB		AB		AB		AB			AB				AB			

	NO SEED
	NO - GERMINATED
N	NORMAL
AB	ABNORMAL