

Annika Wilhelmson

The importance of oxygen availability in two plant-based bioprocesses: hairy root cultivation and malting

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Annika Wilhelmson

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VTT, Bergsmansvägen 3, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 4374

VTT Technical Research Centre of Finland, Vuorimiehentie 3, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 4374

VTT, Tietotie 2, PL 1000, 02044 VTT
puh. vaihde 020 722 111, faksi 020 722 7071

VTT, Datavägen 2, PB 1000, 02044 VTT
tel. växel 020 722 111, fax 020 722 7071

VTT Technical Research Centre of Finland, Tietotie 2, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax +358 20 722 7071

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Abstract

The main aim of this work was to examine the role of oxygen availability in two model processes: the cultivation of *Agrobacterium rhizogenes*-induced hairy roots of the medicinal plant Egyptian henbane (*Hyoscyamus muticus*), and barley (*Hordeum vulgare*) malting. This was accomplished by transferring the *vhb* gene encoding bacterial haemoglobin (VHb) from *Vitreoscilla* to *H. muticus* and to barley with the specific goals of improving the growth and alkaloid production of *H. muticus* hairy roots and the germination rate of barley.

In *H. muticus* hairy roots, heterologous *vhb* expression and an increase in aeration both improved the growth, but not the specific hyoscyamine production of the hairy roots. This suggests that oxygen availability may not be a limiting factor in hyoscyamine synthesis. The effect of VHb on the alkaloid profile of *H. muticus* hairy roots was not identical to that of aeration, indicating that the physiological effects of VHb are probably not related directly to its ability to increase the intracellular effective oxygen concentration. Although VHb production did not have a statistically significant effect on hyoscyamine production due to wide somaclonal variation, the highest production levels were found among the VHb-lines. A problem related to *vhb* expression was the tendency of hairy roots to dedifferentiate to callus. This effect probably counteracted the positive effects of *vhb* expression, because root dedifferentiation leads to impaired alkaloid production.

Barley plants expressing the *vhb* gene did not react to oxygen deficiency by increasing the alcohol dehydrogenase (ADH) activity in the roots, unlike the control plants. This could be due to the ability of VHb to supplement the role of ADH in the recycling of NADH and maintaining glycolysis. In contrast to previous findings, we found that constitutive *vhb* expression did not improve the

germination rate of barley kernels. On the contrary, VHb even retarded germination slightly. Moreover, VHb restricted root growth in young barley seedlings. These effects are probably related to the $\cdot\text{NO}$ dioxygenase activity of VHb. Because nitric oxide (NO) has both cytotoxic and stimulating properties, the effect of *vhb* expression in plants may depend on the level and role of endogenous NO in the conditions studied. In conditions involving excess NO production, such as severe oxygen deficiency, VHb can protect plant cells from the adverse effects of NO. However, in conditions in which NO plays an important role as a signal molecule, such as germination and root growth, *vhb* expression may even have slightly adverse effects on growth and development.

Another aim of the study was to verify the hypothesis that barley embryos suffer from oxygen deficiency in the malting process, regardless of aeration. This was accomplished by studying the effect of aeration on the production of the inducible isoenzymes ADH2 and ADH3, ADH activity, ethanol production and α -amylase activity. Furthermore, the effect of the indigenous microbial community of the barley kernel on embryo oxygen deficiency was studied by modifying the microbial populations in the malting process. Neither aeration nor a reduction in grain microbes alleviated the oxygen deficiency of the barley embryo. An improvement in germination was observed after restriction of bacteria, but this was probably a consequence of facilitated root emergence. Many of the bacterial species dominating in the malting process can produce exopolymeric substances that may form a physical barrier restricting root emergence.

A certain degree of oxygen deficiency in the embryo appears to be an inevitable stage in barley germination. The recent results of other research groups showing that NO is produced in plant cells under oxygen deficiency and that NO stimulates seed germination, together with the present results showing that *vhb* expression slows down barley germination slightly, imply that embryo oxygen deficiency is in fact beneficial or even necessary for germination. Although aeration cannot and need not overcome the oxygen deficiency of the barley embryo, the need for aeration increases as the malting process proceeds. Aeration not only introduces the oxygen needed for the completion of germination, enzyme synthesis and other post-germinative events, but also removes CO_2 that would otherwise inhibit modification of the grain.

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Avainsanat alkaloid, barley, germination, haemoglobin, hairy root, hyoscyamine, *Hordeum vulgare*, *Hyoscyamus muticus*, malting, oxygen deficiency, VHb, *Vitreoscilla*

Tiivistelmä

Työn pääasiallinen tavoite oli tutkia hapen roolia kahdessa malliprosessissa: Egyptin villikaalin (*Hyoscyamus muticus*) juuriviljelmissä sekä ohran (*Hordeum vulgare*) mallastuksessa. Hapen roolia tutkittiin siirtämällä bakteeri-hemoglobiinia (VHb) koodaava *vhb*-geeni *Vitreoscillasta* Egyptin villikaaliin ja ohraan. Geeninsiirron tavoitteena oli parantaa Egyptin villikaalin juuriviljelmien kasvua ja alkaloidituottoa ja nopeuttaa ohran itämistä.

Sekä VHb:n tuotto että ilmastus paransivat juuriviljelmien kasvua, mutta eivät vaikuttaneet hyoskyamiinituottoon. Hapen puute ei siis näytä rajoittavan hyoskyamiinisynteesiä. VHb:n vaikutus Egyptin villikaalin alkaloidiprofiiliin poikkesi ilmastuksen vaikutuksesta, mikä viittaa siihen, ettei VHb:n fysiologinen vaikutus perustu pelkästään sen kykyyn nostaa solunsisäistä happikonsentraatiota. Parhaat hyoskyamiinintuottajalinjat löytyivät VHb-linjojen joukosta, vaikka VHb:n tuotto ei suuren hajonnan takia nostanutkaan hyosmyakiinituottoa tilastollisesti merkitsevästi. VHb-linjat olivat kuitenkin vertailukasveja alttiimpia menettämään juurisolukkorakenteensa ja muodostamaan kallusta. Tämä taipumus heikentää VHb-linjojen käyttökelpoisuutta, koska kalluksen alkaloidituotto on selvästi heikompi kuin juurten.

VHb:tä tuottavien ohrakasvien juuret eivät vertailukasvien tapaan reagoineet hapen puutteeseen lisäämällä alkoholidehydrogenaasiaktiivisuutta (ADH). Tämä voi johtua VHb:n kyvystä korvata ADH:ta glykolyysin ylläpidossa ja NADH:n kierrätyksessä. Aikaisemmista tutkimuksista poiketen VHb ei nopeuttanut ohran itämistä. VHb jopa hidasti itämistä hieman. VHb rajoitti myös nuorten ohra- taimien juurten kasvua. Nämä vaikutukset liittyvät todennäköisesti VHb:n NO dioksygenaasiaktiivisuuteen, sillä sekä itäminen että juurten kasvu ovat typpi- oksidin (NO) säätämiä. VHb:n on aikaisemmin todettu suojaavan kasvisoluja

liiallisen NO-tuoton haitallisilta vaikutuksilta. Olosuhteissa, joissa NO toimii tärkeänä signaalimolekyylinä, kuten siemenen itämisessä ja juurten kasvussa, VHB voi kuitenkin hidastaa kasvua ja kehitystä.

Työn toinen tavoite oli todentaa hypoteesi, jonka mukaan ohra kärsii hapen puutteesta mallastusprosessissa, ilmastuksesta huolimatta. Ilmastuksen vaikutusta alkion hapen puutteeseen tutkittiin mittaamalla indusoituvien ADH-entsyymien ADH2 ja ADH3 ilmestymistä, ADH-aktiivisuutta, etanolituottoa ja α -amylaasi-aktiivisuutta. Lisäksi selvitettiin ohran mikrobiston vaikutus alkion hapen puutteeseen muokkaamalla mallastusprosessin mikrobipopulaatioita. Ilmastus tai mikrobien vähentäminen eivät lieventäneet alkion hapen puutetta. Bakteerien vähentäminen paransi hieman ohran itämistä, mutta vaikutusmekanismi ei liittynyt hapen puutteeseen. Monet mallastusprosessissa esiintyvät bakteerit tuottavat eksopolymeerisiä yhdisteitä, jotka saattavat inhiboida itämistä estämällä juuri-idun kasvua fyysisesti.

Hapen puute näyttää olevan luonnollinen ja väistämätön vaihe ohran itämisessä. Muiden tutkimusryhmien tulokset ovat osoittaneet, että NO:ta muodostuu hapen puutteesta kärsivissä kasvisoluissa ja että NO stimuloi itämistä. Nämä tulokset yhdessä tämän tutkimuksen tulosten kanssa indikoivat, että alkion jonkin asteinen hapen puute saatta olla edullinen tai jopa välttämätön osa itämistä. Vaikka ilmastuksella ei ole mahdollista tai edes välttämätöntä lieventää ohran hapen puutetta, ilmastuksen tarve kasvaa mallastusprosessin edetessä. Ilmastus tuo itämisen loppuun viemiseen tarvittavan hapen ohralle, sekä poistaa jyvän myöhentymistä hidastavan hiilidioksidin ilmatilasta.

Preface

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Espoo, August 2007

Annika Wilhelmson

List of publications

- I Wilhelmson, A., Kallio, P.T., Oksman-Caldentey, K.-M. and Nuutila, A.M. 2005. Expression of *Vitreoscilla* hemoglobin enhances growth of *Hyoscyamus muticus* hairy root cultures. *Planta Med.* Vol. 71. Pp. 48–53.
- II Wilhelmson, A., Häkkinen, S.T., Kallio, P.T., Oksman-Caldentey, K.-M. and Nuutila, A.M. 2006. Heterologous expression of *Vitreoscilla* hemoglobin (VHb) and cultivation conditions affect the alkaloid profile of *Hyoscyamus muticus* hairy roots. *Biotechnol. Prog.* Vol. 22. Pp. 350–358.
- III Wilhelmson, A., Laitila, A., Vilpola, A., Olkku, J., Kotaviita, E., Fagerstedt, K. and Home, S. 2006. Oxygen deficiency in barley (*Hordeum vulgare*) grain during malting. *J. Agric. Food Chem.* Vol. 54. Pp. 409–416.
- IV Laitila, A., Kotaviita, E., Peltola, P., Home, S. and Wilhelmson, A. 2007. Indigenous microbial community of barley greatly influences grain germination and malt quality. *J. Inst. Brew.* Vol. 113. Pp. 9–20.
- V Wilhelmson, A., Kallio, P.T., Oksman-Caldentey, K.-M. and Nuutila, A.M. 2007. Heterologous expression of *Vitreoscilla* haemoglobin in barley (*Hordeum vulgare*). In press.

The author's contribution to the appended publications

- I Annika Wilhelmson was responsible for the experimental work except for construction of the expression vector, which was done by Pauli Kallio. Annika Wilhelmson was responsible for planning of the research, experimental design, interpretation of the results and writing the paper.
- II Annika Wilhelmson was responsible for all the experimental work except alkaloid analysis by gas chromatography, which was done by Suvi Häkkinen. Annika Wilhelmson was responsible for planning of the research, experimental design, interpretation of the results and writing the paper with the exception of the interpretation of the gas chromatography results, which was done together with Suvi Häkkinen.
- III Annika Wilhelmson was responsible for all the experimental work except the micro-malting and FTIR-analysis, which were done by Arvi Vilpola. Annika Wilhelmson was responsible for planning of the research, experimental design, interpretation of the results and writing the paper.
- IV Annika Wilhelmson was responsible for the analysis of grain germination and physiology. Planning of the research, experimental design, interpretation of the results and writing the paper was done together with Arja Laitila, who was also responsible for the microbiological analyses and PCR-DGGE. The authors have agreed that both Annika Wilhelmson and Arja Laitila will use this publication in their doctoral dissertations.
- V Annika Wilhelmson was responsible for the experimental work, except construction of the expression vector, which was done by Pauli Kallio. Annika Wilhelmson was responsible for planning of the research, experimental design, interpretation of the results and writing the paper.

Contents

Abstract.....	3
Tiivistelmä	5
Preface	7
List of publications	9
The author's contribution to the appended publications.....	10
Abbreviations.....	13
1. Introduction.....	14
1.1 Egyptian henbane (<i>Hyoscyamus muticus</i>) as a source of tropane alkaloids.....	14
1.1.1 The production of tropane alkaloids by <i>Agrobacterium rhizogenes</i> -induced hairy roots	14
1.1.2 The effect of oxygen on hairy root growth and alkaloid production	16
1.2 Malting of barley (<i>Hordeum vulgare</i>)	19
1.2.1 The malting process	19
1.2.2 Oxygen deficiency in barley kernels during malting	20
1.3 Nitric oxide (NO) in plants.....	22
1.4 Haemoglobins in plants and bacteria.....	23
1.4.1 The role of haemoglobins in oxygen deficient plant tissues ...	23
1.4.2 Heterologous expression of <i>Vitreoscilla</i> haemoglobin	25
2. The aims of the present study	27
3. Materials and methods	28
3.1 <i>Hyoscyamus muticus</i> hairy root cultivation.....	28
3.1.1 Transformation of <i>H. muticus</i> (Paper I)	28
3.1.2 Characterization of transformed hairy roots (Paper I).....	28
3.1.3 Growth medium optimization (Paper II).....	29
3.1.4 Cultivation in a bioreactor (Paper II)	29
3.1.5 Cultivation with and without aeration (Paper II).....	30

3.2	Barley germination	30
3.2.1	Malting with different steeping conditions (Paper III).....	30
3.2.2	Malting with antibiotics to restrict microbial growth (Paper IV)....	31
3.2.3	Transformation of barley (Paper V)	31
3.2.4	Characterization of transformed barley (Paper V)	31
3.2.5	Analysis of root growth of <i>vhb</i> -expressing barley (Paper V).....	32
3.2.6	Analysis of ADH activity and endogenous Hb in <i>vhb</i> -expressing barley (Paper V)	32
3.2.7	Statistical analysis (Papers I, II, V)	34
4.	Results and discussion	35
4.1	<i>Hyoscyamus muticus</i> hairy root cultivation.....	35
4.1.1	The effect of heterologous <i>vhb</i> expression on growth and alkaloid production of <i>H. muticus</i> hairy root cultures (Paper I)	35
4.1.2	Medium optimization for <i>vhb</i> -expressing <i>H. muticus</i> hairy roots (Paper II)	37
4.1.3	Effect of <i>vhb</i> expression and aeration on the alkaloid profile of <i>H. muticus</i> hairy roots (Paper II).....	39
4.2	Barley germination	41
4.2.1	Effects of steeping conditions on barley oxygen deficiency, germination and malt quality (Paper III).....	41
4.2.2	Effects of the indigenous microbial community on barley oxygen deficiency, germination and malt quality (Paper IV)....	44
4.2.3	The effect of heterologous <i>vhb</i> expression in barley (Paper V)...	46
5.	Conclusions.....	50
	References.....	52

Appendices

Papers I–V

*Appendices I–V of this publication are not included in the PDF version.
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Abbreviations

ADH	alcohol dehydrogenase
ADH1, ADH2, ADH3	barley alcoholdehydrogenase isoforms 1, 2 and 3
DGGE	denaturing gradient gel electrophoresis
EBC	European Brewery Convention
FTIR	Fourier transform infrared spectroscopy
H6H	hyoscyamine-6 β -hydroxylase
Hb	endogenous barley haemoglobin
MPO	<i>N</i> -methylputrescine oxidase
NAD	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
NAD(P)H	nicotinamide adenine dinucleotide phosphate, reduced form
NO	nitric oxide
PCR	polymerase chain reaction
PMT	putrescine- <i>N</i> -methyltransferase
RIA	radioimmunoassay
6BHH	6 β -hydroxyhyoscyamine
SNP	sodium nitroprusside
VHb	<i>Vitreoscilla</i> haemoglobin
<i>vhb</i>	gene encoding <i>Vitreoscilla</i> haemoglobin

1. Introduction

Oxygen availability is a factor that often limits the desired cellular activities in biotechnological processes. This applies to plant cell cultures as well as to microbial cultures, despite the fact that plant cells have lower oxygen requirements than microorganisms. The large-scale cultivation of aerobic plant cells to high cell density is a challenging task. Plant cells are fragile and prone to hydrodynamic shear damage. Therefore, low mixing speeds are commonly used, which in turn leads to non-uniform mixing and low rates of mass transfer, including oxygen transfer.

Oxygen availability is also considered to be limited in germinating seeds (Bewley and Black 1994). This is mainly a consequence of the covering layers of the seed coat acting as oxygen barriers. Seeds may also be subjected to oxygen deficiency in the soil, as a consequence of water lodging. The malting process exploits the germinative metabolism of cereal grains to produce malt, the main raw material of the brewing process. The process includes immersion of the kernels in water for several hours. During these so-called steeping sessions, oxygen is quickly consumed by the kernel and the indigenous microbial community colonizing the kernel, leading to oxygen deprivation. Oxygen deficiency is therefore an important issue in industrial malting.

This thesis examines the role of oxygen deficiency in two model processes: the liquid-submerged cultivation of *Agrobacterium rhizogenes*-induced hairy roots of the medicinal plant Egyptian henbane (*Hyoscyamus muticus* L., Solanaceae), and the germination of barley (*Hordeum vulgare* L., Poaceae).

1.1 Egyptian henbane (*Hyoscyamus muticus*) as a source of tropane alkaloids

1.1.1 The production of tropane alkaloids by *Agrobacterium rhizogenes* -induced hairy roots

H. muticus is a medicinal plant that produces pharmacologically valuable tropane alkaloids. The main alkaloid is hyoscyamine, but smaller amounts of scopolamine

also occur. Other sources of tropane alkaloids include plants belonging to the genera *Atropa*, *Duboisia* and *Datura*. Tropane alkaloids are anticholinergic agents that affect the parasympathetic nervous system and exhibit a wide range of pharmacological activities. Hyoscyamine and scopolamine are the esters of phenyllactic acid and tropane. The conversion of hyoscyamine to scopolamine via 6- β -hydroxyhyoscyamine (Figure 1) is catalyzed by the enzyme hyoscyamine 6- β -hydroxylase (H6H; see review by Sevón et al. 2001).

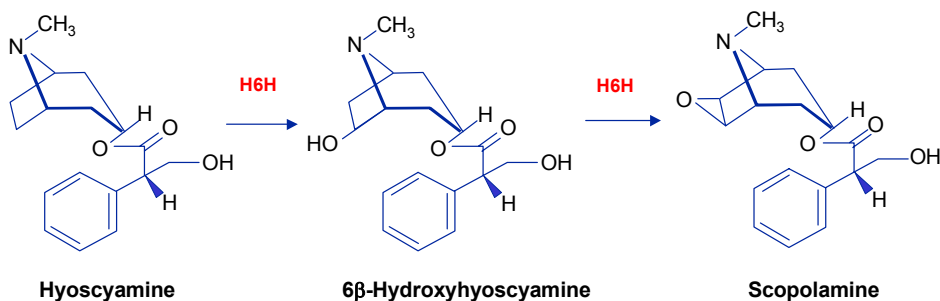


Figure 1. Structure of hyoscyamine, 6 β -hydroxyhyoscyamine and scopolamine.

Tropane alkaloids are among the economically most important pharmaceuticals that are still isolated from plants, as other means of production have not yet become economically feasible (Rates 2001; Raskin et al. 2002). Hairy root cultures offer a potential alternative to isolation from plants. Hairy roots are induced by infection of plants with the soil-borne pathogen *A. rhizogenes*. On infection, part of the DNA (T-DNA) from the Ri- (root inducing) plasmid is transferred from the bacterium into the nuclear genome of the host plant (Chilton et al. 1982). The delivery of the T-DNA into the nucleus of the host plant cell is achieved by the action of the *vir* genes (Sheng and Citovsky, 1996; Zupan et al., 2000). The transformed T-DNA causes the host plant to form adventitious roots at the infection site and to produce opines that serve as nutrients for the bacteria. Hairy root induction and morphology are controlled by the *rol* (A, B, C and D) genes from the *A. rhizogenes* Ri-plasmid (White et al. 1985). The *rol* genes have also been found to affect secondary metabolite production (Sevón et al., 1997; Bonhomme et al., 2000; Bulgakov et al., 2004). Modern *Agrobacterium*-mediated gene transfer to plants utilizes binary vectors in which the T-DNA and the *vir* region can reside on separate plasmids (Hellens et al., 2000).

Transformed roots can be excised and grown *in vitro* as hairy root cultures (Figure 2). *A. rhizogenes*-induced hairy roots generally produce the same alkaloids in similar or higher quantities than intact plants and in significantly greater quantities than undifferentiated plant cells (Jung and Tepfer 1987; Signs and Flores 1990; Sevón and Oksman-Caldentey 2002). Other advantageous characteristics of hairy roots include rapid growth rate, simple medium requirements without phytohormones and high culture stability. Large-scale, high tissue density culture of hairy roots is therefore potentially a very attractive method for commercial production of plant secondary metabolites.

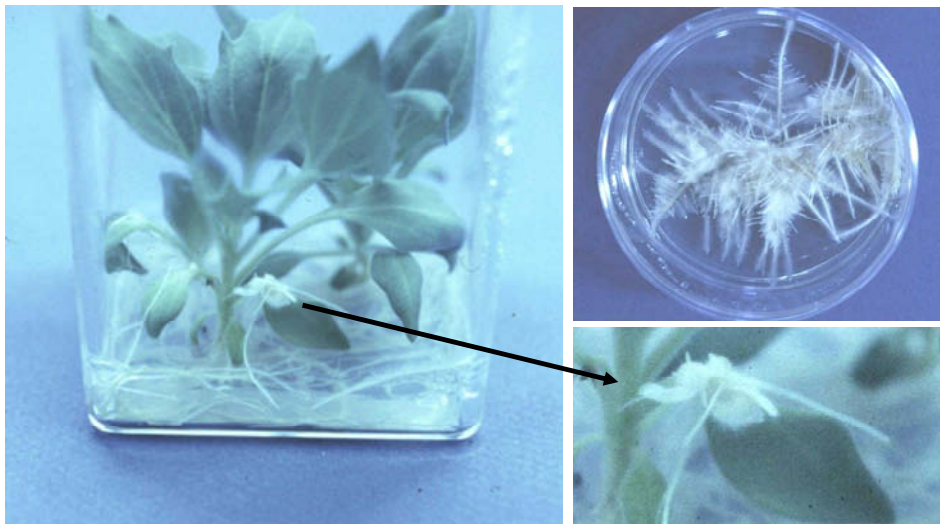


Figure 2. Formation of hairy roots after the infection of *H. muticus* with *A. rhizogenes* (left) and *in vitro* cultivation of hairy roots (top right).

1.1.2 The effect of oxygen on hairy root growth and alkaloid production

Oxygen transfer is poor in root cultures submerged in liquid medium, mainly because the mixing rate must be restricted in order to avoid shear stress (Yu and Doran 1994; Tescione et al. 1997; McKelvey et al. 1993; Williams and Doran 2000). Shear stress is known to alter the morphology of the hairy roots, which usually leads to impaired alkaloid production (Flores et al. 1987). When the

mixing rate is reduced, oxygen and nutrient gradients develop, which in turn may lead to inefficient nutrient uptake and reduced growth and product synthesis rates (Kwok and Doran 1995; McKelvey et al. 1993). A characteristic of hairy root growth is the formation of root clumps, which develop into a tight matrix and remain essentially stationary in the bioreactor (Figure 3), with consequently poor oxygen and nutrient transfer. Hairy roots also require relatively high external critical oxygen tensions for growth and respiration, due to mass transfer resistance within the root tissue (Yu and Doran, 1994).

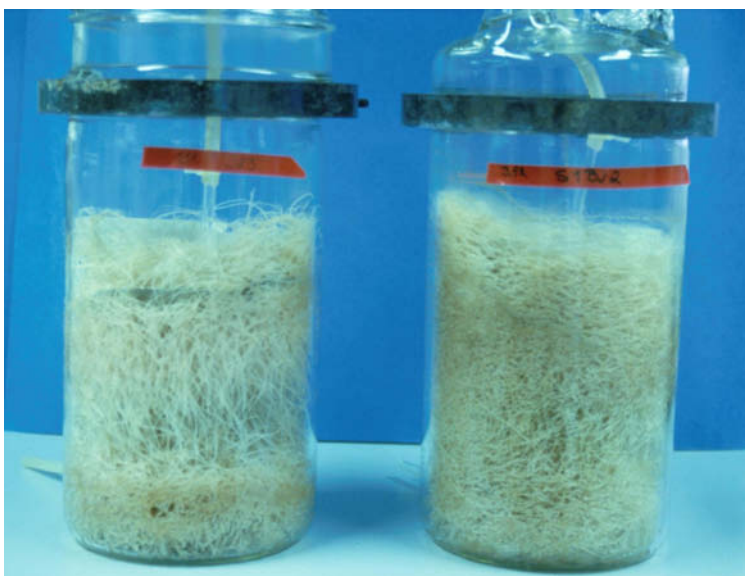


Figure 3. Hairy root matrix after four weeks of cultivation in a 3-litre liquid-submerged bioreactor. VHb-producing line (right) and control (left).

Whereas oxygen deficiency has been shown to limit growth and biomass accumulation of hairy roots even in shake flasks (Kanokwaree and Doran 1997), there is little experimental data available on the effect of oxygen on alkaloid production. Higher oxygen concentration increased ajmalicine production in suspension cultures of *Catharanthus roseus* (Schlatmann et al. 1995) and improved aeration led to higher alkaloid content in cultures of *Berberis wilsonae* (Breuling et al. 1985). Endo and Yamada (1985) suggested that the optimum aeration rate for hyoscyamine and scopolamine production in cultured roots of *Duboisia* may be higher than that for growth. The biosyntheses of hyoscyamine and scopolamine in *H. muticus* have not been totally elucidated, but it is known

that at least two reactions require oxygen: conversion of the hyoscyamine precursor *N*-methyl putrescine to *N*-methylpyrrolinium, catalyzed by the enzyme *N*-methyl putrescine oxidase (MPO), and conversion of hyoscyamine to scopolamine via 6- β -hydroxyhyoscyamine, catalyzed by the enzyme hyoscyamine 6- β -hydroxylase (Sevón *et al.* 2001) (Figure 4).

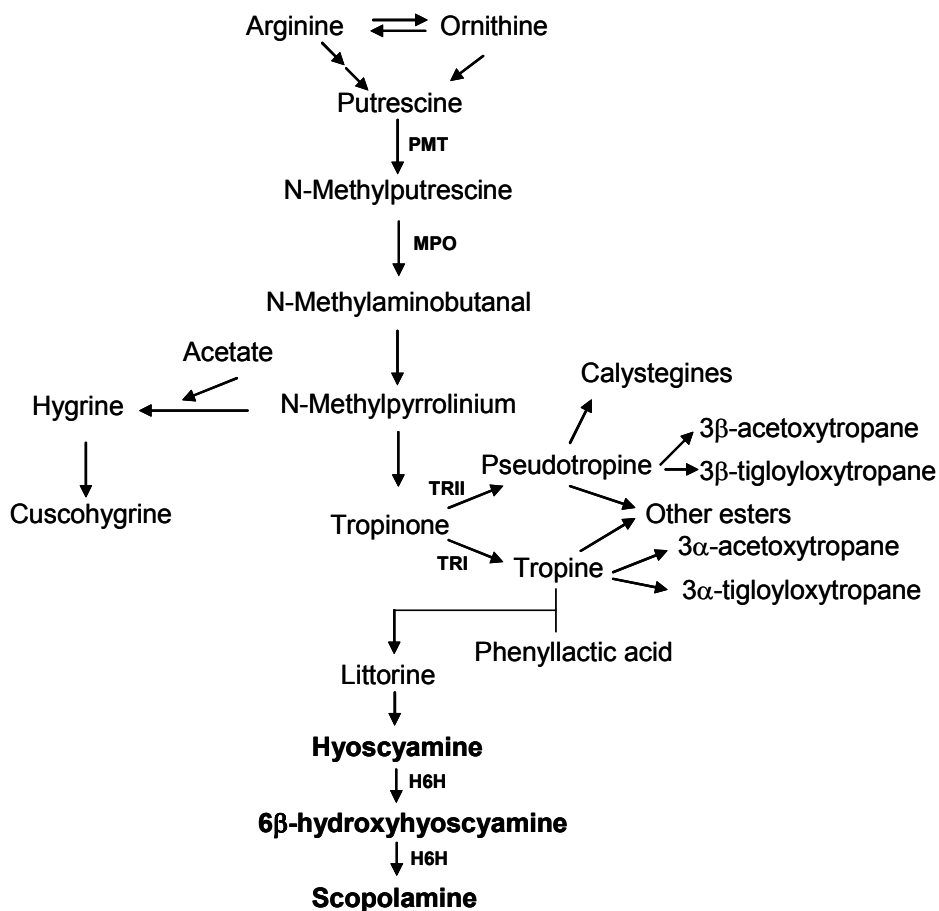


Figure 4. Metabolic pathway of tropane alkaloids. PMT putrescine-*N*-methyltransferase; MPO *N*-methylputrescine oxidase; TRI and TRII tropinone reductases I (tropine-forming) and II (pseudotropine-forming); H6H hyoscyamine-6 β -hydroxylase.

1.2 Malting of barley (*Hordeum vulgare*)

1.2.1 The malting process

Barley is one of the most important grain crops worldwide. It is grown in a range of environments that vary from northern Scandinavia to north-western Africa. Most of the barley is used as animal feed, but some is germinated industrially to produce malt, the main raw material of beer. In 2004, about 22 million tons of barley (14% of the world barley crop) was used for malting purposes (FAO 2007; Braks and Leijh 2005). Barley germination has therefore been subject to extensive research over time.

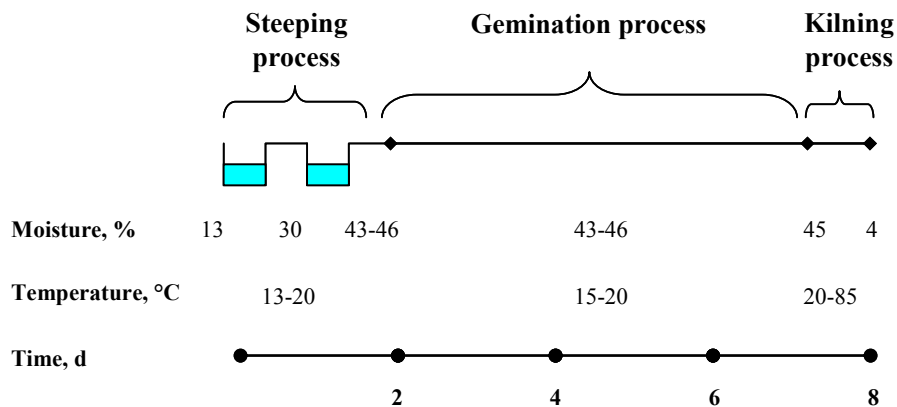


Figure 5. Outline of the malting process.

The aim of barley malting is to convert the hard barley kernels into friable malt containing a package of various amylolytic, glucoamylolytic and proteolytic enzymes. The malting process relies on the metabolic events of germination, which are initiated by hydration of the kernel. Barley is first steeped in water (Figure 5) to raise the moisture content of the grains to at least 43–46% (Hough et al. 1971; Briggs et al. 2004). Steeping involves immersion of the grains in water for several hours twice or three times, usually in cylindroconical tanks. The steep water is generally aerated intermittently. The immersion periods are alternated with so-called air rests, during which the water is drained off, the grain bed aerated and carbon dioxide removed. After steeping, the barley is transferred to a germination box, where it is allowed to germinate and grow for four to six days. During the process, barley aleurone cells produce hydrolytic

enzymes which hydrolyze the cell walls and storage proteins of the starchy endosperm (Figure 6). The moisture content is kept at about 43–46% to avoid excessive growth of the roots and shoot. The barley is then dried in hot air in a step called kilning, and the roots are removed.

According to the correct plant physiological terminology, germination is defined as the chain of events taking place between water uptake and growth of the embryo (Bewley and Black 1994). The first visible sign of completed germination is the protrusion of the testa and pericarp from the growing embryo. The events taking place after this are referred to as post-germinative growth or early seedling development. However, in the malting industry, the term germination is commonly extended also to cover the post-germinative growth (Figure 5). In this thesis ‘germination’ refers to the plant physiological term, and ‘germination process’ to the post-germinative growth step in the malting process.

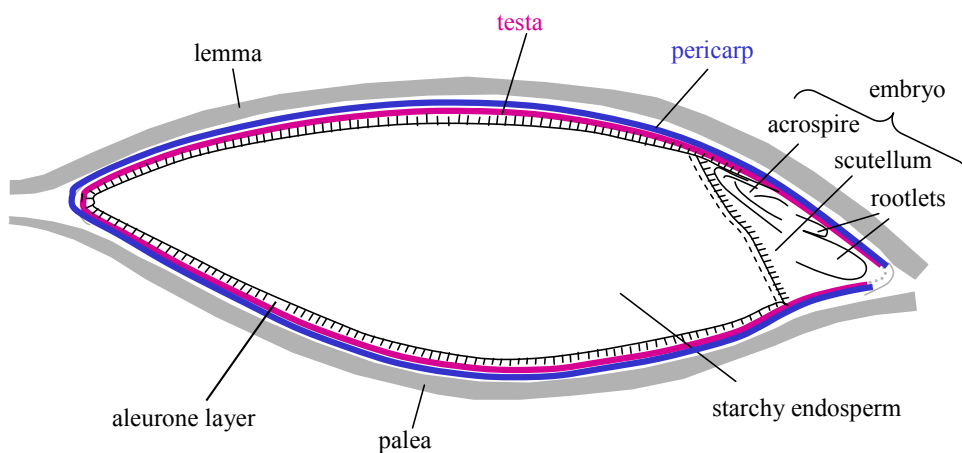


Figure 6. Longitudinal section of the barley kernel.

1.2.2 Oxygen deficiency in barley kernels during malting

The barley embryo is situated at the proximal end of the kernel, under the testa, pericarp and husk (Figure 6). Several findings indicate that the testa and pericarp limit the oxygen availability of the barley embryo and aleurone cells during the time between imbibition and completion of germination (Chapon 1959; Crabb and Kirsop 1969). It has also been suggested that the aerobic microbial

populations normally colonizing the barley kernel may compete with the grain tissues for dissolved oxygen during the steeping phase in industrial scale malting (Doran and Briggs 1993; Kelly and Briggs 1992a). Moreover, oxygen availability may play an important role in seed coat-imposed dormancy, which is the prevailing form of dormancy in cereals (Côme et al. 1984).

Although the barley plant does not cope well with anoxia, it has been demonstrated that barley kernels can survive for days without oxygen (Harberd and Edwards 1982). This survival is largely dependent on the ethanol fermentation pathway, and the existence of the constitutive alcohol dehydrogenase enzyme ADH I (ADH; alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1), which is found in the scutellum, embryo and endosperm of a dry barley kernel. In the ethanol fermentation pathway, pyruvate decarboxylase converts pyruvate to acetaldehyde, which is then converted to ethanol by ADH, with the concomitant regeneration of NAD⁺. This function is vital in avoiding cytoplasmic acidosis and maintaining ATP levels during extended periods of hypoxia (Kennedy et al. 1992; Roberts et al. 1984). When barley is subjected to anoxia, two other ADH enzymes are induced: ADH2 and ADH3 (Harberd and Edwards 1983; Hanson and Brown 1984). The active enzymes are homo- or heterodimers combining the three monomers (ADH1, ADH2, ADH3) into six different isoenzymes that can be separated by non-denaturing gel electrophoresis.

In embryos and aleurone cells the induction of the ADH2 and ADH3 enzymes occurs under anaerobiosis, whereas complete anoxia kills root tissue. The embryo and aleurone cells of barley kernels are therefore well equipped to survive even severe oxygen deficiency. However, as opposed to rice, the barley kernel will not complete germination without oxygen (Perata et al. 1998; Loreti et al. 2002). This may be a consequence of aleurone cells not being able to respond to gibberellic acid in the absence of oxygen (Hanson and Jacobsen 1984). Gibberellic acid -induced changes that do not take place under anoxia include *de novo* production of the starch hydrolysing enzymes α -amylase, limit dextrinase and α -glucosidase (Guglielminetti et al. 1995). As malting relies on the production of these hydrolytic enzymes, a prolonged anoxia in the beginning of germination retards the process.

In industrial malting, the steep water is usually aerated since it is generally accepted that malting should be as aerobic as possible. However, in practice it

has also been claimed that aeration during steeping in fact has only minor effects on malt quality (French and McRuer 1990; Kelly and Briggs 1992b; Hariri et al. 2001). It is questionable whether the air introduced into a steep actually reaches the embryo before protrusion of the covering layers has occurred. Whereas the principles of aerobic steeping were discovered long ago, the effects of different malting procedures on the physiology of barley germination have not been investigated in depth.

A diverse microbial community is associated with the barley grain, and consists of various types of bacteria, yeasts and filamentous fungi (Noots et al. 1999; Petters et al. 1988). Therefore, malting can be considered as a complex ecosystem involving two metabolically active groups: the germinating grains and the diverse microbial community. The majority of the microbial community naturally present on the barley kernel is located between the husk and testa (Petters et al. 1988; Sarlin et al. 2005). It has been proposed that microbes may inhibit germination by competing with the embryo for oxygen (Doran and Briggs 1993; Harper and Lynch 1979; Harper and Lynch 1981; Lynch and Prynne 1977). Dormant kernels are considered to be especially vulnerable to microbial competition (Doran and Briggs 1993; Kelly and Briggs 1992a). However, as van Campenhout and her coworkers pointed out, the existence of microbial respiratory activity does not necessarily mean that there is actually competition for oxygen between plant tissues and microbes (Van Campenhout et al. 1998; 1999).

1.3 Nitric oxide (NO) in plants

The diverse roles of NO in plants have received much attention recently (Igamberdiev and Hill 2004; Delledonne 2005). NO is a highly reactive and toxic atmospheric trace gas that can diffuse through membranes due to its lipophilic nature. The half-life of NO in biological tissue is estimated to be < 6s (Thomas et al. 2001). NO reacts directly with metal complexes and other radicals, and indirectly as a reactive nitrogen species with DNA, proteins and lipids (Wink and Mitchell 1998). In aqueous environments it reacts with oxygen, producing nitrate and nitrite. There are two known enzymatic routes for NO synthesis in plants: by NO synthase from arginine (Cueto et al. 1996; Durner et al. 1998; Ribeiro et al. 1999) and by nitrate reductase from nitrate via nitrite (Stöhr et al. 2001). Recently, Bethke et al. (2004) demonstrated that NO can also

be produced non-enzymatically by the reduction of apoplastic nitrite in barley aleurone cells.

NO is produced in plant cells during hypoxic conditions (Dordas et al. 2003a; Dordas et al. 2004), including seed germination (Simontacchi et al. 2004; Desel and Krupinska 2005; Sarath et al. 2006). Several recent publications propose a role for NO in seed germination. The NO-donor sodium nitroprusside (SNP) has been shown to break seed dormancy in *Arabidopsis thaliana* seeds (Libourel et al. 2006) and to promote the germination of lettuce (*Lactuca sativa* L.) seeds (Beligni and Lamattina, 2000) and warm-season grasses (Sarath et al. 2006). NO has also been proposed to act as a signal molecule in various other physiological processes including plant defence against microbial pathogens (Delledonne et al. 1998; Durner et al. 1998), root organogenesis (Ribeiro et al. 1999), maturation and senescence (Leshem et al. 1998). Recently, Correa-Aragunde et al. (2006) reported that NO modulated the expression of cell cycle regulatory genes in tomato (*Solanum lycopersicum* L.).

1.4 Haemoglobins in plants and bacteria

1.4.1 The role of haemoglobins in oxygen deficient plant tissues

Various plant species, including barley (Duff et al. 1997; Taylor et al. 1994), rice (*Oryza sativa*) (Arredondo-Peter et al. 1997; Lira-Ruan et al. 2001), alfalfa (*Medicago sativa*) (Seregélyes et al. 2000) and *Arabidopsis* (AHb1) (Trevaskis et al. 1997), express haemoglobins in response to oxygen deficiency. These stress-induced haemoglobins are classified as non-symbiotic class 1 haemoglobins. They differ from the non-symbiotic class 2 haemoglobins with respect to their oxygen-binding properties and ways of induction. They have an extremely high affinity for oxygen due to their slow rates of oxygen release (Table 1). Class 1 non-symbiotic haemoglobins are induced by hypoxic stress (Taylor et al. 1994; Trevaskis et al. 1997; Lira-Ruan et al. 2001), oversupply of nitrate, nitrite and NO (Wang et al. 2000; Sakamoto et al. 2004) and deficiency of phosphorus, potassium and iron (Wang et al. 2003). Non-symbiotic class 2 haemoglobins (e.g. *Arabidopsis* Hb2) are inducible by cold (Trevaskis et al. 1997) or cytokinins (Hunt et al. 2001). However, recent studies indicate that the functions of these two classes may slightly overlap (Ross et al. 2004). The oxygen binding properties of

non-symbiotic class 2 haemoglobins are similar to those of the symbiotic haemoglobins, such as soybean leghaemoglobin (Table 1) (Appleby et al. 1983; Gibson et al. 1989). The symbiotic haemoglobins were the first plant haemoglobins to be discovered. They regulate the oxygen supply to nitrogen fixing bacteria in the nodules of plant roots (Appleby 1984). Recently, a third group of plant haemoglobins, the truncated haemoglobins, was identified. The functional roles of these haemoglobins are still not well known (Wittenberg et al. 2002).

Haemoglobins are most commonly known for their ability to act either as oxygen (O₂) carriers or stores to facilitate O₂ delivery, but they are also capable of regulating NO. The class 1 non-symbiotic as well as the truncated haemoglobins have been shown to be capable of modulating the levels of NO and protecting aerobic respiration from NO inhibition (Dordas et al. 2003a; Dordas et al. 2004; Ouellet et al. 2002). Alfalfa root cultures expressing non-symbiotic barley haemoglobin were found to accumulate significantly less NO than an antisense control line (Dordas et al. 2003a). In microorganisms, NO is scavenged by flavohaemoglobins possessing NAD(P)H-dependent NO-scavenging activity (Gardner et al. 1998). Similarly, it has been proposed that the non-symbiotic barley haemoglobin acts as a dioxygenase converting NO to nitrate, consuming NAD(P)H (Dordas et al. 2003b). The expression pattern and oxygen binding properties of the non-symbiotic class 1 haemoglobins support this theory.

Table 1. Oxygen association constants (k'_{on}) and dissociation constants (k_{off}) of various plant and microbial haemoglobins.

Haemoglobin	k'_{on} ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	Reference
Barley	9.5	0.027	a
Rice	68	0.038	b
Arabidopsis Hb1	74	0.12	c, d
Arabidopsis Hb2	1.1	0.14	c
	86	0.14	d
Arabidopsis Hb3	0.2	0.3	d
Soybean leghaemoglobin	120	5.6	e, f
<i>Vitreoscilla</i>	200	4.2 ^h ; 0.15 ⁱ	g

^aDuff et al. 1997; ^bArredondo-Peter et al. 1997; ^cTrevaskis et al. 1997; ^dWatts et al. 2001; ^eGibson et al. 1989; ^fAppleby et al. 1983; ^gGiangiacoimo et al. 2001; ^hfast rate of biphasic reaction counted for 15% of the reaction; ⁱslow rate of the biphasic reaction.

1.4.2 Heterologous expression of *Vitreoscilla* haemoglobin

The problems of oxygen deficiency in hairy root cultures can be tackled by applying novel and advanced bioreactor design methods and techniques (Nuutila et al. 1997; Palazón et al. 2003; Sung and Huang 2006). Correspondingly, oxygen deprivation in the malting process has been dealt with by introducing air and adding hydrogen peroxide into the steeping water (Briggs 1987). In hairy roots, another approach to alleviate the problem of poor oxygen transfer was demonstrated by Shiao et al. (2002). They engineered *Arabidopsis thaliana* hairy roots to overexpress two enzymes of the ethanol fermentation pathway: ADH and pyruvate decarboxylase (PDC). Transformed root lines were able to maintain high growth rates even at low oxygen saturation levels, whereas the growth rates of control hairy root cultures declined significantly.

A different metabolic engineering approach to alleviate the adverse effects of inadequate oxygen availability on bioprocess productivity was first demonstrated by Khosla and Bailey (1988). Their approach was based on the observation that the Gram-negative bacterium *Vitreoscilla* synthesizes large amounts of haemoglobin (VHb) when growing in oxygen-limited conditions. Heterologous expression of *Vitreoscilla* haemoglobin (VHb) has been shown to improve the growth properties and productivity of various microorganisms, as summarized previously (Frey and Kallio 2003). VHb production was particularly beneficial to mycelium-forming microorganisms, which were grown in highly viscous media in which oxygen transfer was limited.

The mechanisms of VHb function in heterologous hosts are not yet fully characterized. The beneficial effects of VHb were originally attributed to its ability to increase the concentration of intracellular oxygen, defined as the sum of the concentrations of free and haemoglobin-bound oxygen in microaerobic conditions (Kallio et al. 1994). This increase is thought to shift the relative activity of the terminal oxidase to the energetically more favourable enzyme cytochrome bo_3 . VHb production has indeed been shown to increase the amount (5-fold) and activity (50%) of cytochrome bo_3 in microaerobic *Escherichia coli* (Tsai et al. 1996). Although several findings indicate that *vhb* expression improves cellular respiration and thus the efficiency of energy generation during oxygen-limited growth, it has recently been speculated that VHb is unlikely to have an oxygen-delivering role, based on a re-evaluation of the rates of oxygen

binding and release (Table 1; Giangiacomo et al. 2001). Recent studies suggest that a more relevant physiological function of VHB, as well as other bacterial globins, is ·NO dioxygenase activity (NOD), which protects cells against nitrosative stress (Frey et al. 2002; Frey et al. 2004; Gardner 2005).

The *vhb* gene has been transferred to and expressed in plants and plant cell cultures. VHB-producing tobacco (*Nicotiana tabacum*) was reported to have accelerated germination and growth, and an increase in both chlorophyll and nicotine content (Holmberg et al. 1997). Li et al. (2005) found that *vhb* expression improved germination in cabbage (*Brassica oleracea* var. *Cabitata*). In-depth follow-up studies (Frey et al. 2004) have failed to reproduce the marked effects on germination and growth in tobacco obtained by Holmberg et al. (1997). However, tobacco suspension cultures showed a shorter lag phase and improved growth at low agitation rates (Farrés and Kallio 2002). VHB production has also been shown to protect tobacco suspension cultures against chemically induced nitrosative stress (Farrés and Kallio 2002; Frey et al. 2004). In *vhb*-expressing *Populus tremula* x *tremuloides* (hybrid aspen), no significant differences were observed in elongation growth or any other phenotypic characteristics under standard greenhouse conditions (Häggman et al. 2003). However, the leaves of *vhb*-expressing plants had a significantly higher starch content relative to controls, indicating a more efficient energy household.

2. The aims of the present study

The main aim of this work was to improve the understanding of the role of oxygen availability in two bioprocesses: the cultivation of *Agrobacterium rhizogenes*-induced hairy roots of the medicinal plant *Hyoscyamus muticus* (Egyptian henbane), and *Hordeum vulgare* (barley) malting. This was accomplished by transferring the *vhb* gene from *Vitreoscilla* to *H. muticus* and to barley with the specific goals of improving the growth and alkaloid production of *H. muticus* hairy roots and the germination rate of barley.

Secondly, the aim was to verify the hypothesis that barley embryos suffer from oxygen deficiency in the malting process, regardless of aeration. This was achieved by studying the effects of aeration on the production of the inducible isoenzymes ADH2 and ADH3, ADH activity, ethanol production and α -amylase activity.

A further aim was to verify the hypothesis that microbes naturally present on the barley kernel can slow down germination by competing for oxygen with the embryo. This was accomplished by studying the ethanol production and ADH and α -amylase activity of barley kernels with modified microbial populations.

3. Materials and methods

Materials and analytical methods are presented in detail in Papers I–V.

3.1 *Hyoscyamus muticus* hairy root cultivation

3.1.1 Transformation of *H. muticus* (Paper I)

VHb-producing hairy root cultures were obtained by transformation of young *H. muticus* leaves or whole plants with *A. rhizogenes* carrying the *vhb* gene under the regulation of the CaMV 35S promoter as described in Paper I (vector pBVHb). Control plants were obtained by infecting *H. muticus* with *A. rhizogenes* carrying the *uidA* gene under the regulation of the CaMV 35S promoter (vector pBI121). The hairy roots that were formed at the infection sites (Figure 2) were excised and cultured on solid medium in illuminated conditions. Three to four months later, roots were transferred to the same medium without casein and with 50% of the original macro- and microsalt content in order to minimize callus formation. The presence of the transferred *vhb* gene was analyzed using polymerase chain reaction (PCR). Southern blot analysis was performed to confirm integration of the gene into the genome. The production of VHb protein was determined using Western blot analysis.

3.1.2 Characterization of transformed hairy roots (Paper I)

The nine root lines that produced VHb protein and the ten control root lines were transferred to liquid medium in 100 ml Erlenmeyer flasks. For initial characterization, the cultures were incubated for 4 weeks on an orbital shaker. The harvested roots were collected by filtration, washed with water and weighed immediately (fresh weight), and subsequently freeze dried and weighed (dry weight). The hyoscyamine content was determined from crude methanol extracts using radioimmunity (RIA).

Based on the results of the initial characterization, six representative lines (three *vhb* lines: V1, V2, V14 and three controls: C16, C23, C51) were selected and

grown in order to define growth rates. The roots were cultured as in the initial characterization and harvested after 4, 7, 11, 14, 21 and 28 days and weighed, dried and analyzed as described above. The 28-day growth experiment was repeated one year later using the same six lines in order to test the stability of the lines with respect to growth and hyoscyamine production.

3.1.3 Growth medium optimization (Paper II)

A growth medium optimization experiment was designed using a central composite rotatable design for four variables: sucrose, phosphate, nitrate and ammonium content of the medium. The design resulted in 25 points representing different media compositions and six replicates at the centre point (Paper II; Table 2). One Vhb line (V2) and one control line (C23) were selected for the medium optimization experiment. The cultures were incubated for 28 days on an orbital shaker. The hyoscyamine content was determined from crude methanol extracts using RIA.

Second order polynomial mathematical models describing the effects of the selected variables on dry weight, hyoscyamine content and volumetric hyoscyamine production were derived from the experimental results using multiple regression analysis (Statgraphics® Plus, Version 3, Manugistics Inc., 1997). Based on the results of the medium optimization experiment, a new medium composition (“optimized medium”) was selected for subsequent cultivations of Vhb and control lines in 100 mL flasks and a bioreactor. In order to test the optimized medium and to study the effect of *vhb* expression on the alkaloid profile, three Vhb lines (V1, V2, and V14) and three controls (C16, C23 and C51) were cultivated in 100 mL flasks. The cultivation was performed as in the medium optimization experiment but using only the optimized medium. Roots were cultured for 21 or 28 days.

3.1.4 Cultivation in a bioreactor (Paper II)

One Vhb line (V2) and one control line (C23) were cultivated in a bioreactor (Figure 3) in order to study the effects of cultivation conditions on alkaloid production and growth of the selected hairy root cultures. An inoculum of 17.5 g

f.w. was used in 3.5 L of medium. Growth medium samples were taken during cultivation for analysis of the sucrose, glucose and fructose, phosphate, nitrate and ammonium concentrations as described in Paper II. After 28 days, the roots were removed from the bioreactor, washed with water, dried on filter paper, lyophilized, and weighed. The alkaloid contents of the roots were analyzed by gas chromatography-mass spectrometry.

3.1.5 Cultivation with and without aeration (Paper II)

In order to study the effect of aeration on the alkaloid profile, two VHB lines (V1 and V2) and two controls (C16 and C23) were cultured in triplicate in 100 mL flasks on an orbital shaker with or without aeration. The cultivation time was limited to 14 days in order to minimize the effect of evaporation in aerated flasks.

3.2 Barley germination

3.2.1 Malting with different steeping conditions (Paper III)

Barley samples of the cultivar Scarlett were malted in a specially designed, computer controlled micro-malting equipment as described in Paper III. In order to subject the barley to oxygen deficiency, samples were sparged with nitrogen gas during the first steep, during the first 8 h of the air rest or during the last 8 h of the air rest. To mimic unevenly distributed aeration in industrial malting conditions, one sample was left unaerated during the air rest to allow the concentration of carbon dioxide in the malting drum to increase. One sample was steeped in hydrogen peroxide (H₂O₂) solution (with aeration) to apply oxidative stress. The different treatments were compared to a continuously aerated control. A Fourier Transform Infrared Spectroscopy (FTIR) multicomponent gas analyzer Gasmeter® (Temet Instruments Ltd, Helsinki, Finland) was used to monitor the ethanol and carbon dioxide concentrations in the head space of each malting drum during the process. The percentage of germinated kernels was calculated daily. Samples were taken during malting for ADH activity assay, separation of isoenzyme dimers by native gel electrophoresis and α -amylase activity analysis. Malt samples were analyzed using the following EBC

recommended methods: fine/coarse extract, friability, soluble nitrogen and free amino nitrogen (Analytica EBC, 1998).

3.2.2 Malting with antibiotics to restrict microbial growth (Paper IV)

Barley samples of the cultivar Scarlett were malted in a specially designed, computer controlled micro-malting equipment as described in Paper IV. The barley samples were steeped in water or in water containing antimicrobials or H₂O₂. The concentration of ethanol in the head space of each malting drum was analyzed continuously using an FTIR multicomponent gas analyzer Gasmeter® (Temet Instruments ltd, Helsinki, Finland). The number of germinated kernels was counted daily until at least 90% had germinated. Samples were taken during malting for ADH and α -amylase activity and microbial analyses. The following microbial groups were analyzed from homogenized barley samples by cultivation on specific media: aerobic heterotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria, and yeasts. Moreover, the bacterial population was analyzed with PCR-DGGE (Denaturing Gradient Gel Electrophoresis) using universal bacterial primers.

3.2.3 Transformation of barley (Paper V)

The *vhb* gene was cloned into the plant expression vector pAHC25 as described in Paper V. Barley was transformed using particle bombardment with the procedure of Wan and Lemaux (1994) as described in Paper V. Putative transgenic regenerants were initially screened for the presence of the transgene by PCR. Stable integration of the gene was confirmed by Southern blot hybridization. The production of VHB protein was verified using Western blot analysis.

3.2.4 Characterization of transformed barley (Paper V)

The germination conditions for the transgenic and control seeds from T0 plants were chosen to mimic industrial malting practice in miniature scale. After germination, the plantlets were potted in soil mix and grown in a greenhouse. The time from immersion to completion of germination was recorded and the

length of the main stem and the total amount of stems were monitored. The germination experiment was repeated with seeds from the T1 and T2 plants. The inheritance of the *vhb* gene was confirmed by PCR, and production of VHB protein production was verified by Western blot analysis. The negative segregants of each line were used as controls.

3.2.5 Analysis of root growth of *vhb*-expressing barley (Paper V)

Root growth was analysed in two growth conditions: 1) in test tubes on solid rooting medium, in darkness and 2) in vermiculite in greenhouse conditions as described in Paper V. For growth in test tubes, kernels representing the VHB-positive lines 3b and 32h and their respective negative segregants were surface sterilized and placed on solid rooting medium in test tubes. The plant was harvested when the leaf had reached the top of the test tube. The roots and leaves were measured, excised and weighed. For growth in vermiculite, kernels were planted in plastic pots containing vermiculite, moisturised with growth medium. After 7–9 days, the plants were carefully removed from the vermiculite, washed with water and dried on filter paper. The roots and leaves were measured, excised and weighed. VHB production was confirmed using Western blot analysis.

3.2.6 Analysis of ADH activity and endogenous Hb in *vhb*-expressing barley (Paper V)

Kernels of VHB-positive lines 3b and 32h and their respective negative segregants were allowed to germinate in petri dishes on moist filter paper as described in Paper V. The dishes were incubated for three days with illumination. In these conditions, only a portion of the kernels germinated within three days. After three days, the embryos of the kernels that had not germinated were excised and used for ADH activity, protein content and Western blot analysis. The ADH activity was also analyzed from quiescent embryos. These embryos were cut from the dry kernels and analyzed as described above.

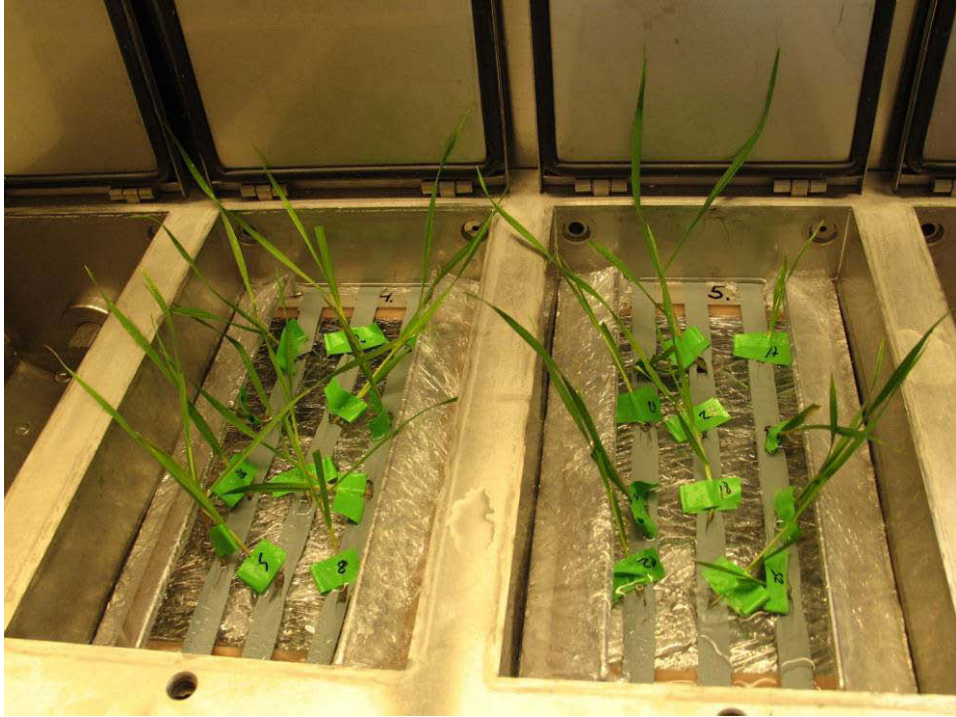


Figure 7. Barley plants were immobilized so that the roots were in growth medium sparged with N_2 gas and the stem and leaves were in air at room temperature.

For analysis of barley Hb expression during germination and early growth, barley kernels were steeped in conditions chosen to mimic industrial malting practice as described in Paper V. After steeping, seeds were placed on moist filter paper on Petri dishes, in darkness for three to four days, until the kernels had germinated and developed three to four rootlets. The embryos and roots were excised and used for Western blot analysis.

For analysis of hypoxic response, kernels were grown in vermiculite and growth medium as described in Paper V. Three weeks after sowing, the plants were carefully removed from the vermiculite and quickly rinsed in water. The plants were then immersed in a water bath containing growth medium sparged with nitrogen gas for oxygen removal. The plants were immobilised so that the roots stayed beneath and the leaves and stem above the liquid surface (Figure 7). After seven hours, the plants were removed from the bath and analyzed for ADH

activity, Vhb and Hb expression. Plants harvested three weeks after sowing without anoxic treatment were used as controls.

3.2.7 Statistical analysis (Papers I, II, V)

The statistical significance of the difference between the results of the *vhb*-lines and those of the controls was calculated using Statgraphics® Plus 5.0 (Statistical Graphics Corp.).

4. Results and discussion

4.1 *Hyoscyamus muticus* hairy root cultivation

4.1.1 The effect of heterologous *vhb* expression on growth and alkaloid production of *H. muticus* hairy root cultures (Paper I)

The *vhb* gene was introduced into *H. muticus* with the ultimate aim of improving growth and alkaloid production of hairy root cultures. Altogether 39 and 11 hairy root lines were obtained after transformation of *H. muticus* with *A. rhizogenes* carrying either the pBVHb or the pBI121 (control) vector, respectively. In 36 lines, the presence of the *vhb* gene was confirmed with PCR; production of VHb protein was detected in 17 lines using Western Blot analysis. Stable integration was confirmed using Southern blot analysis.

Compared to the controls, a large proportion of the *vhb*-expressing hairy root lines were prone to callus formation (Table 2), indicating that *vhb* expression may affect root organogenesis. This can have implications in two ways. First, undifferentiated callus does not in general produce as high levels of alkaloids as hairy roots (Table 2; Sevón et al. 1998). Second, the optimal growth conditions of callus may be different from those of hairy roots. The presence of the *rol* genes (A, B and C) from *A. rhizogenes* was assayed by PCR from the root lines because these genes are known to affect root morphology (White et al. 1985). However, all three *rol* genes (A, B and C) were detectable in all lines except one *vhb* line that lacked the *rolB* gene. Therefore, the presence or absence of the *rol* genes could not explain callus formation.

It has recently been shown that VHb has ·NO dioxygenase activity (NOD), and can thereby protect cells against nitrosative stress (Frey et al. 2002; Frey et al. 2004). However, NO is also an important signal molecule in plants (reviewed by Stöhr and Stremlau 2006; Delledonne 2005; Mur et al. 2006), and constitutive *vhb* expression may therefore affect NO-dependent functions in plants. NO induced lateral root formation by mediating the induction of the *cyd3;1* (cyclin) gene and by repression of a *cdk* (cyclin dependent kinase) inhibitor at the beginning of lateral root primordial formation. Moreover, Pagnussat et al. (2002) have shown that NO is required for root organogenesis in cucumber (*Cucumis*

sativus L.). The fact that constitutive *vhb* expression led to an increase in callus formation in *H. muticus* hairy roots suggests that NO may also regulate hairy root organogenesis.

Table 2. Biomass production and hyoscyamine contents of VHb-expressing and control lines with and without the tendency to form callus.

	Number of lines	Biomass (g d.w.)	Hyoscyamine content (mg/g d.w.) (mg/L)	
Normally growing controls	9	0.186	1.44	13.5
Callus-forming controls	1	0.235	0.00	0.0
Normally growing VHb-lines	9	0.220	1.75	19.4
Callus-forming VHb-lines	8	0.200	0.40	4.1

The growth properties and hyoscyamine production of callus-negative *vhb*-expressing lines and controls were compared after 4 weeks of growth in liquid growth medium. In these conditions, the expression of *Vitreoscilla* haemoglobin was found to improve the growth of *H. muticus* hairy roots. The dry weights of the roots expressing the *vhb* gene were on average 18% higher than those of the controls. Both VHb lines and controls were stable with respect to biomass formation over a time period of three years. Reduced lag phase, previously observed in *vhb*-expressing tobacco cell suspension cultures (Farrés and Kallio 2002), was not observed in VHb-producing *H. muticus* hairy root cultures (Paper I; Figure 4). VHb-production did not increase the specific hyoscyamine production (mg/g d.w.). Although VHb production did not have a statistically significant effect on hyoscyamine production (mg/l) due to the wide somaclonal variation, the highest production levels (31–34 mg/l) were detected among the VHb-producing lines. The most productive VHb-lines achieved high production levels mainly through high biomass production, whereas the control lines achieved their highest production levels through a high specific hyoscyamine production (mg/g d.w.).

Hitherto, haemoglobin genes have not been detected in *H. muticus*, but since class-1 haemoglobins are now believed to be ubiquitous in plants (Perazzolli et al. 2006), *H. muticus* is likely to have one or several haemoglobin genes. If so,

the expression of these genes may be affected by *vhb* expression as was the case for barley (see section 4.2.3).

4.1.2 Medium optimization for *vhb*-expressing *H. muticus* hairy roots (Paper II)

In order to evaluate whether one or more of the main nutrients sucrose, ammonium, nitrate or phosphate were limiting either growth or hyoscyamine production in the previous experiments (section 4.1.1), one VHb line (V2) and one control (C23) were selected for a medium optimization experiment. Second-order polynomial mathematical models describing the effects of the nutrient concentrations on growth and hyoscyamine production were derived from the results. In both lines, the sucrose concentration of the medium had opposite effects on biomass accumulation and specific hyoscyamine production (mg/g d.w.): maximum biomass accumulation was achieved with the highest sucrose concentration, whereas maximum hyoscyamine content was achieved with the lowest sucrose concentration. Similar results were obtained by Oksman-Caldentey et al. (1994). High sucrose content may lead to high glycolysis and respiration rates with accompanying enhanced biomass production, thus overriding the secondary metabolite production.

The optimum nitrate concentration for growth was slightly higher for the VHb line (3125 mg/l) than for the control (2732 mg/l), although both lines used nitrate at similar rates (Paper II; Figure 3). In anoxic maize cell suspension cultures, Dordas et al. (2004) showed that NO was derived from nitrate in the medium. Moreover, *Arabidopsis* plants have been shown to express a non-symbiotic haemoglobin gene during exposure to nitrate ions (Wang et al. 2000). Thus, it appears that exposure to high concentrations of nitrate may require haemoglobin to protect the cells from excess NO. It can therefore be speculated that *vhb*-expressing *H. muticus* root cultures may tolerate higher concentrations of nitrate than the control, due to the detoxifying effect of VHb on nitrate-derived NO.

Based on the medium optimization experiment, an optimized medium was chosen for subsequent shake flask and bioreactor cultivations. Based on visual observations during cultivation, the VHb lines grew faster than the controls in

both conditions. Most of the VHb roots and/or media turned brown during the 28-day experiments, whereas the controls remained yellowish throughout the experiment (Figure 8). The faster growth of the VHb lines was confirmed by faster nutrient usage in the bioreactor experiment (Paper II; Figures 3 and 4). However, the cultivations also revealed that the medium optimization was more successful for the controls than for the VHb lines. In bioreactor conditions, the hyoscyamine production (mg/l) of the control was 48% of the predicted value, whereas that of the VHb line was 33%. The controls also performed better than the VHb lines in shake flasks. The VHb lines may have been limited by the low sucrose content or by the concentration of some other nutrient that was not optimized in this study. In the previous experiment (4.1.1), VHb lines achieved high production rates (mg/l) in particular through high biomass production, which was restricted in the optimized medium. Interestingly, equations 1 and 2 (Paper II; Table 3) predict that a lowering of the medium sucrose content below 30 g l^{-1} would impair biomass accumulation of the VHb line more strongly than that of the control line. At sucrose concentrations above 30 g l^{-1} better biomass accumulation was predicted for the VHb line than for the control.

Whereas the results clearly show that *vhb*-expression accelerated nutrient usage and growth of *H. muticus* hairy roots, they also point out the difficulty in optimizing medium components for hairy roots. Individual hairy root clones may have different optimum concentrations of sucrose and minerals, making medium optimization a very difficult task.

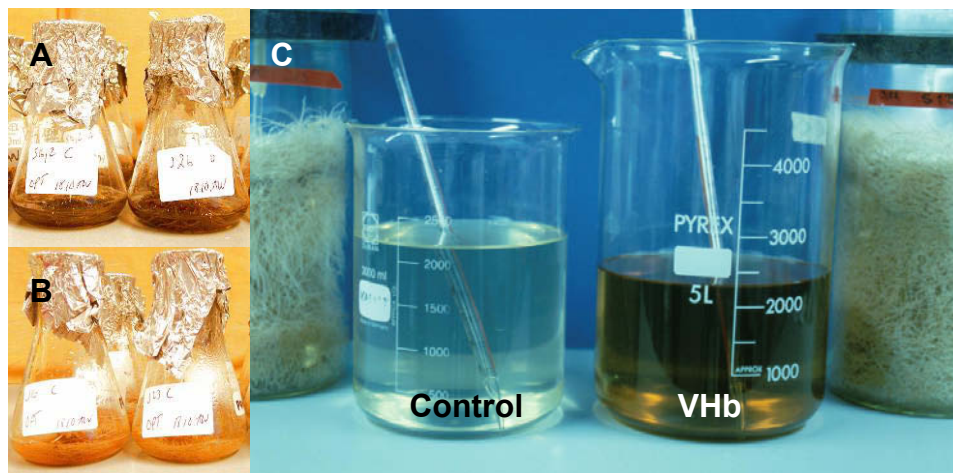


Figure 8. In shake flask cultivation, the faster growing Vhb roots (A) turned brown after 28 days of cultivation, whereas the controls (B) remained yellow. In bioreactor cultivation the growth medium of vhb-expressing roots (C, right) turned brown, whereas the roots remained yellow in both lines.

4.1.3 Effect of vhb expression and aeration on the alkaloid profile of *H. muticus* hairy roots (Paper II)

One aim of the present study was to explore the effect of vhb expression on the alkaloid production of *H. muticus* hairy roots. Apart from the dominating alkaloid hyoscyamine, several of its biosynthetic precursors as well as different tropane alkaloids were also identified and quantified in both shake flask and bioreactor cultivations (Paper II; Table 5). All of these alkaloids except 6 β -hydroxyhyoscyamine have also been detected in *Datura innoxia* plants (Witte et al. 1987). Moreover, pseudotropine, 3 α -acetoxytropine, cuscohygrine and 6 β -hydroxyhyoscyamine have also been detected in *Atropa belladonna* (Hartmann et al. 1986). Four alkaloids that could not be identified with certainty were also quantified (UI1–3 and alkaloid 414). UI1 could possibly be a tropine derivative, since according to Witte et al. (1987) both tropine and pseudotropine also have the main fragment $m/z = 82$. The fragment 84 of UI2 indicates that this alkaloid most probably contains an *N*-methylpyrrolidinyl moiety, whereas UI3 has a very hyoscyamine-like mass spectrum.

Table 3. The effect of *vhb*-expression and aeration on biomass formation (g d.w.) and alkaloid contents (mg/g d.w.) of *H. muticus* hairy roots grown in optimized medium in shake flasks (Paper II).

	<i>vhb</i> -expression	Aeration
Biomass formation	+ 14%	+ 12–29%
3 β -acetoxytropane	- 79%	no effect
(<i>N</i> -methyl-pyrrolidinyl)hygrine	- 74%	- 48–59%
Cuscohygrine	no effect	+ 101–176%
Alkaloid 414 ^a	no effect	- 11–67%
UI1 ^b	- 69%	+74–179 (<i>vhb</i> -lines) +20–56% (controls)
UI2 ^c	- 75%	+80–81% (<i>vhb</i> -lines) +38–40% (controls)

^aalkaloid 414 (m/z (rel.int.) = 84 ([M⁺], 100), 124 (51), 94 (10))

^bUI1 (m/z (rel. int.) = 197 ([M⁺], 1), 82 (100), 140 (71))

^cUI2 (m/z (rel. int.) = 222 ([M⁺], 1), 84 (100), 82 (47))

Heterologous *vhb* expression affected the alkaloid profile of *H. muticus* hairy root cultures. In the 21-day shake flask cultivation, all three VHB lines had lower concentrations of 3 β -acetoxytropane, (*N*-methylpyrrolidinyl)hygrine and two unidentified alkaloids UI1 and UI2, than the control lines (Table 3). The position and role of these alkaloids in the biosynthetic pathway are not known. Interestingly, when the control line C23 was cultivated in a bioreactor, the concentrations of 3 β -acetoxytropane, (*N*-methylpyrrolidinyl)hygrine and the unidentified alkaloid UI2 decreased to the same level as for the VHB lines. Therefore, these alkaloids were affected by *vhb* expression as well as cultivation conditions. The alkaloid profiles of the VHB line and the control were similar when cultivated in bioreactors.

Both VHB lines and controls responded in the same way to aeration in shake flasks. After 14 days of cultivation, the aerated samples had higher concentrations of cuscohygrine and two unidentified alkaloids (UI1 and UI2) and lower concentrations of (*N*-methylpyrrolidinyl)hygrine and alkaloid 414 than the unaerated samples (Table 3). Cuscohygrine biosynthesis diverts from the hyoscyamine synthesis pathway and is synthesized from *N*-methylpyrrolinium

via hygrine (Figure 4). Our results indicate that oxygen availability may limit cuscohygrine biosynthesis. Aeration did not improve the hyoscyamine content, and its effect on volumetric hyoscyamine production was insignificant despite the improvement in biomass accumulation. It therefore appears that hyoscyamine biosynthesis is not limited by oxygen availability.

Scopolamine was not detected in any of the samples in any conditions, although its precursor, 6 β -hydroxyhyoscyamine (6BHH) was present in all roots except the Vhb line V14 that developed callus during the experiment. Aeration had no significant effect on 6BHH, although both the formation of 6BHH from hyoscyamine and the conversion of 6BHH to scopolamine are oxygen dependent. Interestingly, the effect of *vhb* expression on the alkaloid profile of *H. muticus* hairy roots was not identical to that of aeration (Table 3). Vhb production and aeration both decreased the concentration of (*N*-methylpyrrolidinyl)hygrine and alkaloid 414. However, Vhb production did not affect the concentration of cuscohygrine, which was increased by aeration. Therefore, the effect of Vhb is probably not related directly to its ability to increase the intracellular effective oxygen concentration, as has been suggested for *Escherichia coli* (Kallio et al. 1994).

4.2 Barley germination

4.2.1 Effects of steeping conditions on barley oxygen deficiency, germination and malt quality (Paper III)

Barley was malted using a range of steeping conditions with the aim of understanding the effect of external oxygen availability on the degree of oxygen deficiency in barley embryos. This was accomplished by determining the presence of the inducible isoenzymes ADH2 and ADH3. Native, ungerminated barley kernels, embryos and aleurone cells have previously been found to contain mainly ADH1·ADH1 homodimer and traces of ADH1·ADH2 (Hanson et al. 1984; Hanson and Brown 1984; Good and Crosby 1989). In the current study, traces of ADH1·ADH3 were detected as well as ADH1·ADH1 and ADH1·ADH2. This suggests that some of the ADH2 and ADH3 isoenzymes that are expressed during kernel development in the starchy endosperm and pericarp/testa/aleurone (Macnicol and Jacobsen 2001) survive kernel desiccation into the mature kernel.

After the first steep of the malting process (8h), the ADH isoenzyme pattern of the embryo still resembled that of the native barley. However, during the 16-hour air rest, all barley samples showed clear signs of a fermentative metabolism: the ethanol content in the malting drum head-space increased rapidly, the ADH activity increased, and a clear band representing the ADH2 homodimer appeared (Paper III; Figure 6). Moreover, the ADH2·ADH3 and ADH3·ADH3 dimers were detectable in some samples, including the continuously aerated control.

The ADH2 and ADH3 isoenzymes are both induced by oxygen deficiency (Hanson and Brown 1984; Good and Crosby 1989), indicating that the barley embryos were indeed experiencing oxygen deficiency during the steep and/or air rest, in all of the conditions studied. Hanson et al. (1984) found that ADH2 and ADH3 enzymes were expressed in isolated aleurone layers at head-space oxygen concentrations of 5% or lower. Interestingly, Crabb and Kirsop (1969) estimated that when the oxygen concentration of the aeration air was 4%, dehusked barley kernels had similar oxygen uptake and carbon dioxide production to husked barley at an oxygen concentration of 21%. This suggests that the oxygen concentration in the immediate vicinity of the embryo of steeped barley is of the order of 4–5%, a level which in aleurone cells induces the ADH2 and ADH3 isoenzymes. This is in accordance with the results of Benech-Arnold et al. (2006).

After the first 26 h of malting (8 h steep, 16 h air rest, 2 h steep), the aeration rate was doubled and the temperature lowered slightly, in order to mimic industrial process conditions. After this point, the germination percentages of all samples increased sharply to a level of 84–93%. The ethanol content of the head space increased to 30–70 ppm and then declined (Paper III; Figure 2). Ethanol production in industrial malting also followed the same pattern, indicating that the results are applicable to industrial practice. A previous study reported a similar pattern of ethanol production in germinating barley (Cantrell et al. 1981).

The order in which the ethanol concentration declined was consistent with the germination percentage at 1.3 days of malting. The decline in ethanol production is therefore probably a consequence of the down-regulation of the constitutive *adh1* gene by gibberellic acid, as observed previously in aleurone cells (Macnicol and Jacobsen 2001). Moreover, chitting of the kernels; *i.e.* rupture of

the testa and pericarp and emergence of the root and shoot, may lead to better oxygen conditions for the embryo followed by down-regulation of the *adh2* and *adh3* genes. A high germination rate also correlated well with an early increase in α -amylase activity, an enzyme which is up-regulated by gibberellic acid (Chrispeels and Varner 1966). Rapid recovery from oxygen deficiency was therefore related to faster enzyme production and good malt quality.

Hydrogen peroxide (H_2O_2) improved germination, although the barley sample studied was not dormant. H_2O_2 is an active oxygen source that can diffuse into plant tissue and affect germination directly or indirectly through O_2 production. Recent work on *Zinnia elegans* seeds indicated that H_2O_2 itself promotes germination rather than O_2 (Ogawa and Iwabuchi 2001). H_2O_2 may be involved in the degradation of abscisic acid (Wang et al. 1998) or in the oxidation of inhibitors in the pericarp (Ogawa and Iwabuchi 2001). However, the possibility that H_2O_2 affected germination through an increase in oxygen supply cannot be ruled out.

The results of the current study show that oxygen deficiency during the first steep had no effect on germination or malt quality, whereas oxygen deficiency only 8–16 hours later, at the end of the air rest, retarded germination. This study therefore shows for the first time that the timing of externally applied oxygen deficiency determined its effects on malt quality. In these experiments, oxygen deficiency was not applied later than the end of the air rest. If oxygen deficiency were to occur later in the process this would probably affect germination and modification significantly, since less ADH activity would be available to maintain a fermentative metabolism. Therefore, the importance of aeration increases as the process proceeds. Aeration also plays an important role in removing CO_2 that has a negative effect on germination and modification. This is of particular importance in industrial scale malting, in which high CO_2 concentrations may easily develop *e.g.* at the end of the steeping period. Anaerobic pockets have been detected in industrial scale aerated steeping vessels (Aalbers et al. 1983). During air rest periods, air is sucked through the steeping vessel from the top to the bottom and consequently the grain at the bottom of the vessel may become starved of oxygen (Cantrell et al. 1981). However, the germination process is generally considered to be more aerobic.

4.2.2 Effects of the indigenous microbial community on barley oxygen deficiency, germination and malt quality (Paper IV)

The first steeping water was supplemented with mixtures of antibiotics in order to selectively suppress the bacterial or fungal community in the malting process. The ultimate aim was to verify the hypothesis that microbes naturally present on the barley kernel can slow down germination by competing for oxygen with the embryo. The treatments applied were adapted from previous investigations, which also showed that the effects of antibiotics were a result of their direct action on microbial metabolism without disturbing grain activity (Doran and Briggs 1993; Gaber and Roberts 1969; Kelly and Briggs 1992a; Van Campenhout et al. 1998; Van Campenhout et al. 1999).

The results clearly showed interaction between the microbial community and grain metabolism even with good quality, mature malting barley. By restricting bacteria, germination was improved, whereas the restriction of fungi did not affect germination. Even a 2 log reduction of aerobic bacterial counts, mainly consisting of Gram-negative species, was advantageous with respect to both germination and malt quality. The differences in germination were most noticeable after 1.3 days of malting (Paper IV, Table III). At that point, the antibacterial treatments had improved the germination percentage by 13–19%-units and H₂O₂ by 23%-units. It has been proposed that microbes inhibit germination by competing with the embryo for oxygen (Doran and Briggs 1993; Harper and Lynch 1979; Harper and Lynch 1981; Lynch and Prynne 1977). However, the current results showed that in the case with normal malting barley, improved germination was not related to oxygen availability. Although both antibacterial treatments and H₂O₂ improved germination, they gave rise to different physiological responses in barley. H₂O₂, which is an active oxygen source (Ogawa and Iwabuchi, 2001), improved germination and led to a faster disappearance of ethanol from the malting drum headspace and to a faster gibberellic acid dependent α -amylase synthesis. The response of aleurone cells to gibberellic acid is known to be oxygen dependent (Hanson and Jacobsen 1984). Oxygen deficiency, caused by applying N₂ gas during steeping, slowed down germination, and led to a slower disappearance of ethanol and slower α -amylase synthesis. The antibacterial treatments did not influence these parameters, although the treatments improved germination and rootlet growth.

It is possible that the complex microbial community inside the barley layers formed a physical barrier and that reducing especially the bacterial load simply improved root emergence, and not germination. This theory is supported by the results of van Campenhout et al. (1998), who reinfected barley after antimicrobial treatment with *P. agglomerans* and detected adverse effects such as inhibited water uptake and shorter acrospires. Pseudomonads and members of Enterobacteriaceae, also identified as the predominant species in this study, produce extracellular polysaccharides and other exopolymeric substances, and are often involved in the formation of complex microbial biofilms in plant ecosystems (Morris and Monier 2003). As early as 1944, Bishop suggested that bacteria developing at the time of harvest form a slime or mucus covering the embryo, and thereby inhibit germination. Furthermore, the gel-like polymeric matrix in plant-associated biofilms may prevent the normal gas exchange and reduce diffusion of nutrients and other substances (Morris and Monier 2003). Van Campenhout et al. (1998; 1999) and Van Campenhout (2000) reported that heavy microbial loads on barley grain may inhibit the plant contribution to the overall CO₂ release.

The antibacterial treatments also notably improved the filterability of the malt mash, whereas the antifungal treatments had the opposite effect. Suppression of the bacterial community led to approximately 30% shorter filtration time. This is a significant improvement in an important quality criterion from the brewer's point of view. The accelerated wort filtration could be due to the reduction of slime-forming bacteria, but also to enhanced fungal growth and concomitant production of cell wall-degrading enzymes.

The fact that no association between embryo oxygen deficiency and microbial load was detected in this study could also imply that the reduction in microbial load was insufficient to affect this parameter. The viable counts were surprisingly high even after broad-spectrum antimicrobial treatments, indicating that the major part of the microbial community inside the kernel was well protected. Suppression of the bacterial community with antibacterial treatments provided more living space for yeast growth. Approximately 10-fold higher yeast counts were detected after antibacterial treatments. Therefore, a significant reduction of the microbial load is difficult to accomplish unless sterilization procedures are applied. However, sterilization procedures that are severe enough to inactivate microbes situated between the testa and pericarp usually also affect embryo viability.

4.2.3 The effect of heterologous *vhb* expression in barley (Paper V)

The *vhb* gene from *Vitreoscilla* was introduced into barley with the primary aim of improving germination. Bombardment of 1545 immature Golden Promise embryo-halves with the plasmid pAL113 carrying the *vhb* gene resulted in 157 transgenic barley plants representing at least seven separate lines. Of these lines, two were fertile and produced detectable amounts of VHb protein (3b and 32h), and were therefore selected for further studies. The negative segregants of the same lines were used as controls. Based on Western blot analysis of root and leaf tissue, the line 3b produced significantly more VHb protein than the line 32h. Line 3b also had a higher copy number of the *vhb* gene than line 32h, based on the Southern blot.

Although the initially recorded positive effects of *vhb* expression on germination of tobacco seeds (Holmberg et al. 1997) have not been reproduced by other workers (Frey et al. 2004), Li et al. (2005) reported that *vhb* expression improved germination of cabbage seeds (*Brassica oleracea*). Despite this discrepancy, we hypothesized that *vhb* expression could improve germination of barley kernels, because germination is known to be an energy demanding growth stage (Bewley and Black 1994), during which the embryos also suffer from oxygen deficiency (section 4.2.1; Paper III). In these conditions, the embryo could potentially benefit from the two proposed roles of VHb: NO-scavenging and improvement of cellular respiration (Kallio et al. 1994; Frey et al. 2004). However, in the current study, heterologous *vhb* expression did not improve barley germination. On the contrary, the germination of *vhb*-expressing lines was in many cases even slightly slower than that of their respective negative controls.

The slight adverse effect of VHb on barley germination could be related to the ability of VHb to detoxify NO (Frey et al. 2002; Frey et al. 2004; Gardner 2005). Interestingly, Desel and Krupinska (2005) found that γ -tocopherol, another potential NO detoxifier, had a negative impact on barley germination and seedling development. These results as well as the results of the current study suggest that NO is to some extent beneficial and necessary for germination, and that heterologous *vhb* expression could slow down germination through its NO modulating effect.

VHb expression had no significant effect on kernel weight, elongation growth, leaf area or tillering. Although VHb expression did not affect plant growth, it changed the ratio of the total root length (sum of root lengths) to stem length in young seedlings. VHb-producing seedlings had fewer and/or shorter roots than the control plants under both conditions studied. This effect may also be linked to the interference of VHb with NO signalling. Several studies indicate a role for NO in root development and growth. The NO donor SNP stimulated post-germinative root growth in barley (Desel and Krupinska 2005) and induced root tip elongation in maize (Gouvêa et al. 1997). Moreover, Pagnussat et al. (2002) showed that an increase in the NO concentration was required for adventitious root development induced by indole acetic acid in cucumber (*Cucumis sativus* L.). NO is also involved in auxin-dependent lateral root formation in tomato (*Solanum lycopersicum* L.) (Correa-Aragunde et al. 2006). Gupta et al. (2005) showed that root mitochondria were able to reduce nitrite to NO, whereas leaf mitochondria could not. It appears that NO has a specific role in root metabolism and it can be speculated that *vhb* expression could restrict root growth in young barley seedlings by reacting with NO.

Table 4. The effect of *vhb* expression on ADH activity in barley seeds and roots.

Barley line	3b control	3b VHb	32h control	32h VHb
Embryos				
Dry seeds				
- ADH, nkat/embryo	44 ± 8	35 ± 4	50 ± 9	48 ± 13
Imbibed, non-germinated seeds				
- ADH, nkat/embryo	46 ± 14	31 ± 4	47 ± 3	37 ± 5
- ADH, nkat/mg protein	107 ± 12	108 ± 12	114 ± 13	120 ± 14
Roots				
Normoxic conditions				
- ADH, nkat/mg protein	52 ± 2	46 ± 0	52 ± 0	50 ± 3
Hypoxic conditions				
- ADH, nkat/mg protein	64 ± 3	48 ± 0	57 ± 2	49 ± 5

In malting conditions, the ADH activity in germinating barley initially increased, and then decreased after root emergence (Papers III, IV). Moreover, the oxygen tension at the vicinity of the embryo has been estimated to be about 4–5% (Benech-Arnold et al., 2006), which is low enough to induce the expression of the *adh2* and *adh3* genes (Hanson et al. 1984). After imbibition, VHb-producing embryos had lower ADH activity than the control embryos (Table 4). In line 3b, which had a higher VHb production level than line 32h, the ADH activity was also lower in the embryos of dry, quiescent kernels. However, imbibed, non-germinated embryos of *vhb*-expressing kernels also had lower soluble (extractable) protein content than the control embryos, leading to equal ADH activities per mg of soluble protein. A lower amount of soluble protein was also observed in the VHb-positive embryos (including coleoptiles and rootlets) isolated from kernels 3–4 days after imbibition, although the fresh weights of the embryos were similar. We have observed that the protein content of the embryo (including coleoptile and rootlets) initially decreased during the first two days after imbibition, and then started to increase (unpublished data). The initial decrease in protein content may be a consequence of oxygen deficiency, which is known to depress the synthesis of normoxic proteins (Chang et al. 2000). Unfortunately, the small amounts of transgenic kernels available did not allow experiments on a time scale and therefore the effects of *vhb* expression on the dynamics of germination could not be established. Further research will be required to elucidate the relationship between *vhb* expression and soluble protein in the embryo, coleoptile and rootlets.

Plant survival under low oxygen conditions, such as during flooding, is dependent on the ethanol fermentation pathway. The most important function of this pathway is thought to be the generation of NAD⁺ from NADH, because this function is impaired by the inactivation of oxidative phosphorylation (Kennedy et al. 1992). Heterologous *vhb* expression has previously been found to improve the survival of cabbage plants under submergence (Li et al. 2005). In the present study, *vhb*-expressing barley plants were subjected to oxygen deficiency by submerging the plant roots in growth medium, saturated with nitrogen gas, for seven hours. This treatment led to a statistically significant increase in ADH activity in the roots of control plants, whereas *vhb*-expressing plants showed no increase in ADH activity (Table 4). Metabolically engineered maize cells expressing barley Hb were also reported to have lower ADH activity than control cells under hypoxia (Sowa et al. 1998). Interestingly, it has been

proposed that the endogenous barley Hb helps in maintaining the energy status of plant cells by an alternative mechanism to the classic fermentation pathway (Igamberdiev and Hill 2004). Alternative fermentation based on Hb or VHb could possibly supplement the role of ADH in the recycling of NADH. This cycle may also sustain glycolysis and the energy status of plant cells, maintaining a higher level of ATP in haemoglobin-expressing lines under hypoxia (Sowa et al. 1998; Dordas et al. 2003b). VHb-producing *E. coli* cells have been shown to generate a 50% higher proton flux per reduced oxygen molecule, with a concomitant 30% increase in ATP synthase activity and a higher net turnover of ATP (Chen and Bailey 1994; Kallio et al. 1994; Tsai et al. 1996).

Due to the several similarities between the properties and suggested roles of endogenous barley haemoglobin (Hb) and VHb (Giangiacoimo et al. 2001; Duff et al. 1997; Frey et al. 2002; Gardner 2005; Dordas et al. 2003a; Dordas et al. 2004), *vhb* expression was considered likely to affect the expression of endogenous Hb. The effect of VHb production on endogenous Hb expression was evaluated using Western blot analysis, which is semiquantitative but gives an indication of the expression levels. Barley Hb mRNA has previously been detected in root tissue and the transcript levels have been found to increase during flooding (Taylor et al. 1994). In greenhouse conditions, *vhb*-expressing barley roots had slightly lower Hb levels than the control plants. This is in good agreement with the slightly lower ADH activity observed in the same conditions. These results could indicate two things: 1) *vhb* expression affects the energy metabolism in normoxic conditions, or 2) the control growth conditions may have been mildly hypoxic. Hypoxia may occur in metabolically active root tissue even in plants grown in air (Højberg and Sørensen 1993). In anoxic root tissue, *vhb*-expressing plants and control plants had similar Hb levels, possibly indicating that VHb alone was insufficient for the roots to withstand prolonged anoxia.

5. Conclusions

An improvement in alkaloid productivity of hairy root cultures can be achieved through an increase in biomass formation or an improvement in specific hyoscyamine production (mg/g d.w.), or both. In the current study, heterologous *vhb* expression improved the growth, but not statistically significantly the specific or volumetric (mg/l) hyoscyamine production of *A. rhizogenes*-induced hairy roots of *H. muticus*. A problem related to *vhb* expression was the tendency of hairy root cultures to dedifferentiate to callus. This effect probably counteracts the positive effects of *vhb* expression, because root dedifferentiation leads to impaired alkaloid production (Sevón et al. 1998). The tendency of different lines to form callus varied somewhat between cultivations and would in practice be difficult to control.

Although VHb production did not have a statistically significant effect on hyoscyamine production (mg/l) due to wide somaclonal variation, the highest production levels were found among the VHb-lines. This could be of practical importance, because yield improvements in hairy roots are generally stable, and the selection of highly productive clones is a commonly used breeding method for improving alkaloid yields (Sevón and Oksman-Caldentey 2002).

In contrast to previous findings (Holmberg et al. 1997; Li et al. 2005), we found that constitutive *vhb* expression did not improve the germination rate of barley kernels in any of the conditions studied. Moreover, VHb restricted root growth slightly in young barley seedlings. Both germination and root organogenesis are known to be regulated by NO. Because NO has both cytotoxic and stimulating properties, the effect of *vhb* expression in plants may depend on the level and role of endogenous NO in the conditions studied. In conditions involving excess NO production, such as severe oxygen deficiency (Dordas et al. 2003a), VHb can protect plant cells from the adverse effects of NO. However, in conditions in which NO plays an important role as a signal molecule, such as germination and root growth, *vhb* expression even appears to have slightly adverse effects on growth and development. Further studies could be directed towards an improved understanding of the functions of VHb through a metabolomic approach.

This study demonstrated that it is difficult to predict the effects of genetic engineering on the complex physiology of plants. In the current study, the rather moderate effects of heterologous *vhb* expression in barley may be partly explained by the simultaneous decrease in endogenous haemoglobin expression observed in normoxic greenhouse conditions. Commercially interesting properties such as secondary metabolite production and germination rate are difficult to tailor due to the complex control of these events. According to the results of this study, hyoscyamine production is not limited by oxygen availability. A potential method for improving hyoscyamine production of *H. muticus* hairy roots is over-expression of bottleneck genes of the biosynthetic pathways of the tropine alkaloids. However, this approach is limited by the scarce knowledge of these pathways. High-throughput transcript and metabolite profiling methods aiming at recognising key genes involved in secondary metabolite production will open up new possibilities for genetic engineering of specific biosynthetic pathways (Oksman-Caldentey and Inzé 2004).

A certain degree of oxygen deficiency in the embryo appears to be an inevitable stage in barley germination. Neither steep water aeration nor a significant restriction of bacteria naturally colonising the barley kernel alleviated the oxygen deficiency of the embryo. The recent results of other researchers showing that NO is produced in plant cells under oxygen deficiency (Dordas et al. 2003a; Dordas et al. 2004) and that NO stimulates seed germination (Beligni and Lamattina 2000; Sarath et al. 2006), together with the present results, imply that embryo oxygen deficiency is in fact beneficial or even necessary for germination. Although aeration cannot and need not overcome the oxygen deficiency of the barley embryo, the results of this study show that the need for aeration increases as the malting process proceeds. Aeration not only introduces the oxygen needed for the completion of germination, enzyme synthesis and other post-germinative events, but also removes CO₂ that would otherwise inhibit modification of the grain.

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Author(s) Wilhelmson, Annika		
Title The importance of oxygen availability in two plant-based bioprocesses: hairy root cultivation and malting		
Abstract The main aim was to examine the role of oxygen availability in the cultivation of <i>Agrobacterium rhizogenes</i> -induced hairy roots of Egyptian henbane (<i>Hyoscyamus muticus</i>), and in barley (<i>Hordeum vulgare</i>) malting. This was accomplished by transferring the <i>vhb</i> gene expressing bacterial haemoglobin (VHb) from <i>Vitreoscilla</i> to <i>H. muticus</i> and to barley with the specific goals of improving the growth and alkaloid production of <i>H. muticus</i> hairy roots and the germination rate of barley. In <i>H. muticus</i> hairy roots, <i>vhb</i> expression and an increase in aeration both improved the growth, but not the hyoscyamine production. The effect of VHb on the alkaloid profile was not identical to that of aeration. VHb-producing barley plants did not react to oxygen deficiency by increasing the alcohol dehydrogenase (ADH) activity in the roots, unlike the control plants. This could be due to the ability of VHb to supplement the role of ADH in the recycling of NADH. VHb retarded the germination rate of barley slightly and restricted root growth in young seedlings. These effects are probably related to the ·NO dioxygenase activity of VHb. Another aim of the study was to verify the hypothesis that barley embryos suffer from oxygen deficiency in the malting process, regardless of aeration. This was accomplished by studying the effect of aeration on the inducible ADH isoenzymes, ethanol production and α -amylase activity. Furthermore, the effect of the indigenous microbial community of the barley kernel on embryo oxygen deficiency was studied by modifying the microbial population. Neither aeration nor a reduction in grain microbes alleviated the oxygen deficiency of the barley embryo. A certain degree of oxygen deficiency in the embryo appears to be an inevitable stage in barley germination. Although aeration cannot and need not overcome the oxygen deficiency of the barley embryo, the need for aeration increases as the malting process proceeds. Aeration introduces the oxygen needed for the completion of germination, and removes CO ₂ that would otherwise inhibit grain modification.		
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Series title and ISSN VTT Publications 1235-0621 (soft back ed.) 1455-0849 (URL: http://www.vtt.fi/publications/index.jsp)		Project number
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Keywords alkaloid, barley, germination, haemoglobin, hairy root, hyoscyamine, <i>Hordeum vulgare</i> , <i>Hyoscyamus muticus</i> , malting, oxygen deficiency, VHb, <i>Vitreoscilla</i>		Publisher VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland Phone internat. +358 20 722 4404 Fax +358 20 722 4374



Tekijä(t) Wilhelmson, Annika		
Nimeke Hapen saatavuuden merkitys kahdessa kasvipohjaisessa bioprosessissa: juuriviljelmissä ja mallastuksessa		
Tiivistelmä Työn pääasiallinen tavoite oli tutkia hapen roolia kahdessa malliprosessissa: Egyptin villikaalin (<i>Hyoscyamus muticus</i>) juuriviljelmissä sekä ohran (<i>Hordeum vulgare</i>) mallastuksessa. Hapen roolia tutkittiin siirtämällä bakteerihemoglobiinia (VHb) koodaava <i>vhb</i> -geeni <i>Vitreoscillasta</i> Egyptin villikaaliin ja ohraan. Geeninsiirron tavoitteena oli parantaa Egyptin villikaalin juuriviljelmien kasvua ja alkaloidituottoa sekä nopeuttaa ohran itämistä. Sekä VHb:n tuotto että ilmastus paransivat juuriviljelmien kasvua, mutta eivät vaikuttaneet hyoskyamiinituottoon. VHb:n vaikutus Egyptin villikaalin alkaloidiprofiiliin poikkesi ilmastuksen vaikutuksesta. VHb:tä tuottavien ohrakasvien juuret eivät vertailukasvien tapaan reagoineet hapen puutteeseen lisäämällä alkoholidehydrogenaasiaktiivisuutta (ADH). Tämä voi johtua VHb:n kyvystä korvata ADH:ta glykolyysin ylläpidossa ja NADH:n kierrätyksessä. VHb hidasti hieman ohran itämistä ja rajoitti nuorten ohrataimien juurten kasvua. Nämä vaikutukset liittyvät todennäköisesti VHb:n NO-dioksygenaasiaktiivisuuteen, sillä sekä itäminen että juurten kasvu ovat typpioksidin (NO) säättämiä. Työn toinen tavoite oli todentaa hypoteesi, jonka mukaan ohra kärsii hapen puutteesta mallastusprosessissa, ilmastuksesta huolimatta. Ilmastuksen vaikutusta alkion hapen puutteeseen tutkittiin mittaamalla indusoituvien ADH-entsyymien ilmestymistä, ADH-aktiivisuutta, etanolituottoa ja α -amylaasiaktiivisuutta. Lisäksi selvitettiin ohran mikrobiston vaikutus alkion hapen puutteeseen muokkaamalla mikrobipopulaatioita mallastuksen aikana. Ilmastus tai mikrobien vähentäminen eivät lieventäneet alkion hapen puutetta. Hapen puute näyttää olevan luonnollinen ja väistämätön vaihe ohran itämisessä. Vaikka ilmastuksella ei ole mahdollista tai edes välttämätöntä lieventää ohran hapen puutetta, ilmastuksen tarve kasvaa mallastusprosessin edetessä. Ilmastus antaa ohralle itämisen loppuun viemiseen tarvittavan hapen, sekä poistaa jyvän myöhentymistä hidastavan hiilidioksidin ilmatilasta.		
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