Nodulation and biomass development of yellow and blue lucerne under differential pH conditions

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Nodulation and biomass development of yellow and blue lucerne under pH stress

Hur olika pH-förhållanden påverkar nodulering och tillväxt hos blå- och gullusern

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SAMMANFATTNING

Vall odlas på ungefär 77% av jordens åkermark. Vallodling är grunden till all djurproduktion och en viktig del av människans livsmedelförsörjning. Därför är det viktigt att vallodlingen är så hållbar och biologiskt mångfacetterad som möjligt. Det är viktigt att det finns en god balans mellan fiber-, energi- och proteininnehåll i djurföda. Baljväxter är viktiga för en god vallfoderkvalitet och de förser sig själva med kväve, vilket minskar behovet av att kvävegödsla.

Medicago sativa L. (lusern) odlas på över 30 miljoner hektar globalt och är en viktig baljväxt för jordbruket. Lusern fixerar kväve i symbios med bakteriearten *E. meliloti*, som bildar rhizobiumknölar (noduler) på lusernrötter. *E. meliloti* är känslig för sura miljöer och vid pH<6 minskar noduleringen. Detta leder till minskad tillväxt och sämre näringskvalitet hos lusern.

Målet med den här studien var att undersöka och jämföra pH-känsligheten hos två underarter av lusern, blålusern (BL) och gullusern (YL). Studien utgick från tre hypoteser: 1) YL tål lägre pH än BL men vid höga pH-värden producerar BL mer biomassa än YL; 2) Noduleringen minskar med lägre pH för både BL och YL; och 3) Lågt pH minskar biomassaproduktionen för BL och YL.

En mineraljord kalkades med olika mängder kalk för att ta fram sex behandlingar med pH-värden mellan 5.7-6.5. BL- och YL-frön inockulerades med Nitragin Gold (en kommersiell blandning av olika *E. meliloti*-sorter) och planterades i 60 krukor. Krukorna placerades ut i randomized block design i en växthuskammare och skördades 75-81 dagar efter sådd. Icke-destruktiva mätningar gjordes under tillväxt och vid skörd utfördes destruktiva mätningar. I en statistisk analys testades signifikansen för fyra faktorer; lusernsort, pH, lusernsort*pH och tid*lusernsort, för 11 responsvariabler kopplade till biomassa och nodulering. Kalkningseffekt på biomassa och nodulering utvärderades i en regressionsanalys med kalkmängd som förklaringsvariabel. Under experimentets gång ökade pH i jordarna efter sådd i samtliga behandlingar, inklusive en okalkad behandling. Detta ledde till att pH nivåerna blev högre än beräknade, mellan pH 6.5-6.8. Det gjorde att det inte gick att studera vilket effekt pH-stress kan ha på BL och YL. De statistiska analyserna både bekräftade och motbevisade hypotes 2) och 3). I slutändan kunde dock ingen av hypoteserna prövas ordentligt på grund av att rätt förutsättningar saknades och därmed varken bevisas eller motbevisas.

Studien visade att Nitragin Gold-inockulum är kompatibelt med lusernsorterna SW Nexus (BL) och Karlu (YL). *E. melilotis* höga pH-känslighet framgick också. Fortsatt behövs det mer forskning på YL och dess pH-tålighet, samt på vilka *E. meliloti*-symbionter den är kompatibel med. Sådana studier kan med fördel göras under nordsvenska förhållanden över flera odlingssäsonger, gärna med olika YL-sorter.

Nyckelord: Lusern, blålusern (BL), gullusern (YL), *E. meliloti*, nodulering, pH

ABSTRACT

Approximately 77% of global arable land use is devoted to forage cultivation, which is the basis for animal production and global food security. Therefore a sustainable and biologically diverse forage cultivation is needed. Feed for cattle needs to contain a good balance between fibre, energy and protein. Legumes are important for forage quality and fix their own nitrogen, reducing the need for N-fertilization.

Medicago sativa L. (lucerne) is an agronomically important legume species that is cultivated on over 30 million hectares worldwide. Lucerne fixates N_2 in symbiosis with bacterial species *E. meliloti*, which attaches to the root and forms nodules. *E. meliloti* is acid sensitive and at pH<6 nodulation is reduced. This leads to decreased biomass production and quality.

The aim of this study was to evaluate the pH tolerance of two lucerne subspecies; Blue lucerne (BL) and yellow lucerne (YL). The hypotheses were 1) YL will be more tolerant than BL against low pH, but BL will produce more biomass than YL in high pH conditions; 2) A low pH will decrease the nodulation formation and quality for BL and YL; and 3) A low pH will decrease biomass quantity for BL and YL.

A mineral soil with different rates of lime was used to create six treatments with pH 5.7-6.5. 60 pots containing YL and BL cultivars inoculated with Nitragin Gold, a commercial *E. meliloti* mix were placed in a randomized block design in a greenhouse chamber. After 75-81 days the pots were harvested. Non-destructive measurements were made during growth and destructive measurements were made at harvest. The significance of four factors; cultivar, pH, cultivar*pH and time*cultivar, were statistically tested for 11 response variables connected to biomass and nodulation. The effect of liming on biomass and nodulation was also evaluated in a regression analysis using lime rate as an explanatory variable. The soil pH kept rising after sowing, even for the non-limed soil, and ended up higher than intended at 6.5-6.8. This meant it was not possible to properly evaluate the effect of pH stress on BL and YL. The statistical analyses partly confirmed and partly contradicted hypotheses 2) and 3). In the end, however, none of the hypotheses could be confidently confirmed since the right conditions for the study were lacking. One practical finding of this study is that Nitragin gold is compatible with both SW Nexus and Karlu. The pH sensitivity of *E. meliloti* is also highlighted. Future directions for research would be to continue researching YL response to pH stress, as well as *E. meliloti* strain compatibility, perhaps in a Northern Swedish climate over several seasons, using several cultivars.

Keywords: Lucerne, blue lucerne (BL), yellow lucerne (YL), *E. meliloti*, nodulation, pH

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Abbreviations

Lucerne	Alfalfa, *Medicago sativa* species complex
BL	Blue lucerne, *Medicago sativa* ssp. *sativa*
YL	Yellow lucerne, *Medicago sativa* ssp. *falcata*
E. meliloti	Rhizobium bacterial species, *Ensifer meliloti*

1. Introduction

1.1 Forage cropping

Approximately 77% of global arable land is devoted to forage cultivation (Richie & Roser 2013). In Sweden, forage farming occupies 44% of arable land (Jordbruksverket 2022a). Globally, at least 1.3 billion people depend on livestock production for their livelihood. In developing countries livestock represents approximately 20% of the total agricultural production. For developed countries the corresponding number is 40%. Additionally, livestock contributes to 34% of total protein supply worldwide. The global annual dry matter forage consumption is around 6 billion tonnes. Grass constitutes about 50% and cereal about 13% (FAO 2022). Meat production is expected to increase as a consequence of economic growth, better access to good quality food and human population growth (National Research Council 2015; FAO 2021). Based on this, it can be argued that a sustainable and resilient forage production is not only an important basis for animal health production but also for global food security. These facts point to the importance of increased sustainability for animal-sourced food production.

"While agricultural development contributes to food security, unsustainable agricultural expansion, driven in part by unbalanced diets, increases ecosystem and human vulnerability and leads to competition for land and/or water resources (high confidence)." (IPCC 2022)

Here, a balanced diet refers to plant-based foods combined with sustainably produced, low GHG emitting food, from animal source systems (IPCC 2022).

1.1.1 The role of legumes

Legumes have various benefits that increase forage quality and nutrition (Fogelfors 2016a; Kulkarni et al 2018). They form symbiotic relationships with rhizobium bacteria that have the ability to fixate atmospheric nitrogen and convert it to organic nitrogen. The symbiosis provides the plants with nitrogen and the bacteria with carbohydrates from the photosynthesis process. It is estimated that legumes in intercropping systems with grass fix up to 682 kg N ha^{-1} $year^{-1}$, reducing the need for N-fertilization. In general, legumes have high crude protein contents and high yield potential. (Ledgard & Steele 1992; Fogelfors 2016a).

Legumes also make animal feed more palatable and digestible, which benefits the health and welfare of animals. As a result, forages typically include both grass and legumes (Kulkarni et al. 2018; Fogelfors 2016a). According to a 2015 study, intercropping grain and legumes boosted the yield, degradability, and quality of animal feed. Each crop in the intercropping systems had a higher yield per unit of land compared to the same crop being grown in sole cropping systems (Zhang et al 2015). Other studies have shown that milk yield from cows that consume a mix

of grass and red clover is 10% higher than the yield from cows that only consume grass. The optimum share of legume dry matter in a feeding mix is 30-50%, depending on animal and plant species compatibility (Fogelfors 2016a).

It is indicated by the amount of produced and imported seed that the three most popular forage legume species in Sweden are red clover (44%), white clover (27%) and blue lucerne (8%). In total, the *Trifolium* genus stands for approximately 77% and the *Medicago* genus for 10% of all Swedish forage legume seed production and import (Jordbruksverket 2022b).

1.2 Lucerne

Medicago is a genus of predominantly cross pollinating perennial and annual legumes, consisting of 83 species (Quiros & Bauchan 1988; Small and Jomphe 1989). *Medicago sativa* (alfalfa or hereafter, lucerne) is a species complex consisting of a variety of perennial subspecies (Quiros & Bauchan 1988). Lucerne flower colour varies between violet, yellow and white as well as a mix between colours (i.e. variegated colour) (Teuber & Brick 1988). Pod shapes are straight to coiled and the growth style ranges from bushy and decumbent to upright stems (Quiros & Bauchan 1988; Teuber & Brick 1988). Root morphology differs between a long, prominent tap root to a more branched and fibrous root system (Garver 1922). The area above the primary tap root from which above-ground biomass grows is called the root crown. Shape and depth of the root crown varies between subspecies (Gosse 1988; Teuber & Brick 1988). Lucerne prefers a high soil pH, and in order to maximize growth a pH of 6.5 or higher is needed (Tesar & Marble 1988; Fogelfors 2016a).

General morphological traits of lucerne are deep tap roots, a high stress tolerance and a high crude protein content (Fogelfors 2016a; Bruins 2017). One reason for the durability of lucerne is the fact that it has indeterminate growth, meaning that it keeps developing both vegetative and reproductive tissue throughout most of its life (Barnes et al 1972). The lucerne complex consists of both diploid and tetraploid forms, though tetraploidy is standard for most produced cultivars (Barnes et al 1972; Brummer et al 1991; Sullivan 1992). Self pollination is possible but not as common as cross pollination (Quiros & Bauchan 1988). Lucerne develops more pods with a higher seed number with cross pollination compared to self pollination (Viands et al 1988). In fact, there are a number of mechanisms, such as flower tripping, that hinder self pollination and favour cross pollination (Chen et al 2018).

Cross pollination between subspecies occurs naturally and hybrid forms are common in natural populations (Sullivan 1992; Inostroza et al 2021). This gives access to a large pool of genetic resources within the lucerne species complex which is used in cultivar production (Quiros & Bauchan 1988). Two examples of lucerne subspecies are the blue-flowered lucerne (*Medicago sativa ssp sativa,* hereafter BL) and the yellow-flowered lucerne (*Medicago sativa ssp. falcata*, herafter YL) (Barnes et al 1972). Subspecies are recognized by morphological traits and ploidy level (Quiros & Bauchan 1988).

Medicago sativa ssp x *varia*, hereafter *M.* x *varia*, is a result of crossing BL and YL (Quiros & Bauchan 1988; Small and Jomphe 1989; Schoch et al 2020;

Inostroza et al 2021). Hybrid offspring of BL and YL that inherit genes for both flower colours develop flowers with variegated colour (Barnes et al 1972; Teuber & Brick 1988). Since hybridization is common and subspecies readily cross breed, strict taxonomic definitions within the lucerne complex can be tricky.

1.2.1 Agricultural importance

Lucerne has a long history of domestication that is thought to have begun around 8000 years ago in the Mediterranean area (Quiros & Bauchan 1988). It is cultivated on over 30 million hectares worldwide (Michaud et al 1988; FAO 2012). Lucerne has been described as the world's most important legume, with large yields of up to 28 tonnes DM ha^{-1} $year^{-1}$ (Quiros & Bauchan 1988; Brown et al 2005). In Europe lucerne yields between 10-20 tonnes DM ha^{-1} $year^{-1}$ in irrigated systems (FAO 2012). The synonymous name for lucerne, alfalfa, is believed to originate from the ancient Persian word aspo-asti, which translates to horse fodder (Russelle 2001). Lucerne is estimated to fix between 50-463 kg N_2 ha^{-1} $year^{-1}$ depending on several circumstances, the quality of the bacteria-cultivar symbiosis being the most influential factor (Vance et al 1988). Other important factors are yield and fertilization; N-fixation decreases with increasing N-fertilization and high yields are directly related to a high N_2 fixation. In turn, management, precipitation, temperature and other local conditions affect the yield (Vance et al 1988; Carlsson & Huss-Danell 2003). More recent studies show similar results, with a fixation rate ranging between 0-350 kg N_2 ha^{-1} $year^{-1}$ in Europe and North America (Carlsson & Huss-Danell 2003). In Sweden at latitude 60° N the fixation rate in lucerne monocultures ranged between 79-319 kg N_2 ha^{-1} $year^{-1}$ (Mårtensson & Ljunggren 1984; Wivstad et al 1987).

1.3 Blue lucerne

1.3.1. Morphology of blue lucerne

Generally, BL has a distinct tap root with little branching and a small and upright crown (Garver 1922). Stems have an upright growth and the flower colour is blue/violet (Quiros & Bauchan 1988). BL prefers a pH of at least 6.5, and reportedly stops growing at pH 5.6 or below (Lantbrukssällskapet 2020). The seed pods are tightly coiled and about 2-5 mm in diameter (Quiros & Bauchan 1988).

1.3.2. History and agricultural importance of blue lucerne

Cultivated tetraploid BL originates from wild diploid *M. sativa caerulea* and has spread globally as a result of domestication, hybridization and breeding (Quiros & Bauchan 1988; Sullivan 1992; Julier et al 1995; Yu et al 2017). In literature, the term lucerne (or alfalfa), usually refers to cultivated BL and it is often clearly specified when the term also concerns other subspecies within the lucerne complex.

BL is cultivated as a forage crop due to its high nutritional value, drought tolerance and high protein content (Fogelfors 2016a; Bruins 2017). Some breeding targets for BL are increased tolerance to various stresses such as salinity, cold, drought and disease (Bruins 2017).

1.3.3 Blue lucerne in Scandinavia

BL originates from middle eastern climates and is well adapted to warm and dry conditions. Due to successful evolution and domestication it can grow in many kinds of environments. It is, however, less well adapted to low temperatures and waterlogged conditions (Michaud et al 1988; FAO 2012). Swedish BL cultivation is concentrated around the southern parts of the country, where the climate is warmer and winters are milder compared to Northern Sweden. BL cultivation is recommended between 55-61° N latitude (Michaud et al 1988; Olssons frö 2022). Between years 2011-2020 in Swedish field trials up to 60° N, BL has yielded around 8-9 tonnes DM ha^{-1} $year^{-1}$ (Halling et al 2021).

The survival rate of a dormant BL cultivar, WL354HQ, was tested by Xu et al (Xu et al 2020; W-L Alfalfas 2021). Following a two-day cold acclimation period under 12-hour photoperiod conditions, the cultivar had a 50% survival rate at approximately -9 ℃. Interestingly, waterlogging under the same acclimation conditions improved the number to a 50% survival rate at -12 ℃ (Xu et al 2020). In another study, two BL cultivars, one with higher dormancy than the other, were compared. The results showed that the least winter hardy cultivar had less accumulation of cryoprotectants after leaf abscission in the autumn (Dhont et al 2006).

1.4 Yellow lucerne

1.4.1 Morphology of yellow lucerne

YL characteristics are yellow flowers, decumbent growth and smaller seeds than BL. Its creeping growth makes it tolerant against grazing and trampling (Misar et al 2015). YL is highly dormant and winter-hardy (Quiros & Bauchan 1988; Julier et al 1995; Cui et al 2019). The seed pods are not coiled like BL pods but rather curved to straight (Quiros & Bauchan 1988; Julier et al 1995). The typical YL root system has clear morphological differences from BL; it has a higher abundance of branch roots, a wider angle between tap- and branch roots, an abundance of shallow, fibrous roots and a more well developed root crown with numerous rhizomes. The tap root is generally less distinct (Garver 1922).

YL is more tolerant to low pH than BL (Lantbrukssällskapet 2020). It is, however, not further specified by the source which pH range YL tolerates. Jung & Larson describe a direct correlation between a sufficient pH level in the sap and YL cold tolerance. It is suggested that substances, such as proteins and enzymes, active in cold stress response systems are positively affected by a high pH environment. (Jung & Larson 1972b).

1.4.2. History and agricultural importance of yellow lucerne

YL is native to the Eurasian continent and wild populations can be found at high latitudes, for example in Sweden and Siberia (Michaud et al 1988; Sormunen-Cristian et al 1998; Royal botanic gardens 2021). YL is an important gene donor for tolerance characteristics in cultivated BL populations, contributing with cold tolerance, drought tolerance and disease resistance traits (Michaud et al 1988; Cui et al 2019; Ghaleb et al 2021). The yield potential is lower for YL compared to BL (Mackie et al 2005; Lantbrukssällskapet 2020). A study by Misar et al 2015 evaluated survival rates and yields of BL and YL stands under periods of heavy grazing between 2007-2010. The results showed that YL stands had higher final survival rates (>38% compared to <19% for BL) and yields than BL (Misar et al 2015).

1.4.3. Yellow lucerne in Scandinavia

YL is well adapted to the climatic conditions of Northern Scandinavia (Garver 1922; Michaud et al 1988; Cui et al 2019). According to a study, YL populations had a survival rate of more than 90% at -10 ℃ following a period of cold acclimation (Zhang et al 2011).

In a Finnish study by Sormunen-Cristian et al (1998), YL cultivar “Karlu” was cultivated at 60° latitude and tested for yield and silage qualities in a 2:1 lucerne/timothy seed mix. The DM yield was 7.4 tonnes ha^{-1} at first cutting in June and approximately 3.6 tonnes ha^{-1} at second cutting in August. The fresh yield contained, on average, 70.5% and 88% lucerne biomass respectively for the June and August harvests. Crude protein content was high on both occasions; 199 and 206 g kg^{-1} in June and August, respectively. It is worth noting that the YL seeds were not inoculated in this study (Sormunen-Cristian et al 1998). The study proves that YL cultivation has the potential to contribute to high quantities of good quality forage yields in Scandinavia.

When this is written there is at least one YL lucerne cultivar, Karlu, available on the Swedish market. Cultivation is recommended up to at least latitude 63° N, further North than the BL cultivars (Olssons frö 2022).

1.5 pH effect on *E. meliloti* nodulation

Lucerne yield and quality is directly linked to the symbiosis with a nitrogen fixating bacterial species called *Ensifer meliloti* (formerly *Sinorhizobium meliloti* or *Rhizobium meliloti*) (Vance et al 1988; De Lajudie et al 1994; Zahran 1999; Young 2003). Therefore it is important to understand the symbiosis process and how *E. meliloti* is affected by pH stress.

E. meliloti infects lucerne root hairs where they form enclosed living spaces called nodules. *Nod* genes are genes responsible for signal exchanges between bacteria and plant. At infection, *nod* genes are activated and nodules begin to form (Göttfert 1993). Formation is normally initiated within a week after *E. meliloti* inoculation (Dudley et al 1987; Hirsch 1992; Soto et al 2004; Checcucci et al

2017). Neutral pH conditions are required and if pH gets below 5, formation usually stops. A probable reason for this is that Ca is required at infection and that acidic conditions limits Ca availability. Where Ca and nitrate levels are adequate, nodulation is possible at pH 4.0 (Munns et al 1970; Vance et al 1988). This is, however, rarely the case in a low pH environment. In a study by Rice et al, pH effect on lucerne nodulation was evaluated. Their results showed that pH(H_2O)>6 (Rice et al 1977; Kabala et al 2016) was insignificant for nodulation, meaning that size, position, colour and amount of nodules were largely unaffected and developed normally. At pH(H_2O)<6, however, total nodule score (an amalgamated score consisting of the score of the four categories) significantly decreased, indicating that nodules were becoming smaller, lighter in colour, positioned lower down on the root and less frequently occurring (Rice et al 1977).

Other studies show that the level of acid sensitivity varies between *E.meliloti* strains (O'Hara et al 1989; Vance at al 1988; Soto et al 2004). *E. meliloti* performs poorly when its intercellular pH is not alkaline. In a study, six acid tolerant strains maintained alkaline interiors at pH 5.6-7.2. Two commercial strains, as well as four acid-sensitive ones, were able to maintain alkalinity at pH 6.5 or higher (O'Hara et al 1989). Soto et al found that a low pH medium impaired *nod* gene expression for commercial *E. meliloti* strains but not for an acid tolerant strain called LPU83 (Soto et al 2004). LPU83 is an inefficient nitrogen fixator compared to acid-sensitive *E. meliloti* strains. This means that LPU83 is a better competitor for plant partners at low pH but falls behind commercial *E. meliloti* as pH rises (Soto et al 2004).

At low soil base saturation, H^+ and Al^{3+} ions are available for plant uptake. Therefore, acidity is often connected to Al^{3+} availability (Fogelfors 2016b). Al-toxicity inhibits plant growth and *nod* gene expression (Richardson et al 1988; Zahran 1999; Fogelfors 2016b). Al^{3+} can stimulate plant growth at low concentrations (Bojorquez-Quintal et al 2017), but has been reported to inhibit lucerne growth at concentrations below 5 ppm (Morton & Moir 2018). Other sources suggest a toxicity threshold of <1.0 mg Al kg^{-1} soil for successful *E. meliloti* nodulation (Berenji et al 2017). Tolerance to Al^{3+} varies for different plant species and *E. meliloti* strains (Zahran 1999; Wigley et al 2018). The addition of Ca reduces the negative effect of Al^{3+} on nodulation and plant growth in leguminous plants (Wigley et al 2018; Morton & Moir 2018).

As N-fixation is an interaction between two organisms, the efficiency of the symbiosis also largely varies based on cultivar-bacteria compatibility (Burton 1972; Vance et al 1988).

1.6 pH measurement techniques

There are different methods for making pH measurements in soils (Karastogianni et al 2016; Horiba n.d.). Common techniques are litmus paper tests, metal electrode measurements and glass electrode measurements. Glass electrode-meters have two glass electrodes; one that measures the voltage of a buffer solution and another that measures the voltage of a sample solution. The buffer solution is a mix between a conjugate weak acid and base. It has a

stabilized pH value, usually set to 4, 7 or 10. The electrode converts the voltage difference into a pH value relative to the known buffer (Horiba n.d.)

Water pH measurements specifically measure H^+ ion activity and are usually made in deionized water with a glass electrode-meter (Karastogianni et al 2016). In a 0.01 M $CaCl_2$ or a 1 M KCl solution the H^+ concentration, rather than activity, is measured. The three methods generate different pH result ranges where $pH(H_2O)>pH(CaCl_2)>pH(KCl)$. $CaCl_2$ measurements are on average 0.43 units lower than $pH(H_2O)$ measurements made in 1:5 soil/water ratio (Kome et al 2018). Other studies show that pH $CaCl_2$ results are around 0.5 units lower than $pH(H_2O)$ (Schofield & Taylor 1955). The variation is connected to the free ion content of a solution. Free ions generate H^+ release from soil surfaces, which lowers the pH readings. $CaCl_2$ and KCl provides higher amounts of free ions compared to deionized water. For $pH(H_2O)$ there is a correlation between larger solution volumes and higher pH readings. The reason for this is that larger volumes means that the soil samples become more diluted (Kome et al 2018).

The International Organization for Standardization (ISO) is an organization that provides standard methods, including methods for soil pH measurements. The ISO 10390:2007 and ISO SS-EN 15933:2012 methods both have in common that soil samples are air dried and that the soil:water/$CaCl_2$/KCl ratio is 1:5. This also applies to the current standard, ISO SS-EN 10390:2022. (SIS 2007; SIS 2012; SIS 2022).

2. Aims and Objectives

This thesis is part of a larger SLU project which aims to establish YL as a forage crop in Northern Sweden. A larger range of available legumes would increase biodiversity and potentially also the resilience of forage cultivation. Wild YL populations grow naturally in Northern Sweden (SLU Artfakta 2023) and are highly winter dormant. Cultivated YL could be a valuable addition to Northern forage legume cultivation, which today is dominated by red clover.

The objective of this study was to evaluate the effects of differential soil pH conditions on YL and BL. By evaluating nodulation and growth an experiment was conducted, to examine the effect of pH on the growth and nodulation of lucerne subspecies. The hypotheses are:

1) YL will be more tolerant than BL against low pH, but BL will produce more biomass than YL in high pH conditions.
2) A low pH will decrease the nodulation formation and quality for BL and YL.
3) A low pH will decrease biomass quantity for BL and YL.

3. Materials and Methods

3.1 Soil and liming

For the experiment, a loamy sand provided by Econova, with pH(H_2O) 5.7, a 5.6% OM content and 1.7 mg NO_3-N/100 g air-dried soil was used. A low pH was necessary in order to create a low starting point and then raise the pH with lime. The soil was retrieved 19/1-22.

Omya Calciprill, granulated CaO, was used as liming material to create eight soils with different pH levels. The product was chosen because it is easily soluble and has a fast-acting effect. Based on the desired end pH, the amount of Calciprill per level was calculated according to recommendations on the user instructions. For a loamy sand, the application rate for a 0.1 pH increase was 60 kg Calciprill ha^{-1} soil, or 0.024 g $liter^{-1}$ soil. The liming material was mixed into the soils on date 4/2-22 and 44 days later, 20/3, the soils were put into marked pots. The soils were regularly watered and mixed during this period, in order to increase contact between soil and lime and to stimulate the liming effect.

3.2 pH measurements

pH meter readings using fresh soil were made before the greenhouse experiment started in order to track the liming effect in the soils. The expected final pH was an estimate based on the Calciprill instructions for a sandy loam soil. The pH measurements were made in a 1:5 soil-water ratio using 5 g of fresh soil in 25 g of deionized water. The samples were shaken for 30 minutes at 1000 rpm and stood overnight for approximately 14 h. The measurements were made using a handheld pH-meter with a glass electrode, model Metria PHM93, method A, at 22°C. Based on data from the measurements, six soils with appropriate pH levels were chosen for the greenhouse experiment, 44 days after liming. The measurements were made at three occasions: 21, 32 and 41 days after liming (25/2, 8/3 and 17/3).

In addition to the pre-experiment pH measurements, soil samples were sent to two professional labs at SLU and Eurofins. Both pH(H_2O) and pH($CaCl_2$) analyses were made at the start and at the end of the experiment. The pH(H_2O) analyses were made with ISO 10390:2007 and ISO SS-EN 15933:2012. At the end of the experiment an Al^{3+} analysis was made to evaluate the amounts of free Al^{3+} in each soil.

3.3 Cultivar choice and germination test

SW Nexus is a blue-flowered cultivar with a deep tap root (Halling et al 2021). Karlu is a cultivar with yellow flowers and a branched root system that produces rhizomes (Olssons frö 2022). Both are described as cold tolerant cultivars suitable for Swedish conditions. (Seppänen et al 2018; Olssons frö 2022; Halling et al 2021). Based on this, SW Nexus and Karlu were chosen to represent BL and YL in this study.

A germination test was made using petri dishes, filter paper and 100 seeds each of cultivars Nexus (BL) and Karlu (YL). Two petri dishes with wet filter paper were prepared. One dish contained 100 Nexus seeds and the other contained 100 Karlu seeds. The dishes were kept at room temperature for four days until maximum germination was completed. The germination percentage rate was calculated.

3.4 Greenhouse experiment setup

3.4.1 Inoculation

Nitragin gold powder, a commercial *E. meliloti* strain mix, was used for inoculation. Approximately 1 g of Nitragin Gold was added to a can containing 20 g of seed. The can was lightly shaken until an even layer of powder was observed on the seeds.

3.4.2 Potting

In total, 60 pots of lucerne were cultivated. A treatment consisted of a specific combination between factors "cultivar" and "pH". For cultivar there were two options, BL and YL. For pH there were six options, soils 1-6. In total there were 12 treatments, A-L. Each treatment consisted of 5 replicates (pots). The treatments were put in a randomized complete block design with one treatment per block, resulting in 5 blocks.

Table 1. Experimental setup for treatments A-L.

Treatment	Factor combination	Pot numbers	Perlite (ml)	Soil (ml)	Total volume (ml)
A	soil 1 * BL	1-5	300	700	1000
B	soil 1 * YL	6-10	300	700	1000
C	soil 2 * BL	11-15	300	700	1000
D	soil 2 * YL	16-20	300	700	1000
E	soil 3 * BL	21-25	300	700	1000
F	soil 3 * YL	26-30	300	700	1000
G	soil 4 * BL	31-35	300	700	1000
H	soil 4 * YL	35-40	300	700	1000
I	soil 5 * BL	41-45	300	700	1000
J	soil 5 * YL	46-50	300	700	1000
K	soil 6 * BL	51-55	300	700	1000
L	soil 6 * YL	56-60	300	700	1000

1.4-L pots (10x10x17 cm) were used for potting. 5 seeds per pot were sown in order to, later on, be thinned out to 3 plants per pot. 0.3 litres of perlite (i.e. pH neutral volcanic rocks in pellet form) was added to the bottom of the pots and 0.7 litres of soil was added on top. The purpose of adding perlite was to fill out the pot volume, as there was limited soil. The distance between the upper soil layer

and the edge of the pots was 1-2 cm. The pots were placed in a greenhouse chamber in 18 hour daylight conditions at 20 ℃, in order to promote rapid plant growth. The plants were watered every day with deionized water. The sowing date was 23/3-22, marking the start of the greenhouse experiment.

3.4.3 Fertilization

Approximately two weeks after sowing, lucerne plants had started to grow and the pots were fertilized with macro- and micronutrients. P and K were added in liquid form using fertilizer Canna PK 13-14 from Hygrogarden. The recommended dosage of 30 kg P per hectare was recalculated to 0.6 ml P per pot. The fertilization was done by mixing 1.5 ml PK fertilizer with 1 liter water, and watering each pot with 200 ml of the solution. Canna Mono Trace mix containing micronutrients Fe, Mo, Cu, Mn, B and Zn was also added a few days after the macronutrients were added. According to instructions, a solution of 1-2 ml fertilizer per litre water was mixed. Each pot was watered with 100 ml of the solution. No N was added, as plant available N interferes with the *E. meliloti* symbiosis and inhibits nodule formation (Zahran 1999).

3.5 Non-destructive measurements during plant growth

At three different occasions during the greenhouse study, 29/4, 12/5 and 30/5 (37, 50 and 68 days after sowing) plant height and chlorophyll content data were collected. The height was measured in cm using a regular straight ruler. Chlorophyll content measurements were made with a SPAD 502 Plus Chlorophyll Meter (Konica Minolta Sensing, Japan).

3.6 Harvest and destructive measurements

Between dates 1-11/6, 75-81 days after sowing, the plants were harvested and destructive measurements were made. The destructive measurements consisted of drying and weighing root biomass and above-ground biomass as well as counting and scoring root nodules. The nodule scoring method was based on a system devised by Rice et al (1977). In this study, a modified version of this system was used (Table 2). Nodule count, nodule cluster count, nodule colour and nodule position were scored.

Table 2. The nodule assessment criteria.

Characteristics	Criteria	Score
Nodule Number	>20	3
	5-20	2
	1-5	1
	0	0
Cluster number	-	Same as the number of clusters
Colour	Dark pink (clearly visible from outside of nodule)	5
	Pink (visible from outside)	4
	Light pink (not visible from outside)	3
	White with slight pink tint	2
	Green-white	1
	No nodules	0
Position	60-100% crown	2
	20-59% crown	1
	0-19% crown	0

Colour was assessed for the general nodule colour in a root system, not for each nodule individually. Nodules on the first 5 cm of taproot below the crown, or on lateral roots within 1 cm of this taproot region were considered crown-positioned.

3.7 Statistical Analysis

A statistical analysis was made using the Statistical Analysis System (SAS) software. For analyses at harvest, Proc Glimmix was used, with pH, cultivar, pH*cultivar and block as fixed factors. Denominator degrees of freedom were estimated using the between-within method. For repeated measures over time, time was added as a random variable, with plot as the subject, and a heterogeneous first-order autoregressive covariance structure. The significance of the factors was tested for 11 variables; Above ground biomass, Root biomass, Total biomass, Nodule count, Nodule colour, Nodule score, Nodule position, Cluster total, Cluster adjusted, Chlorophyll content (SPAD) and Plant height. In the cluster total category, each cluster counted as one regardless of size. For cluster adjusted, big clusters counted as two. Where there were significant F values for pH, differences between means were determined using Fisher's

Protected LSD. Where there were significant interactions, the SLICE function was used to compare effects at each time point.

In another analysis, Proc Glimmix was used to regress response variables on the level of Calciprill (i.e. a regression analysis). Cultivar was used as a categorical variable. Analysis of covariance was used to compare the slopes of the cultivars.

4. Results

4.1 Soil and liming

The six soils used in the experiment had pH(H_2O) values between 5.68 and 6.44, according to pH-meter measurements made 21, 32 and 41 days after liming (Table 3 and Figure 1).

Table 3. Calculation data for the Calciprill liming rates of six soils chosen for the lucerne experiment. The experiment concerns two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH. In bold letters are the expected pH(H_2O) according to the Calciprill label instructions, the total amounts of Calciprill used for each pH-level, and the final measured pH(H_2O).

Soil	Calciprill kg/ha	**Calciprill g/l**	*pH change*	***Expected Final pH(H_2O)***	Soil liter	Total Calciprill g/soil	**Final pH(H_2O)**	Factor
1	0	**0.00**	0.00	**5.70**	9.38	0.00	**5.68**	pH 1
2	250	**0.10**	0.42	**6.11**	9.38	0.94	**5.94**	pH 2
3	500	**0.20**	0.83	**6.53**	9.38	1.88	**5.99**	pH 3
4	1000	**0.40**	1.67	**7.36**	9.38	3.75	**6.24**	pH 4
5	2000	**0.80**	3.33	**9.03**	9.38	7.50	**6.27**	pH 5
6	3000	**1.20**	5.00	**10.70**	9.38	11.26	**6.44**	pH 6

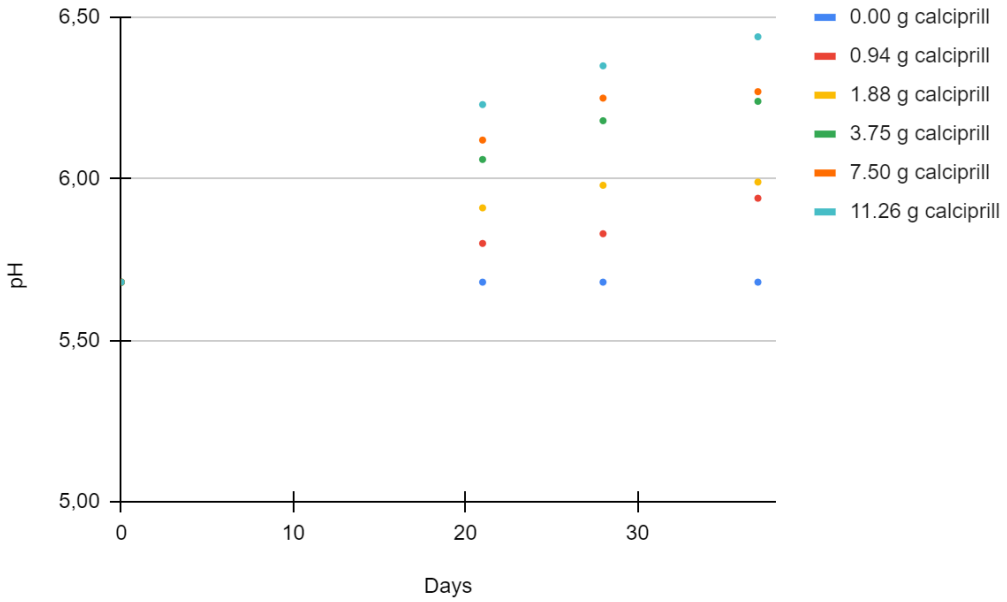

Figure 1. The results of the pH-meter measurements of the soils days 21, 32 and 41 (25/2, 8/3 and 17/3) after liming (4/2). The soils were used in an experiment concerning two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH.

By the end of the experiment, soil 1 had a pH(H_2O) around 6.5, soils 2-4 had 6.5-6.7, soil 5 had 6.5-6.7 and soil 6 had 6.7-6.8.

The pH($CaCl_2$) was 5.2 for soil 1, 5.5 for soil 2, 5.3 for soil 3, 5.5 for soil 4, 5.6 for soil 5 and 5.7 for soil 6.

At the start of the experiment, the difference between the pH(H_2O) and pH($CaCl_2$) results were between 0.7-1.0 units. At the end of the experiment the difference was between 0.9-1.3 units.

The aluminium content of the soils was 56-64 mg 100 g^{-1} dried soil (Table 4).

Table 4. Results from the pH and Al^{3+} analyses of the soils from the SLU and Eurofins labs. ISO 10390:2005-12 and ISO 10390:2007 are two names for the same standard method (SIS 2007; SIS 2012). These methods have since been replaced with ISO standard SS-EN ISO 10390:2022 (SIS 2022). The soils were used in an experiment concerning two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH.

	Pre-Calciprill addition 3/2-22	*Start of experiment 23/3-22*		*End of experiment 1/6-22*			
	pH(H_2O)	**pH($CaCl_2$)**	**pH(H_2O)**	**pH($CaCl_2$)**	**pH(H_2O)**	**pH(H_2O)**	**Al^{3+} (mg/100 g dried soil)**
Lab	SLU	Eurofins	Eurofins	Eurofins	Eurofins	SLU	Eurofins
Standard method	ISO 10390:2007	Em: NIRS	SS-EN 15933 2012*	Em: NIRS	ISO 10390: 2005-12*	ISO 10390:2007	SS 028310:1995-12
Soil 1	5.7	5.1	6.0	5.2	6.5	6.5	59
Soil 2	5.7	5.5	6.2	5.5	6.7	6.5	60
Soil 3	5.7	5.4	6.1	5.3	6.7	6.5	59
Soil 4	5.7	5.3	6.3	5.5	6.7	6.5	56
Soil 5	5.7	5.6	6.3	5.6	6.8	6.5	64
Soil 6	5.7	5.4	6.4	5.7	6.8	6.7	60

4.2 Germination test

The germination rates were 80% for BL and 21% for YL after three days. The fourth day the test resulted in a germination rate of 83% for BL and 86% for YL.

4.3 Statistical analysis

Of the 11 response variables, “cultivar” or “pH” had a significant effect on four. Cultivar had a significant effect on root biomass and pH had a significant effect on cluster total, cluster adjusted and nodule count. As cluster adjusted and cluster total are two versions of the same category, only one of them is included as a figure. The “pH*cultivar” factor was not significant for any of the variables. “Time*cultivar” was significant for plant height (Table 5).

Table 5. The results from the Proc Glimmix analysis testing the significance of factors Cultivar, pH, Cultivar*pH and Cultivar*Time on 11 measured variables connected to lucerne biomass and nodulation development. The table shows the p-values for the variables measured in the study. Values were significant at $p<0.05$. Significant p-values are bolded. The experiment concerns two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH.

				P-values			
Measured variables	**Units**	**Mean**	**Standard error**	**Cultivar**	**pH**	**Cultivar * pH**	**Cultivar * Time**
Above ground biomass	g DM/pot	4.0	0.090	0.288	0.093	0.965	-
Root biomass	g DM/pot	3.9	0.12	**0.026**	0.194	0.874	-
Total biomass	g DM/pot	7.9	0.17	0.306	0.146	0.990	-
Nodule count	Nodules	103	3.56	0.191	**0.015**	0.146	-
Nodule colour	Colour	4.5	1.10	0.898	0.727	0.560	-
Nodule score	Nodules (modified)	2.9	0.030	1.00	0.509	0.078	-
Cluster total	Clusters	3.7	0.43	0.387	**<0.001**	0.490	-
Cluster adjusted	Clusters	4.2	0.47	0.237	**<0.001**	0.329	-
Nodule position	Distance from crown	0.3	0.057	0.388	0.300	0.504	-
SPAD	SPAD unit	58.3	0.63	0.095	0.263	0.424	0.262
Height	cm	30.1	0.85	0.0820	0.318	0.522	**0.002**

In the regression analysis, Calciprill addition significantly affected above ground biomass and cluster count for BL, as well as nodule count and cluster count for YL. There was no significant difference between cultivar response to any of the variables (Table 6).

Table 6. Regression analyses using Calciprill as an explanatory variable for five biomass and nodulation variables. Values were significant at $p<0.05$ and significant p-values are bolded. The experiment concerns two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH.

variable	units	b_{BL}	p (b_{BL} = 0)	b_{YL}	p (b_{YL}= 0)	p (b_{BL} = b_{YL})
Above ground biomass	g DM/pot	-0.406	**0.045**	-0.303	0.133	0.713
Root biomass	g DM/pot	0.144	0.612	0.00744	0.979	0.734
Nodule count	Nodules	14.1	0.098	31.0	**<0.001**	0.162
Nodule score	Nodules (modified)	0.0270	0.708	0.133	0.069	0.300
Cluster count	Clusters	-3.81	**0.001**	-3.55	**0.001**	0.860

4.3.1 Cultivar effect on root biomass

Root biomass was significantly higher for BL compared to YL. The mean weight was 4.14 g DM for BL and 3.75 g DM for YL. The error bars mark the confidence interval of 0.24 g DM (Figure 2).

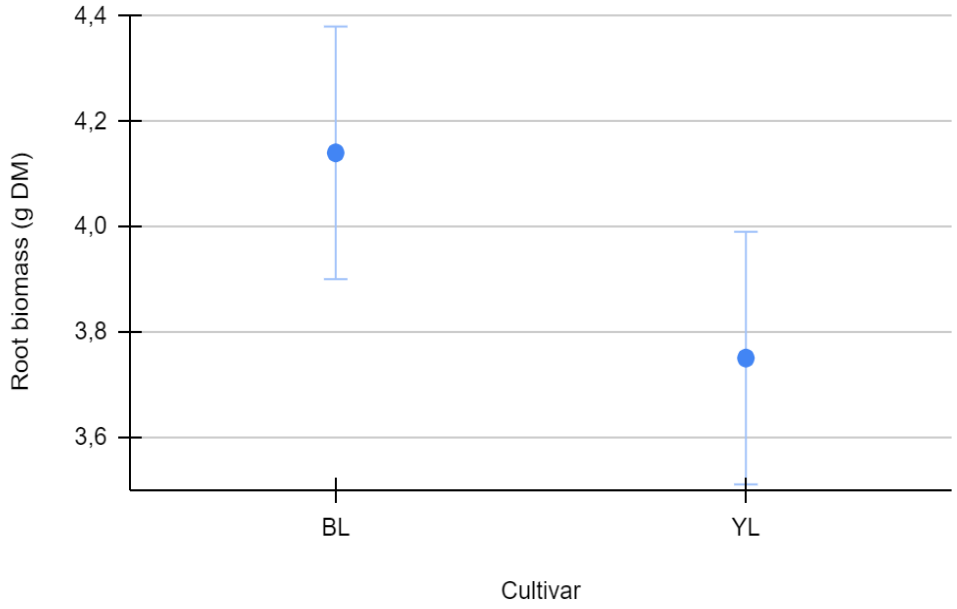

Figure 2. Mean dry matter root biomass weights for two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH. The error bars mark the 95% confidence intervals.

4.3.2 Height development between the cultivars

YL started off slower than BL in terms of growth over time. By the end of the experiment, however, both cultivars ended up being around the same height. At 35, 49 and 69 days the height of BL was 20.8, 34.0 and 38.3 cm. For YL the height was 17.8, 31.1 and 38.5 cm (Figure 3).

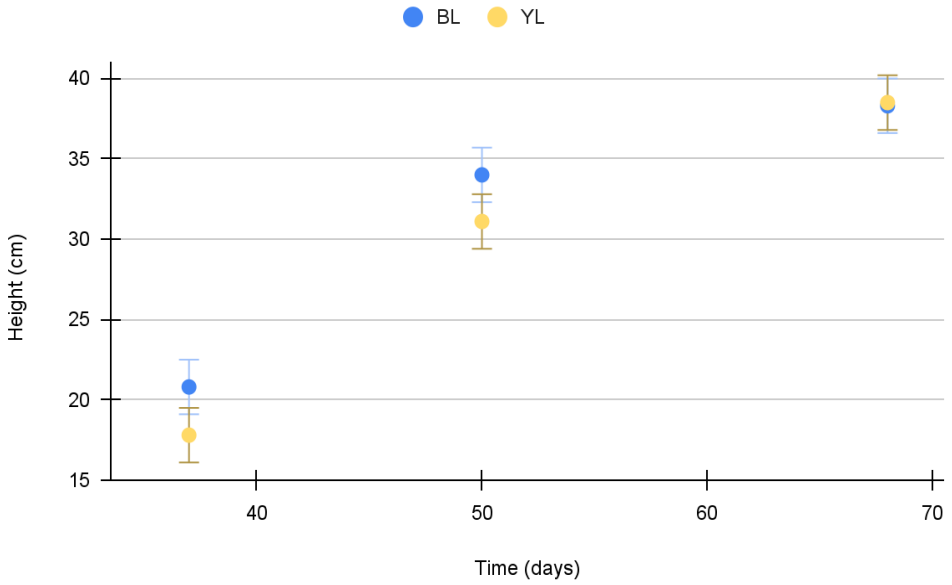

Figure 3. Mean height development over time for two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH. The error bars mark the 95% confidence interval. Height at days 37 and 50 had p-values 0.014 and 0.018, meaning that height was significantly different for BL and YL. At 68 days, p=0.85 showed that there was no longer a difference between cultivars.

4.3.3 pH effect on number of nodule clusters

Soil 1 had on average 6.1 clusters, soil 2 had 5.2, soil 3 had 4.6, soil 4 had 2.4, soil 5 had 3.0 and soil 6 had 1.1 clusters. The error bars mark the confidence interval (1.6 clusters). The trend was that the number of nodule clusters decreased as the pH rose. (Figure 4).

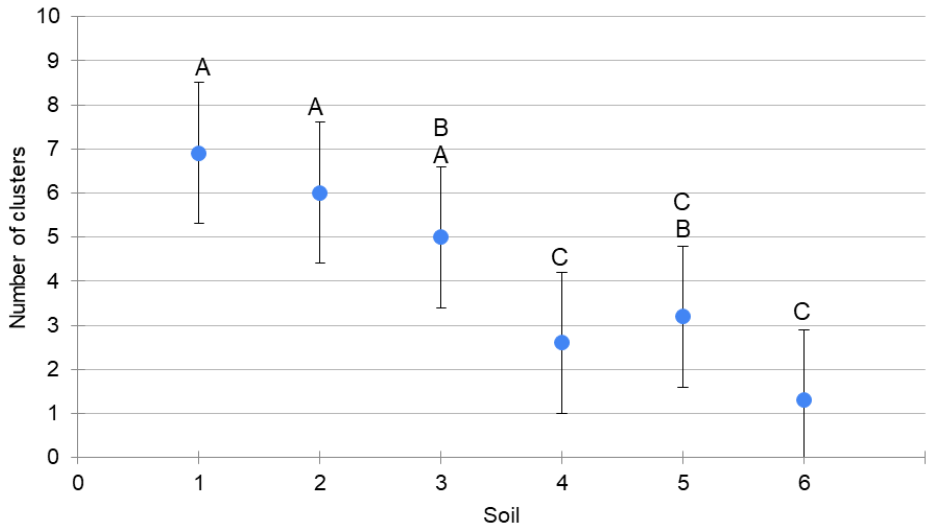

Figure 4. Mean number of clusters for the soils used in an experiment with two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH. Soils with the same letter are not significantly different to each other. The error bars mark the 95% confidence interval.

4.3.4 pH effect on number of nodules

Soil 1 had on average 95.1 nodules, soil 2 had 89.0, soil 3 had 99.6, soil 4 had 101.4, soil 5 had 112.9 and soil 6 had 118.2 nodules. The error bars mark the confidence interval (12.4 nodules). The trend was that number of nodules increased along with the pH (Figure 5).

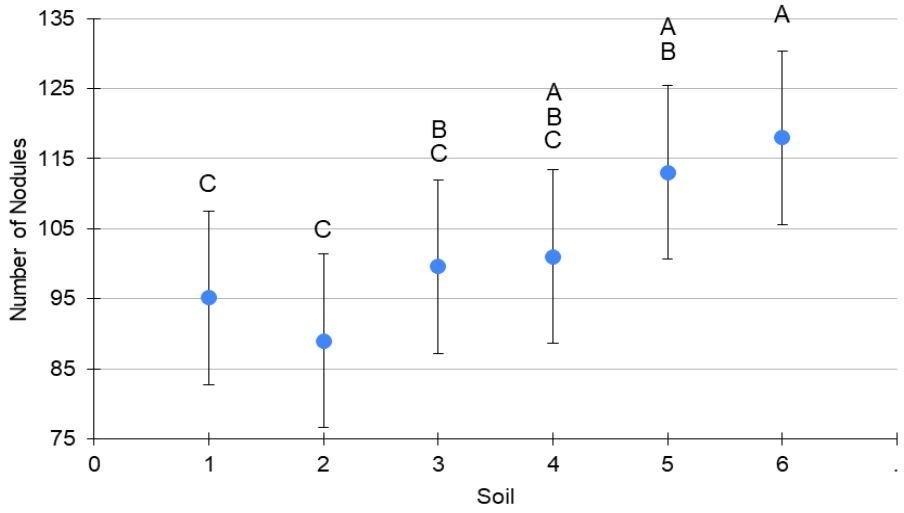

Figure 5. Mean number of nodules for each soil in an experiment with two lucerne cultivars (YL and BL) pot-grown in Sweden in soils with different pH. Soils with the same letter are not significantly different to each other. The error bars mark the 95% confidence interval.

5. Discussion

This thesis is part of a larger SLU project which aims to establish YL as a forage crop in Northern Sweden. A larger range of available legumes would increase biodiversity and potentially also the resilience of forage cultivation. Wild YL populations grow naturally in Northern Sweden (SLU Artfakta 2023) and are highly winter dormant. Cultivated YL could be a valuable addition to Northern forage legume cultivation, which today is dominated by red clover.

The objective of this study was to evaluate the effects of differential soil pH conditions on YL and BL. By evaluating nodulation and growth an experiment was conducted, to examine the effect of pH on the growth and nodulation of lucerne subspecies. The hypotheses were:
1) YL will be more tolerant than BL against low pH, but BL will produce more biomass than YL in high pH conditions.
2) A low pH will decrease the nodulation formation and quality for BL and YL.
3) A low pH will decrease biomass quantity for BL and YL.

5.1 YL will be more tolerant than BL against low pH, but BL will produce more biomass than YL in high pH conditions

The results imply that cultivars Karlu (YL) and SW Nexus (BL), when inoculated with Nitragin gold, develop similarly in terms of nodulation and biomass under favourable temperature and light conditions. There were no significant differences in nodulation and growth between cultivars. However, the pH equalized between treatments during the experiment and the final range in pH was small.

When adjusting the pH for this sort of experiment there are two options: either starting with a high pH soil and adding acidifying material, or starting with a low pH soil and raising the pH with lime. Soil properties, such as clay content or organic matter, affect how the soil reacts to liming and acidifying materials, and how much material is needed to change the pH (Kuepfel et al 2012).
Two common ways to reduce soil pH are to add aluminium sulfate or sulfur. Aluminium sulfate is fast-acting, producing acid as it dissolves and immediately begins to reduce the pH. On the negative side, aluminium addition can inhibit plant growth. Sulfur produces acid as bacteria starts to break it down, a process that can take months depending on factors such as soil quality, abiotic conditions and the texture of the sulfur material (Kuepfel et al 2012). In our study, by starting out with a low pH soil, using a well-established liming material to raise the pH, the problem regarding aluminium addition was avoided. A fast-acting material with a precise effect was preferred and Calciprill was chosen since it fulfils both criteria. Based on the pre-experiment pH readings, the Calciprill addition effectively created different pH levels.

However, both the pH(H_2O) and pH($CaCl_2$) show that there was little difference between the soils in terms of pH by the end of the experiment. In contrast with other studies, the general difference between pH(H_2O) and pH($CaCl_2$) results were greater than 0.4-0.5 units, before and after the experiment.

Unexpectedly, the soil with no lime showed a 0.5-1.0 pH unit increase during the experiment, according to the final lab analyses. Logically, it should have been the same value as from the start, pH(H_2O) 5.7. This indicates that there was a pH increase independent of liming. There are a few possible explanations for this:

- The soil might have contained an unknown lime source from the start.
- Some of the added products (i.e fertilizers, water, perlite) might have contained liming materials.
- The pH analyses were incorrect.

The latter is not very likely, as several analyses show the same tendency. After double checking product descriptions and water sources, it is also not likely that the addition of products affected the pH. The fertilizers contained no lime (Growland 2022a; Growland 2022b). Perlite has a neutral to slightly alkaline pH of 7-7.5 and contains 0.8% CaO. It is, however, chemically inert and has no effect on soil pH (Papadopoulos et al 2008; Chaney 2018). Perlite might have raised the pH of the final soil samples in case it was accidentally mixed in during sample collection. That would have diluted the samples and altered the soil:water/$CaCl_2$ ratios, decreasing H^+ presence in relation to solution volumes. During harvest it was hard to completely separate soil from perlite and fine roots, making this a possible part-explanation for the final high pH results. In this case, the sample pH does not reflect the actual pot pH. It is, however, unlikely that perlite alone could explain the result, based on the fact that overall pH had started to rise before perlite was added. In hindsight, it would have been interesting to test the pH of leftover soil that was not treated or potted, to know if there was an increase based on soil property rather than external treatment. However, this was not possible, as all of the soil was used in the experiment.
Another possibility is that Econova, the garden company, had mixed in some kind of slow-acting liming material into the soil. Due to lack of evidence, however, the reason for the unexpected pH increase remains unclear.

All soils came from the same soil batch and therefore they should have been fairly similar in terms of texture, structure, density and nutrient content. However, there will always be micro climatic differences within a soil. The question is if these differences could have been enough to statistically affect the nodulation/biomass outcome between soil treatments. The nutrient analyses (table A1) show that both at the start and at the end of the experiment, N content and delivery capacity were generally similar between soils. This was also the case for available P and K content as well as the C/N ratio. The N status of the soils should not have been of importance, considering that the plants fix their own N. The similar pH most likely led to similarities in nutrient availability between treatments.

The lab analyses showed that the soils had Al^{3+} contents of 560-640 mg Al^{3+} kg^{-1} dried soil. It was, however, not reported in which form Al^{3+} was present or whether these levels were considered high, low or optimal for plant growth. There were no plant symptoms indicating Al-toxicity in terms of biomass and nodulation. The high pH likely meant that even in case Al^{3+} was present in high concentrations, it would not have been not plant available, in line with the studies by Rice et al 1977 and Berenji et al 2017. A clear reduction of nodulation and biomass would have been the expected outcome at pH<6 in case the Al levels were toxic.

The result can be compared to a study by Athar & Johnson (1996). The nodulation and biomass development of one YL cultivar (Anik), one BL cultivar Spredor 2 and one BL accession (Punyal) in symbioses with different *E. meliloti* strains was evaluated. Plant accession/cultivar , water availability and *E. meliloti* strain significantly impacted number of nodules, total dry weight and shoot nitrogen pools of all three cultivars. Number of nodules were significantly higher for both tested BL compared to the YL cultivar, for all *E. meliloti* strains. The same observation was made for biomass dry weight. That means that the Athar & Johnson results were different to the results of the present study, in which there were no significant differences between the BL and the YL cultivar in terms of biomass and nodulation. In both studies, one or two cultivars/accessions were chosen to represent BL and YL as subspecies, which means that it is questionable if the results give a fair picture of general BL and YL properties. It does, however, give an idea of the production potential and *E. meliloti* strain compatibility of the tested cultivars/accessions.

Based on this discussion an alternate conclusion can be reached, that each soil had a similar effect on nodulation and biomass for BL cultivar Nexus and YL cultivar Karlu. In a future study the pH tolerance of completely wild YL populations would be interesting to examine since such information could provide insights about genetic resources and be valuable for plant breeders.

5.2 A low pH will decrease the nodulation rate and quality for BL and YL nodulation

In this study there was a significant effect of soil treatment on nodulation, although the pH differences were small. At the start of the experiment the pH differences were greater than at the end of the experiment. As reported in the literature (Munns et al 1970; Vance et al 1988), the pH conditions at the time of the *E. meliloti* infection are highly impactful on nodulation development. Therefore, it is likely that the soil pH conditions at the start of the experiment were most important for the final nodulation result. Since pH(H_2O) at this point was above 6.0 in all soils, the normal nodulation development is in line with results from Rice et al (1977) and Berenji et al (2017). Rice et al showed lucerne nodule score reductions at pH(H_2O)<6 and after pH 6 nodulation became constant up to at least 7.5, probably as a result of repressed Al^{3+} availability (Rice et al 1977). Berenji et al conducted a study with five soil pH levels between 5.5-6.5, using the nodule scoring system by Rice et al. They also found that the overall nodulation score increased with liming and peaked at pH 6. At pH>6 nodulation response to liming became weak and almost constant (Berenji et al 2017). Since all soils in the present experiment ended up with a pH(H_2O)>6, the lack of significance for total nodule score is in line with the results from Berenji et al (2017) and Rice et al (1977). Unfortunately, neither of these studies provide details about nodulation other than the overall score. If there were any contradictory trends between categories (position, number, clusters and colour) it was not mentioned, and this is an area where our research provides more detail.

Considering the high pH, it was surprising to see that pH still had some effect on nodulation. Nodule count was positively correlated with increasing pH while nodule cluster count was negatively correlated with pH. The fact that pH had

some effect on nodulation but none on plant growth may be evidence of the pH-sensitive nature of the *E. meliloti* strains in the Nitragin gold mix. That could mean that small pH fluctuations with no real impact on biomass can be visually indicated by studying root nodulation.

Athar & Johnson (1996) show the importance of rhizobial strain for nodulation. Seven *E. meliloti* strains; UL 61, UL 115, UL 136, UL 167, UL 210, UL 222, as well as a commercial strain 102F51a were compared as inoculants for YL and BL. All but one tested strains came from BL hosts, whereas strain UL 167 was derived from a YL host. Interestingly, nodulation of YL was lower than for the two BL cultivars using strain UL167. The three most effective *E. meliloti* strains originated from BL hosts. Unfortunately for our study, it has not been possible to find specific information about which strain(s) the Nitragin gold mix contains. With that information it would have been possible to compare the strains from this study with the study by Athar & Johnson (1996).

5.3 A low pH will decrease biomass quantity for BL and YL biomass

There was no significant effect of pH on any of the biomass variables but cultivar had a significant effect on root biomass, with BL Nexus producing more than YL Karlu. One contributing cause for the result could be that the finer roots of YL were more easily destroyed when separated from the soil at harvest. This might have led to a loss of root biomass that could not be separated and measured. Above-ground biomass and total biomass were not affected by cultivar. This was surprising considering BL is expected to produce larger yields compared to YL. In the end, the second part of hypothesis 1 could not be confirmed, since there was not enough evidence to prove it. None of the other variables were significantly affected by cultivar. The fact that nodulation was similar for YL Karlu and BL Nexus indicates that Nitragin Gold inoculum is compatible with both cultivars.

Visually, all 60 pots looked similar in terms of leaf colour. This was confirmed with data from the SPAD measurements, showing high and similar chlorophyll values for most plants. The exceptions were a few random plants with stress symptoms such as leaf spots and twisted leaves. The result is consistent with Rice et al (1977) and Berenji et al (2017), where biomass was more or less constant at pH>6, likely as a consequence of adequate nodulation. It is also possible that biomass development might have been positively affected by a high nutrient availability for roots, regardless of nodulation quality.

In the regression analysis, Calciprill was used as an explanatory variable, and analysis of covariance was used to examine whether the effect was significantly different between the two cultivars (Table 6). Based on the analysis, there was no evidence that the explanatory variable (Calciprill) affects BL and YL differently for any of the response variables. However, the analysis showed that Calciprill had an effect on above ground biomass for BL, nodule count for YL and total cluster count for both BL and YL, as follows:

Above ground biomass: more Calciprill → less AG biomass development for BL;

Nodule count: more Calciprill → nodule count goes up for YL, supported by the Proc Glimmix analysis where higher pH increased the number of nodules;

Cluster total: more Calciprill → less clusters for BL and YL, supported by the Proc Glimmix analysis where number of clusters decreased with rising pH.

Calciprill addition increased nodule number and decreased cluster number. Since Calciprill can be directly connected to pH increase, the results for both nodule and cluster count support the results of the Proc Glimmix analysis. Unexpectedly, Calciprill addition had a negative effect on above ground biomass for BL. This is a strange result. The significance was, however, marginal, with a p-value of 0.045 (the significance level threshold was $p<0.05$). It is worth mentioning that the *difference* between cultivar response to Calciprill was *not significant* for above ground biomass. This, combined with the fact that Calciprill was *not significant* for YL biomass, can be reasons for questioning whether the significance for BL actually reflects a true effect.

5.4 Conclusions

The statistical analysis partly confirmed and partly contradicted hypotheses 2 and 3. Ultimately, however, there was not enough evidence to confidently draw conclusions about hypotheses 1, 2 or 3. The most obvious shortcoming of this study was that the soil pH was too high, making it impossible to properly evaluate the effect of low pH on BL and YL. The objectives of the study were not fully met, and further research is needed to address these questions.

5.5 Implications and outlook

One practical finding of this study is that Nitragin gold is compatible with both SW Nexus and Karlu. The pH sensitivity of *E. meliloti* is also highlighted. The research also indicates that YL Karlu has a production potential that can match the potential of BL Nexus, at least under controlled conditions before a hypothetical first cutting. A high producing YL (Karlu) with an effective known inoculant mix can be a good place to start for Swedish YL cultivation trials.

There are still knowledge gaps in the lucerne research field when it comes to the acid tolerance and *E. meliloti* strain compatibility of YL cultivars. Previous studies on the subject are few. Information about acid tolerance of YL cultivar Karlu and BL cultivar SW Nexus would clarify the requirements for YL and whether they differ to that of BL. Such information is useful for farmers, crop advisors, and plant breeders. Therefore it is important to keep researching YL inoculants as well as acid tolerance of cultivars/bacterial strains in order to properly understand what makes YL cultivation effective.

Future directions for research would also be to continue researching the endurance and growth of Karlu, as well as several other YL cultivars, under field conditions over a period of several seasons. Such studies exist in Finland (Sormunen-Cristian 1998; Paasikallio & Sormunen-Cristian 2002), where yield

and protein content after several cuttings has been studied. Similar studies need to continue to be made in Sweden.

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Popular science summary

Blue-flowered lucerne (BL) is the most popular legume worldwide. It is cultivated on over 30 million ha, contributing to food security and animal health. Cultivated BL originates from the Meditteranian area and is popular for its drought tolerance, high production potential and protein content. Thanks to cross breeding with the resilient and cold tolerant yellow-flowered lucerne (YL) it is possible to cultivate BL in Scandinavia. In Sweden BL cultivation is recommended up to 60° N, where winters are relatively mild and warm compared to Northern conditions.
The most common forage legume in Sweden is red clover followed by white clover. Forage production in Northern Sweden would benefit from a larger legume variety to increase biodiversity and resilience of forage production. Wild YL populations grow naturally in Northern Sweden and are highly winter dormant. Cultivated YL could be a valuable addition to Northern forage legume cultivation, which today is dominated by red clover.

Lucerne species in general are pH sensitive, largely as a consequence of their symbiosis with a nitrogen fixing bacterial species called *E. meliloti*. In order for a successful infection and symbiosis to happen, Ca is required. As Ca availability normally correlates with an alkaline pH, studies have shown that most *E. meliloti* strains reduce their ability to infect and form root nodules at around pH<6. As part of a larger project which aims to introduce YL as a forage crop option, the goal of this study was to find out which lucerne subspecies is most pH tolerant: YL or BL.

In order to test this, a low pH mineral soil was used to create six pH levels between 5.7-6.5. This was made by adding different amounts of a liming material called Calciprill to all but one soil. The combination of a cultivar (YL/BL) and a pH level (soil 1-6) constituted a treatment, resulting in 12 unique treatments. YL cultivar Karlu and BL cultivar Nexus were chosen based on morphological traits typical for their subspecies.

The seeds were inoculated with a commercial *E. meliloti* mix called Nitragin Gold. The treatments were cultivated in a greenhouse chamber for 75-81 days in light and warm conditions. During growth, the plant height and chlorophyll content was measured. At harvest, the root- and shoot biomass was weighed and the *E. meliloti* nodulation was scored according to tested criteria. Soil pH measurements were made before sowing, at sowing and after harvest to track the pH development.

A statistical analysis was made for the significance of factors “pH”, “cultivar”, “pH*cultivar” and “time*cultivar” for 11 variables connected to nodulation and biomass. Additionally, a regression analysis was made with Calciprill as a covariance factor. Unfortunately, the soil pH ended up between 6.5-6.7 instead of 5.7-6.5. This meant that there were overall high pH conditions a narrow pH span. Proven by the fact the uncalcified soil had a pH increase of almost 1 unit, there was an unexpected pH rise independent of the Calciprill addition. Most likely, there was an accidental input of liming material but it remains unclear from what source.

Despite high pH conditions, the results showed that nodule number and nodule cluster count was correlated to pH. There were fewer nodules but more nodule clusters at lower pH. At a higher pH there were more nodules but fewer clusters. The result highlights the pH sensitive nature of *E. meliloti* and indicates an opposite trend for nodule number/cluster. YL had slower initial growth than BL and BL produced more root biomass than YL. Based on this study it was not possible to draw any conclusions about pH tolerance in YL or BL, the main reason being the lack of pH stress.

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I would like to express a warm thank you to all of you!

Appendix

Figure A1. Calciprill effect on above-ground biomass for BL and YL. The effect was significant for BL (i.e. the slope was significantly different from zero).

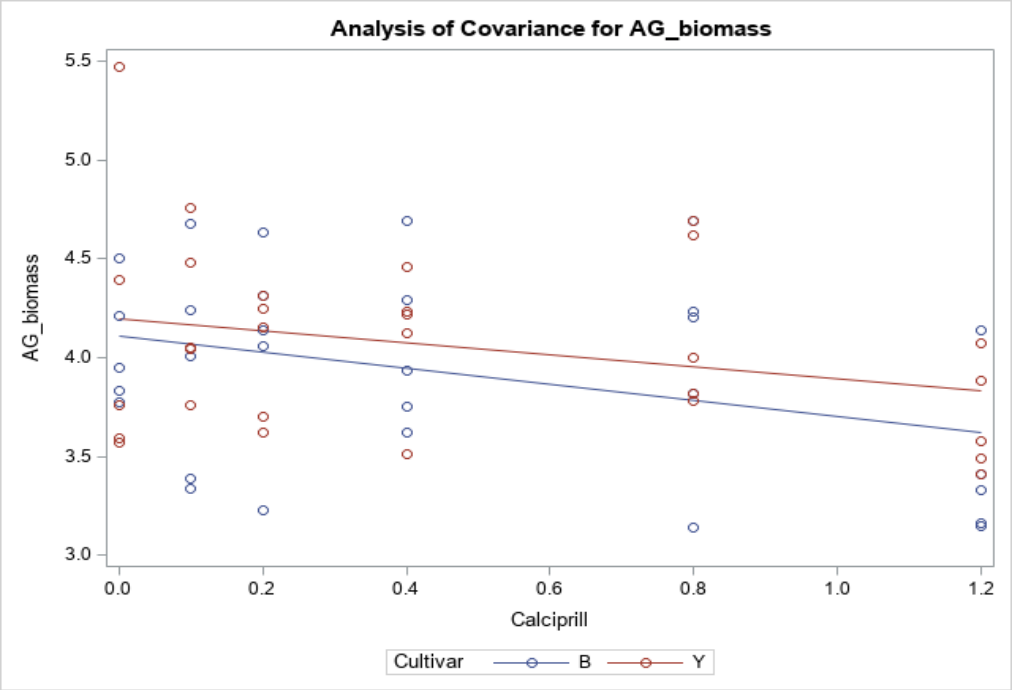

Figure A2. Calciprill effect on nodule count. The effect was significant for YL (i.e. the slope was significantly different from zero).

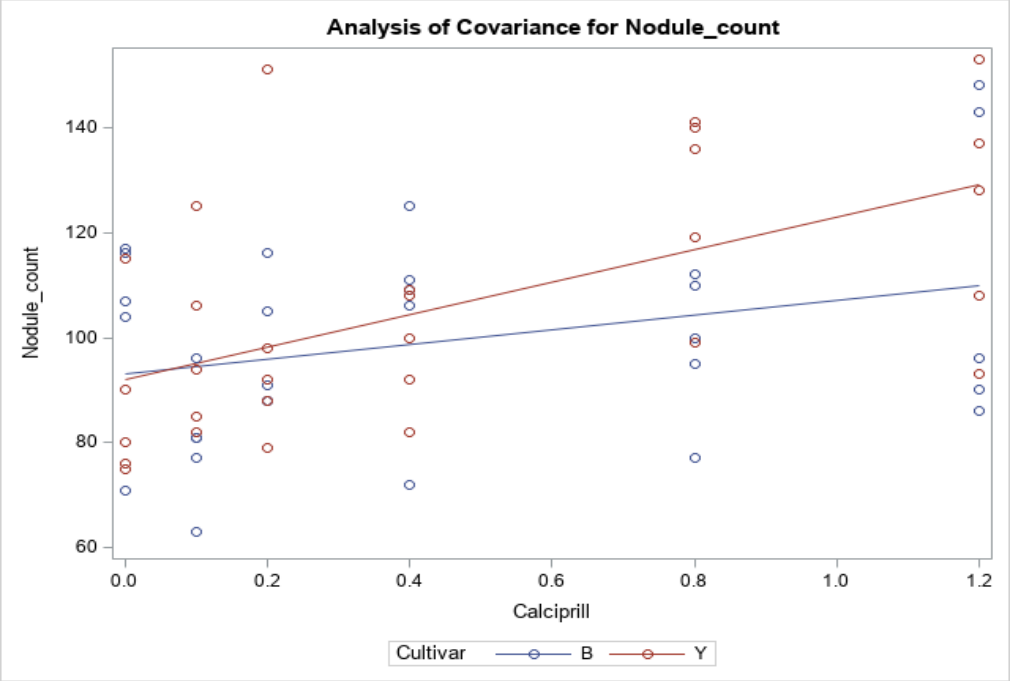

Figure A3. Calciprill effect on cluster number. The effect was significant for both BL and YL (i.e. the slopes were significantly different from zero).

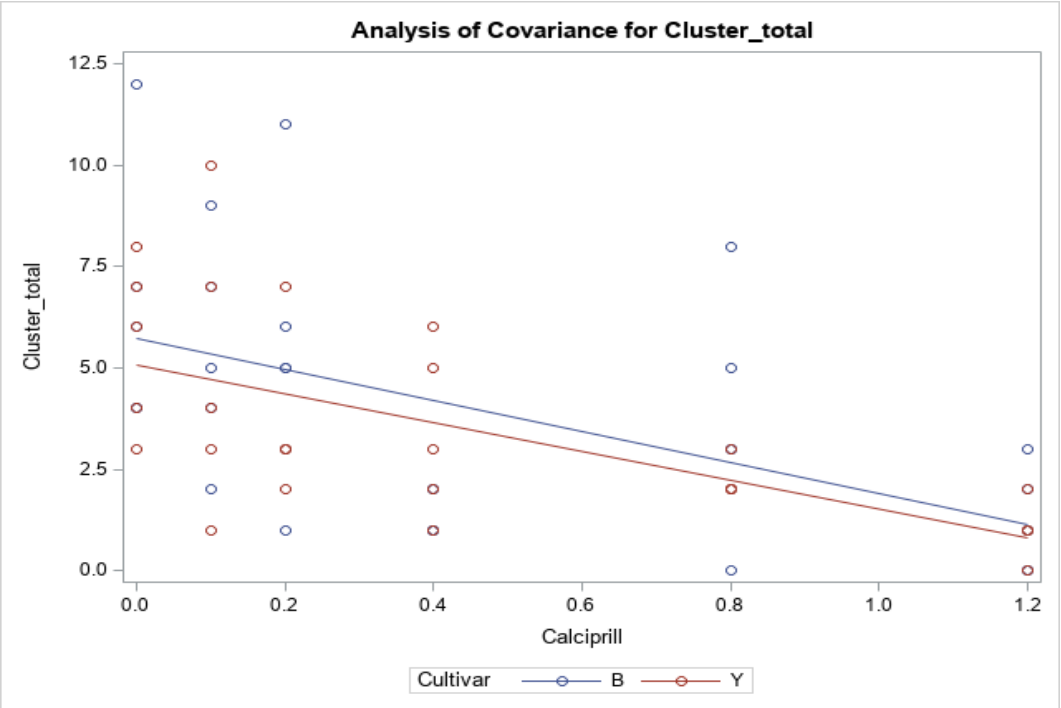

Table A1. Nutrient status of the soils at the start of the experiment. The data comes from soil sample analyses from Eurofins. The five categories are low (red), moderately low (orange), good (green), moderately high (turquoise) and high (blue).

	Total N content kg/ha	N delivery capability kg/ha	C/N ratio	Available S kg/ha	Available P kg/ha	Available K kg/ha	Available Ca kg/ha	Available Mg kg/ha	Available Na kg/ha
	Start of experiment								
Soil 1	6210	50	21	61	1.7	190	160	350	100
Soil 2	5610	60	18	59	1.7	175	250	290	85
Soil 3	4690	25	24	90	1.7	235	440	410	120
Soil 4	6400	70	18	89	1.7	225	300	400	120
Soil 5	5850	40	22	238	1.3	205	320	375	115
Soil 6	6150	60	19	100	2.0	200	465	350	105
	End of experiment								
Soil 1	6270	70	18	78	2.0	140	215	575	130
Soil 2	6100	65	18	58	2.7	115	300	570	130

Soil 3	8730	105	16	41	2.0	125	290	635	145
Soil 4	6390	65	19	54	2.6	130	135	610	150
Soil 5	6720	75	18	148	1.3	130	240	575	165
Soil 6	6830	75	18	51	1.3	140	240	585	155
	Low	Moderately low	Good	Moderately high	High				

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