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ALKALOID PRODUCTION BY HAIRY ROOT CULTURES

by

Bo Zhao

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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2014

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ABSTRACT

Alkaloid Production by Hairy Root Cultures

by

Bo Zhao, Doctor of Philosophy

Utah State University, 2014

Major Professor: Dr. Foster Agblevor
Department: Biological Engineering

Alkaloids are a valuable source of pharmaceuticals. As alkaloid producers, hairy roots show rapid growth in hormone-free medium and synthesis of alkaloids whose biosynthesis requires differentiated root cell types. Oxygen mass transfer is a limiting factor in hairy root cultures because of oxygen's low solubility in water and the mass transfer resistance caused by entangled hairy roots. In addition to limited oxygen supply, alkaloids accumulating primarily in the root tissue present a bioprocessing challenge because the root tissue must be homogenized and the phytochemical extracted and purified from a chemically complex solution. The present research focused on improving oxygen mass transfer with microbubbles and enhancing alkaloid release in hairy root cultures.

Hairy roots are heterotrophic and require sucrose and oxygen for respiration. The tobacco hairy roots were able to grow with glucose and/or fructose, but the growth was only 10% of that with sucrose as the carbon source. Oxygen was a limiting nutrient for tobacco hairy root growth. In a 1-liter fermentor, microbubbles stabilized by 50 mg l⁻¹

Triton X-100 increased oxygen mass transfer, hairy root growth, and nicotine production in medium. Basification of the culture medium associated with root growth resulted in a dramatic reduction in nicotine accumulation in the medium, which was reversed by adjusting the medium pH from 8 to 6.

Confocal microscope observation showed that the *H. niger* hairy root cells with less aeration were much wider than the cells with sufficient aeration. The combination of microbubble generator and a novel ground-joint column bioreactor improved the oxygen mass transfer and consequently the growth of *H. niger* hairy roots. When the medium pH was adjusted from 6 to 3, the concentration of hyoscyamine in hairy root culture medium increased from zero (undetectable) to 42 mg l⁻¹, whereas the scopolamine in medium increased from 0.1 to 11 mg l⁻¹. Medium pH 3 inhibited *H. niger* hairy root growth but the root branches appeared to survive short time medium pH 3 treatment. In comparison to the wild-type *H. niger* hairy root line, the transgenic line showed about 10-fold increase in total tropane alkaloid released at the medium pH3.

(165 pages)

PUBLIC ABSTRACT

Alkaloid Production by Hairy Root Cultures

Bo Zhao

In the present research, nicotine alkaloid production by *Nicotiana tabacum* (tobacco) hairy roots and tropane alkaloid production by *Hyoscyamus niger* hairy roots were investigated. The first objective of this research was to improve the oxygen mass transfer in hairy root cultures with microbubbles. Oxygen was shown as a critical nutrient for the growth of tobacco and *H. niger* hairy roots. In a 1-liter fermentor, microbubble dispersion improved the oxygen mass transfer, tobacco hairy root growth, and nicotine production in the medium. In a novel ground-joint column bioreactor, microbubbles enhanced the oxygen mass transfer and the growth of *H. niger* hairy roots. The second objective of this research was to enhance the release of alkaloids from the hairy roots into the culture medium. In a 1-liter fermentor, nicotine concentration in medium was improved by adjusting the medium pH to 6. Unlike the nicotine alkaloid, hyoscyamine concentration in medium was not detectable at medium pH 6, whereas hyoscyamine in medium increased to 42 mg l⁻¹ at medium pH 3. Similar to the hyoscyamine, scopolamine in medium increased from 0.1 to 11 mg l⁻¹ when the medium pH was adjusted from 6 to 3. The release of alkaloids into culture medium provides opportunities to isolate a high-value alkaloid directly from the culture fluid, and reduces the cost of product recovery.

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Bo Zhao

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CHAPTER 1

INTRODUCTION

1. Overview

Hairy roots are generated by infecting plant leaf or stem tissue with *Agrobacterium rhizogenes* (Guillon et al. 2006). As novel phytochemical producers, hairy roots show rapid growth in hormone-free media and promote the synthesis of phytochemicals whose biosynthesis requires differentiated root cell types.

Oxygen is usually a limiting nutrient in hairy root cultures because of its low solubility in water and the mass transfer resistance caused by entangled hairy roots (Yu and Doran 1994). At given mass transfer coefficient and volume, volumetric oxygen mass transfer coefficient (k_La) increases with increasing gas-liquid interfacial area, which is inversely proportional to the size of gas bubbles. Smaller gas bubbles also have an increased residence time in the bioreactor, which is beneficial to oxygen dissolution. Microbubbles are bubbles with diameter on the order of 100 μm , which is less than one-tenth of the diameter of conventional air bubbles (3–5 mm) (Bredwell et al. 1995). Microbubbles are coated and stabilized by surfactant layers and disperse thoroughly in medium, which form microbubble dispersion (MBD). MBD significantly increases the k_La in microbial fermentation (Hensirisak et al. 2002; Weber and Agblevor 2005; Zhang et al. 2005), but has not been evaluated for hairy root cultures.

In addition to oxygen mass transfer that usually limits hairy root growth, the utility of producing a phytochemical in hairy root cultures depends upon the expense associated with the bioprocessing required to isolate the chemical of choice.

Phytochemicals accumulating primarily in the root tissue present a bioprocessing challenge because the root tissue must be homogenized and the phytochemical extracted and purified from a chemically complex solution. On the other hand, phytochemicals that accumulate mostly in the culture medium are in a chemically less complex milieu than those which are retained within the hairy root tissue. Therefore, phytochemicals that accumulate in the culture medium require substantially less bioprocessing effort to isolate and purify.

Hairy root cultures produce secondary metabolites such as alkaloids. Nicotine alkaloid is a potent natural insecticide and potential anti-inflammatory agent. Hyoscyamine and scopolamine are the most intensively studied tropane alkaloids, probably because of their anticholinergic activity on the parasympathetic nerve system. Hyoscyamine and scopolamine are used in the treatment of motion sickness, cardiac diseases, and gastric disorder.

2. Objectives

- Show the effects of nutrients on *Nicotiana tabacum* hairy root growth and nicotine production.
- Investigate the effects of aeration and medium pH on *N. tabacum* hairy root growth and nicotine release.
- Utilize microbubble generator to improve *N. tabacum* hairy root growth and nicotine production in medium.
- Show the effects of aeration on *Hyoscyamus niger* hairy root growth and the production of hyoscyamine and scopolamine.

- Extend the utilization of microbubble generator to *H. niger* hairy root cultures in a novel ground-joint column bioreactor.
- Improve the release of hyoscyamine and scopolamine into the culture medium.
- Compare a wild-type and transgenic *H. niger* hairy roots for tropane alkaloid release.

3. Format of dissertation

Chapter 2 is a literature review on alkaloids and hairy root cultures. Chapter 3 describes the effects of nutrients on the growth of *N. tabacum* hairy roots and nicotine production. Chapter 4 details the effects of oxygen and medium pH on *N. tabacum* hairy root growth and nicotine release. Chapter 5 shows the utilization of microbubble generator improves oxygen mass transfer in hairy root cultures. Chapter 6 details the effects of oxygen on *H. niger* hairy root growth and tropane alkaloid production, and hairy root cultivation in a novel ground-joint column bioreactor connected to a microbubble generator. Chapter 7 describes the release of hyoscyamine and scopolamine by a transgenic *H. niger* hairy roots, and the preliminary comparison between wild-type and transgenic *H. niger* hairy roots for tropane alkaloid release. Chapter 8 is a summary of this dissertation.

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CHAPTER 2

LITERATURE REVIEW

1. Alkaloids from plants as a source of pharmaceuticals

Secondary metabolites in plants are defined as compounds that are not essential to plant growth and exist in low abundance (Bennett and Bentley 1989; Bourgaud et al. 2001). Secondary metabolites in plants are usually classified according to their molecular structures and three groups are considered: phenolics, terpenes, and nitrogen-containing compounds (Bourgaud et al. 2001). In plants, secondary metabolites are involved in the defense against microorganisms, insects, herbivorous animals, and neighboring competing plants (Bennett and Wallsgrove 1994). Secondary metabolites also serve as growth regulators (hormones), attractants (for organisms like pollinating insects), structural support (lignin), and pigments (Walton and Brown 1999). For humans, secondary compounds from plants represent an important source of food additives, fragrances, and pharmaceuticals (Razdan 2003).

Alkaloids as secondary metabolites in plants belong to nitrogen-containing compounds (Wink 1997). According to Pelletier (1983), an alkaloid is “a cyclic compound containing nitrogen in a negative oxidation state.” Alkaloids are diverse and are classified into several groups according to their structures such as tropane alkaloids, pyridine alkaloids, indole alkaloids, etc. Alkaloids are a prominent class of defense chemicals in plants against vertebrates, invertebrates, and microorganisms (Roberts and Wink 1998). Because of their defense functions, alkaloids typically show toxicity and pharmacological activities, particularly in mammals, by affecting the chemical

transmitters in the nervous system (Roberts and Wink 1998). The toxicity of alkaloids has been traditionally used by humans in hunting and warfare and nowadays in treating diseases (Luca and Pierre 2000).

As a typical pyridine alkaloid, nicotine is useful as a potent natural insecticide (Isman 2006; Kircher and Lieberman 1967; Richardson and Busbey 1937; Smith and Goodhue 1943) and potential anti-inflammatory agent (Mabley et al. 2011). Hyoscyamine and scopolamine are the most intensively studied tropane alkaloids, probably because of their anticholinergic activity on the parasympathetic nerve system (Yun et al. 1992). Hyoscyamine and scopolamine are useful in the treatment of motion sickness, cardiac diseases, and gastric disorders (Yun et al. 1992; EL Jaber-Vazdekis et al. 2008).

The biosynthetic pathways of nicotine, hyoscyamine, and scopolamine in plants are summarized by Lee et al. (2005) and Palazon et al. (2008). As shown in Figure 2.1, amino acid ornithine is the precursor of 1-methyl-pyrrolinium cation, which is an intermediate for both nicotine and hyoscyamine biosynthesis in plants. Nicotine is formed by combining 1-methyl-pyrrolidinium cation with nicotinic acid, whereas the condensation of 1-methyl-pyrrolidinium cation with acetate moiety forms tropine, which is esterified with tropic acid to yield hyoscyamine. Scopolamine is formed by direct oxidation of hyoscyamine. Hyoscyamine 6 β -hydroxylase (H6H) is an oxoglutarate-dependent dioxygenase that mediates the two-step reaction to generate scopolamine.

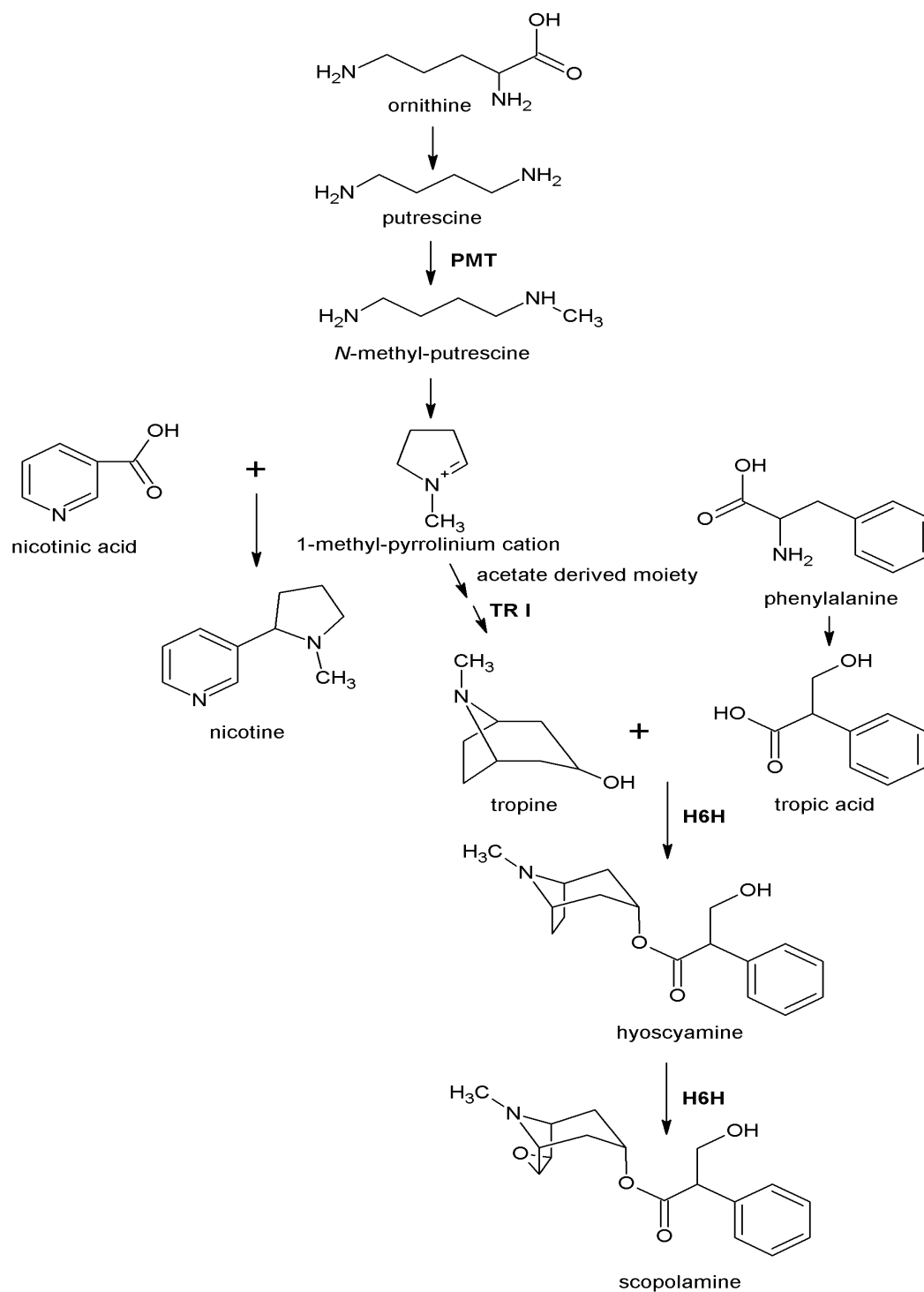


Figure 2.1. Biosynthetic pathways of nicotine, hyoscyamine, and scopolamine (modified from Lee et al. 2005). PMT: putrescine N-methyltransferase; H6H: hyoscyamine 6 β -hydroxylase; TRI: tropinone reductase I.

2. Hairy roots as phytochemical producers

Secondary metabolites in plants, or phytochemicals, are traditionally extracted from whole plants. Traditional agriculture requires months to years for plant growth, and phytochemical concentrations in plants vary considerably due to pathogen and weather conditions. In addition to whole plants, plant cell suspension cultures and hairy root cultures have been developed as alternative phytochemical producers.

Plant cell suspension cultures have advantages of rapid growth and independence of geographical and seasonal variations (Rao and Ravishankar 2002). During the cultivation, plant suspension cells remain undifferentiated, while the biosynthesis of secondary metabolites is often inefficient and unstable in undifferentiated plant suspension cells (Sevon and Oksman-Caldentey 2002).

As shown in Table 2.1, hairy root cultures have several advantages over undifferentiated plant suspension cell cultures (Flores et al. 1999). Hairy roots are genetically stable and grow in hormone-free culture media (Ono and Tian 2011). Hairy roots show rapid growth and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Flores et al. 1999; Sevon and Oksman-Caldentey 2002; Shanks and Morgan 1999).

As shown in Figure 2.2, hairy roots are developed by the infection of plants with *Agrobacterium rhizogenes*, which is a gram-negative soil bacterium containing a root-inducing (Ri) plasmid (Huffman et al. 1984). Phenolic compounds released from plant wound activate the Ri plasmid (Stachel et al. 1985), which produces a group of virulence proteins and a segment of transfer-DNA (T-DNA). The T-DNA transfers from the *Agrobacterium* into the plant cell together with the virulence proteins. In plant cell,

Table 2.1 Comparison of whole plants, plant suspension cell cultures, and hairy root cultures as phytochemical producers

Property	Whole plants	Plant suspension cell cultures	Hairy root cultures
Growth	Slow	Fast	Fast
Control	Limited	Yes	Yes
Stability	High	Low	High
Differentiation	Yes	No	Yes

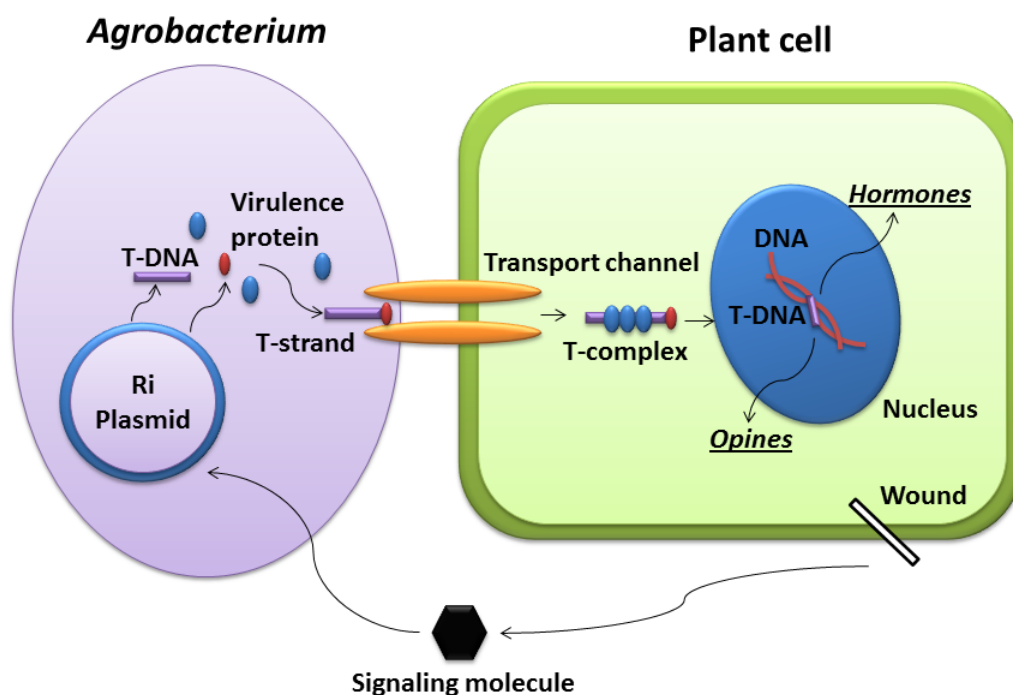


Figure 2.2. Infection of plant cell with *Agrobacterium* (adapted from Gelvin 2005; Pitzschke and Hirt 2010).

T-DNA and virulence proteins form a T-complex which transfers the T-DNA into the nucleus, where the T-DNA is integrated into the plant chromosome (Gelvin 2005). The T-DNA carries a set of genes that encode enzymes for the biosynthesis of opines and hormones such as auxins (Blakesley and Chaldecott 1993). Opines are a group of low

molecular weight compounds derived from condensation of amino acids with keto acid or sugars (Dessaux et al. 1993). Opines can be used exclusively by the *Agrobacterium* as carbon sources and give them a selective advantage (Murphy et al. 1987). The new balance of hormones in the transformed plant induces the formation of adventitious roots, called hairy roots, at the plant wound (Guillon et al. 2006).

3. Phytochemical exudation by hairy roots

A research area of great interest is the secondary metabolite exudation from hairy roots into the surrounding growth medium. Extracellular secondary metabolites can be extracted from a less chemically complex hairy root culture medium, rather than a cytoplasmic content mixture from costly cell disruption. This repartition of secondary metabolites relieves feedback inhibition on metabolite biosynthesis (Brodelius and Pedersen 1993). In addition, exudation of secondary metabolites allows the implementation of in situ product removal that simultaneously recovers the released products by extraction or absorption units connected to bioreactors (Sim et al. 1994). In situ product removal requires the immobilization of suspension cells, whereas hairy roots immobilize themselves by branching. Thus, the efficiency of secondary metabolite production by hairy root cultures can be significantly improved by phytochemical exudation.

RNAi is a phenomenon of sequence-specific gene silencing triggered by double-strand RNA (dsRNA) (Hannon 2002). RNAi is initiated by the cleavage of dsRNA into ~22-nucleotide small interfering RNAs, which then incorporate into RNA-induced silencing complex (RISC). The RISC is a multicomponent nuclease that cleaves

substrate mRNAs. Reviews of RNAi have been given by Hannon (2002), Hutvagner and Zamore (2002), Mello and Conte (2004). Nicotine uptake permease (NUP1) is a plant-specific purine uptake permease-like transporter and the expression of NUP1 in yeast cells preferentially transported nicotine relative to other pyridine alkaloids (Hildreth et al. 2011). Tobacco hairy root lines with reduced expression levels of NUP1 (*NUP1-RNAi* hairy roots) were reported to result in increased levels of nicotine in the culture media (Hildreth et al. 2011). The repartitioning of nicotine from the *NUP1*-reduced expression hairy root line to the culture media provides opportunities to isolate a high-value alkaloid directly from the culture fluid.

4. Nutrients for hairy root growth

The growth of hairy roots requires mineral salts as nutrients. Hairy roots are heterotrophic and need sucrose as carbon and energy sources, and oxygen as the electron acceptor in respiration. Gamborg's B5 medium was developed for soybean callus culture (Gamborg et al. 1968), and is commonly used for hairy root cultures. Gamborg's B5 medium contains macro-nutrients, micro-nutrients, and vitamins. KNO_3 is the most abundant nutrient in Gamborg's B5 medium, which accounts for 78% of the total weight. Sucrose of 20 g l^{-1} is typically used as both the carbon and energy sources.

Oxygen is usually a limiting nutrient in hairy root cultures because of several reasons. Firstly, the solubility of oxygen is low in comparison to other nutrients in hairy root culture medium. Mineral nutrients and sucrose are highly soluble in water and sufficient amounts can be initially incorporated in medium. Oxygen, however, needs to be supplied continuously due to its low solubility in water. The solubility of oxygen in

water is about 8 mg l⁻¹ at 25°C with air, and 41 mg l⁻¹ at 25°C with pure oxygen (Doran 1995). Oxygen enriched air (30% v/v oxygen) improved hairy root growth, whereas 100% oxygen inhibited the growth of hairy roots (Yu et al. 1997). The Secondly, the elongation and branching of hairy roots form an entangled root clump, which considerably reduces the convective flow of liquid medium (McKelvey et al. 1993). Lastly, root hairs and mucilage on the surface of hairy roots provide additional barriers to oxygen mass transfer (Shiao and Doran 2000; Williams and Doran 1999).

5. Microbubble dispersion

Diffusive mass transfer can be expressed as Fick's first law:

$$flux = -D \times dc/dx \quad (1)$$

where the *flux* is defined as mol s⁻¹ m⁻², *D* is the diffusion coefficient (diffusivity, m² s⁻¹), and *dc/dx* is the concentration gradient. Equation (1) can be rewritten as:

$$flux \times A/V = -D/dx \times A/V \times dc \quad (2)$$

where *A* is the interfacial area (m²) and *V* is the volume (m³). The unit of *flux* × *A/V* is mol m⁻³ s⁻¹ (mol s⁻¹ m⁻² × m²/m³ = mol m⁻³ s⁻¹), which is the same as molar concentration divided by time (*dC_L/dt*). For oxygen mass transfer in liquid medium, equation (2) can be expressed as:

$$dC_L/dt = k_L \times a \times (C^* - C_L) \quad (3)$$

where *k_L* is the mass transfer coefficient ($-D/dx$), *a* is the interfacial area per volume (*A/V*). The product of *k_L* and *a* (*k_La*) is called volumetric oxygen mass transfer coefficient. *C** is the saturated dissolved oxygen concentration, *C_L* is the actual dissolved oxygen concentration, and *t* is time.

At given k_L and volume (V), $k_L a$ increases with increasing gas-liquid interfacial area (A), which is inversely proportional to the size of gas bubbles. Smaller gas bubbles also have an increased residence time in the bioreactor, which is beneficial to oxygen dissolution (Weber and Agblevor 2005; Zhang et al. 2005). Conventionally, contactors and stirrers are used to reduce the size of sparged air bubbles. However, stirring results in a dramatic increase in power consumption, especially for large-scale systems, because the power consumption is proportional to the impeller rate to the third power and the impeller diameter to the fifth power (Bredwell et al. 1995; Weber and Agblevor 2005).

Microbubbles are bubbles with diameter on the order of 100 μm , which is about one-tenth of the diameter of conventional air bubbles (3–5 mm) (Bredwell et al. 1995). A microbubble generator was previously described by Zhang et al. (2005). As shown in Figure 2.3, the chamber of microbubble generator is filled with aqueous medium. Air is introduced into the chamber and the air bubbles are sheared into microbubbles by a disk spinning at 4000 rpm. In addition to mechanical agitation, microbubbles can be generated by acoustic and hydrodynamic (pressure variations) methods (Kozyuk 2008; Xu et al. 2008).

The generated microbubbles are stabilized by surfactants dissolved in the medium. The surfactant layer surrounding a microbubble generates a diffuse electrical double layer, which repels other microbubbles and prevents coalescence (Jauregi and Varley 1999). The surfactants must be nontoxic to the cells and have no detrimental effects on the biosynthesis of the desirable products (Bredwell et al. 1997). The stabilized microbubbles disperse throughout the culture medium to make uniform white foam called microbubble dispersion (MBD). MBD exhibits colloidal properties and can be

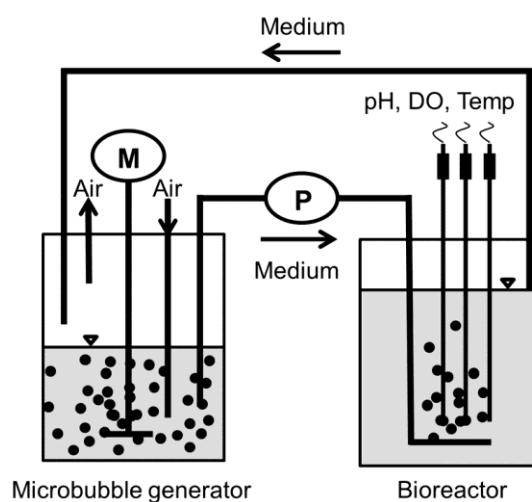


Figure 2.3 Microbubble generator connected to a bioreactor

transferred by a peristaltic pump from the microbubble generator into a bioreactor (Jauregi and Varley 1999), where the microbubbles rise slowly and the oxygen in the bubbles dissolves in the medium. Culture medium is circulated back to the microbubble generator to maintain a constant volume in the microbubble generator and the bioreactor.

MBD improved oxygen mass transfer in microbial fermentations. In a fermentative production of cellulose by *Trichoderma reesei*, MBD showed fivefold increase in k_La and nearly twofold increase in cell mass production in comparison to conventional air sparging (Weber and Agblevor 2005). In human serum albumin production by recombinant *Pichia pastoris*, MBD was found to improve oxygen mass transfer under reduced agitation speed (Zhang et al. 2005). In comparison to conventional air sparging, MBD needed extra power in the microbubble generator, but the overall power consumption of MBD was less than conventional air sparging, which was due to the lower agitation speed required to supply adequate oxygen (Hensirisak et al. 2002).

6. Bioreactors for hairy root cultures

Kim et al. (2002) summarized the three types of bioreactors for hairy root cultures: liquid-phase bioreactors, gas-phase bioreactors, and a combination of both. In liquid-phase bioreactors, hairy roots are immersed in aqueous medium. In gas-phase bioreactors, hairy roots are exposed to gas and nutrients are delivered to hairy roots by droplets.

Liquid-phase bioreactors include agitated bioreactors, airlift bioreactors, and bubble column bioreactors. Since hairy roots can be damaged by mechanical agitation, traditional stirred tank bioreactors were modified to include meshes around the impellers (Muranaka et al. 1993; Sudo et al. 2002). However, the modified agitated bioreactors were not suitable for hairy root species highly sensitive to shear stress (Nuutila et al. 1994). In airlift bioreactors, the medium is kept mixed and gassed by introducing air at the base of a column equipped with a draught tube. Airlift bioreactors provide low shear stress but insufficient mixing for high density cultures (Choi et al. 2006). Bubble column bioreactors are reactors in the shape of a column, in which the medium is mixed and aerated by introducing air into the bottom of the column. Advantages of bubble column bioreactors are low shear stress, simple design and construction, and low energy input (Paek et al. 2001).

Mist bioreactors are gas-phase bioreactors. In mist bioreactors, small liquid particles (about 4 μm in diameter) are produced by sound waves and expelled into the air as mist, which is carried by a flow of air into the growth chamber (Buer et al. 1996). In gas-phase bioreactors, the hairy roots must be manually distributed on frames (Kim et al. 2002).

As shown in Figure 2.4, a novel ground-joint column bioreactor was developed for hairy root cultures. The dimension of the novel column bioreactor is 12 cm (outer diameter) by 68 cm (height, including inlet and outlet glass tubing). The middle part of the column is divided into two chambers for hairy root growth. The unique design of the bioreactor is that the three parts of the column (top, middle, and bottom) are connected by ground joints. The ground joint connection makes the column assembly much easier and facilitates the handling of hairy roots. The ground joints provide a reliable seal to avoid contamination. In addition, this design provides modularity and flexibility for scaling up.

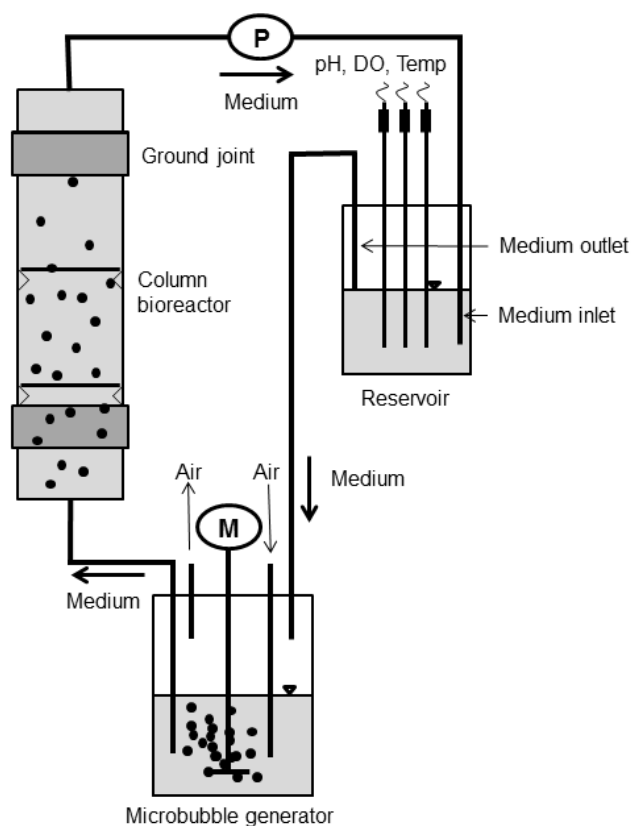


Figure 2.4 Microbubble generator connected to a novel ground-joint column bioreactor

The column bioreactor, microbubble generator, and reservoir were connected by silicon tubes. The medium was moved by a peristaltic pump from the top column bioreactor to the reservoir, where the extra medium was automatically transferred to the microbubble generator by positive pressure. The medium in the microbubble generator flowed into the column bioreactor by negative pressure. The medium level in the column bioreactor remained constant because the medium was pumped out from the top and simultaneously absorbed into the column from the bottom. The medium level in the reservoir was constant because the vessel was sealed, except for the medium inlet and outlet. The medium outlet was a short silicon tube connected to the top of the reservoir (Figure 2.4). When the medium level was lower than the bottom of the outlet tube, air was pushed out as liquid medium was pumped into the reservoir. When the medium level reached the bottom of the outlet tube, liquid medium was pushed out and the medium level remained at the bottom of the outlet. The medium level in the microbubble generator was constant because the medium transferred into the generator was equivalent to the medium transferred out of the chamber (absorbed into the column bioreactor).

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CHAPTER 3

EFFECTS OF NUTRIENTS ON TOBACCO HAIRY ROOT GROWTH AND NICOTINE PRODUCTION

1. Abstract

Box-Behnken design was used to investigate the effect of various parameters on tobacco hairy root growth. Sucrose stimulated the growth of tobacco hairy roots. Although nicotine production (mg g^{-1} fresh root weight) was not significantly affected by nutrients, nicotine release was stimulated by low sucrose concentration. The culture medium with hairy roots showed accumulation of glucose and fructose, which were caused by sucrose hydrolysis. Sucrose hydrolysis ceased when the hairy roots were removed from the culture medium. The tobacco hairy roots were able to grow with glucose and/or fructose as carbon sources, but the root growth was significantly lower than that with sucrose. The data showed that the low root growth was unlikely caused by osmotic stress or carbon source starvation.

2. Introduction

Secondary metabolites from plants (phytochemicals) are a valuable source of pharmaceuticals, dyes, fragrances, and flavors (Oksman-Caldentey and Inze 2004). Phytochemicals can be produced by whole plants, plant cell suspension cultures, and plant tissue cultures. Hairy roots are developed by the infection of wounded plants with *Agrobacterium rhizogenes* (Gelvin 2005; Guillon et al. 2006; Pitzschke and Hirt 2010). As phytochemical producers, hairy roots have advantages such as genetic stability and rapid growth in hormone-free media (Flores et al. 1987; Shanks and Morgan 1999). In

addition, hairy roots promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types.

Plants in the genus *Nicotiana* predominately produce nicotine alkaloid (Saitoh et al. 1985). Nicotine can be used as a potent natural insecticide (Kircher and Lieberman 1967; Richardson and Busbey 1937; Smith and Goodhue 1943) and potential anti-inflammatory agent (Isman 2006; Mabley et al. 2011). Nicotine biosynthesis in *Nicotiana tabacum* (tobacco) is exclusively located in root tips and root growth is an absolute requirement for net nicotine production (Baldwin 1988; Dawson 1942a, b; Dawson and Solt 1959; Solt 1957). In a previous investigation, *N. tabacum* hairy root lines with reduced expression levels of a nicotine uptake permease (*NUPI*) showed increased nicotine levels in the culture media (Hildreth et al. 2011; Zhao et al. 2013). *NUPI* is a plasma membrane-localized nicotine-specific uptake transporter that is mostly expressed in root tips, where nicotine synthesis is also localized.

For a novel hairy root line utilized as phytochemical producer, parametric study could be a first step towards bioprocess control and optimization. The objective of this study was to show the effects of nutrients in Gamborg's B5 medium on a *NUPI-RNAi* reduced expression tobacco hairy root line.

3. Materials and methods

3.1 Hairy root line and culture medium

T13-8-101 is a homozygous *NUPI-RNAi* reduced expression hairy root line developed from *N. tabacum*. T13-8-101 was generated by inoculating sterile leaves (in

the case of T13-8-101, homozygous T3-generation T13-8 transgenic leaves) with *A. rhizogenes* ATCC15384 (Hildreth et al. 2011).

T13-8-101 was maintained by subculturing every three weeks in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 20 g l⁻¹ sucrose and 3 g l⁻¹ phytigel. The Gamborg's B5 mineral mixture and sucrose were dissolved in distilled water and the pH was adjusted to 5.7 with 1 M KOH. After autoclaving, Gamborg's B5 vitamin mixture (Research Products International Corp., Mount Prospect, IL, USA) was added to the medium at 0.112 g l⁻¹ concentration.

3.2 Response surface methodology

A response surface methodology is a mathematical and statistical technique used to optimize the response by finding the relationship between the response and a set of independent variables. In the present study, Box-Behnken response surface methodology was used because it requires fewer combinations of three factors than a central composite design (Mante and Agblevor 2011). The three factors investigated in the Box-Behnken design were sucrose (*A*), Gamborg's B5 mineral mixture (*B*), and Gamborg's B5 vitamin mixture (*C*) (Table 3.1). The responses were tobacco hairy root growth, nicotine production, and release of nicotine into the culture medium.

Data from 15 experiments including 3 central points were generated and analyzed by SAS software (SAS Institute Inc., Cary, NC, USA). Table 3.1 shows the factor levels in the Box-Behnken design. A second-order equation was used to describe the relationship between responses (*Y*) and three variables (*A*, *B*, *C*):

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2$$

Table 3.1 Box-Behnken experimental design

Design pattern	Factor (g l^{-1})		
	Sucrose (A)	Mineral mixture (B)	Vitamin mixture (C)
--0	10	1	0.1
-+0	10	5	0.1
+ -0	50	1	0.1
++0	50	5	0.1
0--	30	1	0
0-+	30	1	0.2
0+-	30	5	0
0++	30	5	0.2
-0-	10	3	0
+0-	50	3	0
-0+	10	3	0.2
+0+	50	3	0.2
000	30	3	0.1
000	30	3	0.1
000	30	3	0.1

3.3 Hairy root cultivation with various carbon sources

Gamborg's B5 medium was supplemented with various sugars: 1 g l^{-1} sucrose (1S), 20 g l^{-1} sucrose (20S), 40 g l^{-1} sucrose (40S), 20 g l^{-1} glucose (20G), 20 g l^{-1} fructose (20F), and a mixture of 10 g l^{-1} glucose and 10 g l^{-1} fructose (10G10F), respectively. Five 10-day-old root tips (about 1.5 cm in length) of T13-8-101 were inoculated in each 250-ml Erlenmeyer flask with 30 ml medium and cultivated on a rotary shaker at 100 rpm and 25°C in dark. Three replicates were carried out for each carbon source and roots were harvested after 10 days cultivation.

3.4 Analytical methods

Fresh hairy roots were rinsed with deionized water, blotted with tissue paper, and the fresh root weight was measured. For dry weight measurement, the fresh hairy roots were dried in oven at 60°C for 12 h and weighed.

High performance liquid chromatography (HPLC) (SCL-10 A, Shimadzu Scientific, Columbia, MD, USA) was used for the quantification of nicotine in hairy root tissue and in culture medium. Fresh hairy roots were added to 15 ml methanol/water (40/60, v/v), ground with mortar and pestle, and extracted on a shaker at 100 rpm and 25°C for 12 h. Culture broth and extracted nicotine samples were filtered with 0.2 µm membrane and analyzed on a Waters, Resolve C18 column (5 µm, 90 Å, 3.9 × 150 mm) with a guard column (Waters, Resolve C18, 5 µm, 90 Å, 3.9 × 20 mm). The isocratic mobile phase consisted of 40/60 (v/v) methanol/water and 2% (v/v) phosphoric acid. The pH of the mobile phase was adjusted to 7.25 with triethylamine. The flow rate of mobile phase was 0.5 ml min⁻¹ and the injection volume was 20 µl. Nicotine was measured with a UV detector at 254 nm. Nicotine standards were prepared in mobile phase at 1, 10, 25, 50, 75, and 100 mg l⁻¹.

Sucrose, glucose, and fructose were measured on Shimadzu HPLC system equipped with a Prevail Carbohydrate ES (5 µm, 4.6 × 150 mm) analytical column and a Prevail Carbohydrate ES (5 µm, 4.6 × 7.5 mm) guard column. The separated sugars were detected by an evaporative light scattering detector (ELSD-LT II, Shimadzu Scientific, Columbia, MD, USA) at 50°C and 350 kPa. Acetonitrile: water (75:25, v/v) was used as mobile phase at constant flow rate of 1 ml min⁻¹. The injection volume was 10 µl.

Standards of sucrose, glucose, and fructose were prepared in deionized water at 1, 5, 10, 15, 20 g l⁻¹.

4. Results and discussion

4.1 Effects of nutrients on the growth of tobacco hairy roots

Table 3.2 shows the responses (root fresh weight, total nicotine, and release ratio). Although the medium pH at the end of cultivation was neither a variable nor a response in the Box-Behnken design, it was listed in Table 3.2 because it could affect the release of nicotine. Table 3.3 shows the analysis of variance on the hairy root growth. The regression was evaluated by the lack-of-fit test. The *p*-value of lack-of-fit was 0.3590 and insignificant at 95% confidence interval (*p*-value > 0.05), suggesting that the regression was adequate to describe the observed data. As shown by the *p*-values, the hairy root growth was significantly affected by sucrose (*A*) (*p*-value < 0.05), but insignificantly influenced by minerals (*B*) and vitamins (*C*) (*p*-values > 0.05). The *p*-value of *A*² was 0.0276 (< 0.05), which implies that the effect of sucrose on root growth was nonlinear.

As shown in Figure 3.1a, the root biomass increased significantly with increasing sucrose concentration but the increase slowed down at high level of sucrose, possibly caused by osmotic stress. The high mineral level slightly improved the root growth at low sucrose concentration, whereas low level of minerals promoted higher root growth at high sucrose concentration (Figure 3.1a). As shown in Figure 3.1b, high levels of vitamins led to high root growth at low sucrose level but lower vitamin concentration was needed to maximize the root growth at high sucrose level. Figure 3.1c shows the

interaction between minerals and vitamins. Low mineral level was favorable for root growth when vitamins were not supplemented (vitamin level at -1, Figure 3.1c), whereas higher mineral level led to increased root growth at high vitamin concentration (vitamin level at 1, Figure 3.1c). This interaction suggests a possible synergetic effect between minerals and vitamins. It is notable that the vitamin mixture was not indispensable for the growth of tobacco hairy roots, because the tobacco hairy roots were able to grow without vitamins (vitamin level at -1 in Figure 3.1c).

Table 3.2 Responses and medium pH of Box-Behnken experimental design

Design pattern	Response			Medium pH
	Root fresh weight (g)	Total nicotine (mg g ⁻¹ FW)	Release ratio	
--0	0.173	1.74 (1.04) ^a	1.48	6.3
-+0	0.092	1.79 (1.54)	6.16	5.4
+ -0	0.731	1.70 (0.72)	0.73	7.2
++0	0.528	2.53 (2.00)	3.77	5.7
0--	0.390	1.95 (0.98)	1.01	7.1
0-+	0.477	1.70 (0.77)	0.83	7.2
0+-	0.363	2.55 (1.96)	3.32	5.7
0++	0.590	2.01 (1.57)	3.57	5.8
-0-	0.105	1.37 (1.09)	3.90	5.7
+0-	0.580	2.21 (1.58)	2.51	6.3
-0+	0.122	2.16 (1.83)	5.55	5.6
+0+	0.502	3.43 (2.38)	2.29	6.5
000	0.507	2.39 (1.66)	2.26	6.5
000	0.662	1.95 (1.21)	1.64	6.4
000	0.550	2.09 (1.37)	1.90	6.3

FW: root fresh weight. Release ratio = nicotine in medium/nicotine in roots. ^a: the

numbers in the brackets show the nicotine in medium

Table 3.3 Analysis of variance for root fresh weight

Term	Degree of freedom	Sum of squares	Mean of squares	<i>F</i> value	Pr > <i>F</i>
A (sucrose)	1	0.427	0.427	42.811	0.0012
B (minerals)	1	0.005	0.005	0.491	0.5148
C (vitamins)	1	0.008	0.008	0.802	0.4117
AB	1	0.004	0.004	0.373	0.5682
AC	1	0.002	0.002	0.226	0.6545
BC	1	0.005	0.005	0.491	0.5148
A ²	1	0.094	0.094	9.454	0.0276
B ²	1	0.004	0.004	0.382	0.5637
C ²	1	0.027	0.027	2.728	0.1595
Model	9	0.566	0.063	6.305	0.0283
Lack-of-fit	3	0.037	0.012	1.932	0.3590
Pure error	2	0.013	0.006		
Total	14	0.616			

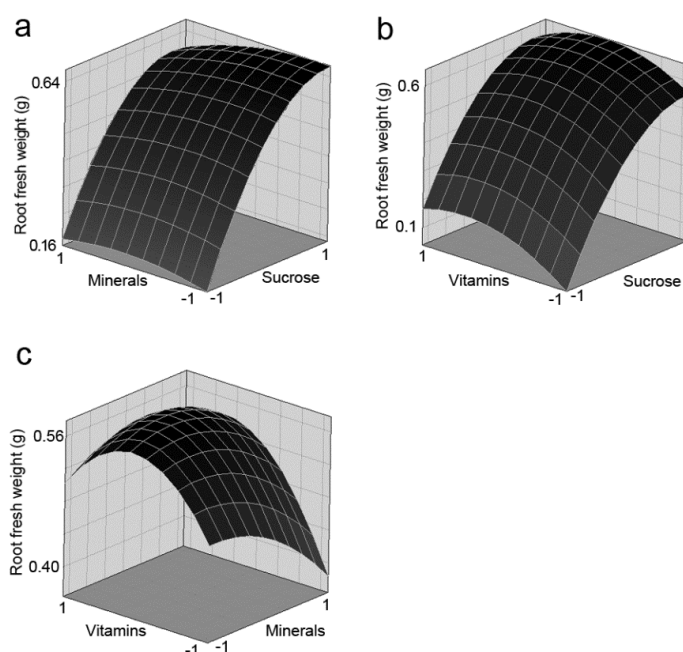


Figure 3.1 Response surface plotting showing the effects of sucrose, minerals, and vitamins on hairy root growth. a: sucrose and minerals; b: sucrose and vitamins; and c: minerals and vitamins

4.2 Effects of nutrients on nicotine production and release

As a model alkaloid product from tobacco hairy roots, nicotine was produced and partly released into the culture medium. In order to show the effects of nutrients on nicotine production, the amount of nicotine in roots and in culture medium were combined and normalized by dividing the amount of nicotine by the root fresh weight (mg g^{-1} root fresh weight). As shown in Table 3.4, none of the variables were significant for the normalized total nicotine production at 95% confidence interval (p -values > 0.05). The absolute amount of nicotine (mg), therefore, mainly depended on the growth of tobacco hairy roots.

The release of nicotine into the culture medium, which was indicated by a ratio of nicotine in medium to nicotine in roots, was significantly influenced by sucrose and mineral mixture (p -values < 0.05 , Table 3.5). The regression for the nicotine release is as follows:

$$\text{Nicotine release ratio} = 1.940 - 0.955A + 1.578B + 0.193C - 0.350AB - 0.490AC + 0.095BC + 1.243A^2 - 0.178B^2 + 0.408C^2 \quad (1)$$

According to Table 3.5, C , AB , AC , BC , B^2 , and C^2 were not significant at 95% confidence interval (p -value > 0.05). Thus, equation (1) was rewritten as:

$$\text{Nicotine release ratio} = 1.940 - 0.955A + 1.578B + 1.243A^2 \quad (2)$$

According to equation (2), the nicotine release ratio decreases at low levels of A (sucrose) and increases at high levels of A , because of the quadratic nature of A . It is notable that three of the four runs with low level of minerals had medium pH higher than 7 (Table 3.2). This high medium pH might be due to the depletion of ammonium, which was preferably utilized by hairy roots (Shin et al. 2003), and the uptake of nitrate, which

Table 3.4 Analysis of variance for total nicotine production

Term	Degree of freedom	Sum of squares	Mean of squares	<i>F</i> value	Pr > <i>F</i>
A (sucrose)	1	0.987	0.987	3.711	0.1120
B (minerals)	1	0.401	0.401	1.506	0.2744
C (vitamins)	1	0.186	0.186	0.700	0.4410
AB	1	0.152	0.152	0.572	0.9482
AC	1	0.046	0.046	0.174	0.6940
BC	1	0.021	0.021	0.079	0.7898
A ²	1	0.001	0.001	0.005	0.9482
B ²	1	0.181	0.181	0.682	0.4464
C ²	1	0.063	0.063	0.238	0.6465
Model	9	2.058	0.229	0.860	0.6034
Lack-of-fit	3	1.229	0.410	8.104	0.1118
Pure error	2	0.101	0.051		
Total	14	3.388			

Table 3.5 Analysis of variance for nicotine release ratio

Term	Degree of freedom	Sum of squares	Mean of squares	<i>F</i> value	Pr > <i>F</i>
A (sucrose)	1	7.296	7.296	20.628	0.0062
B (minerals)	1	19.908	19.908	56.285	0.0007
C (vitamins)	1	0.296	0.296	0.838	0.4019
AB	1	0.490	0.490	1.385	0.2922
AC	1	0.960	0.960	2.715	0.1603
BC	1	0.036	0.036	0.102	0.7623
A ²	1	5.700	5.700	16.116	0.0102
B ²	1	0.116	0.116	0.329	0.5911
C ²	1	0.613	0.613	1.733	0.2451
Model	9	35.374	3.930	11.113	0.0082
Lack-of-fit	3	1.581	0.527	5.625	0.1547
Pure error	2	0.187	0.094		
Total	14	37.143			

Release ratio = nicotine in medium/nicotine in roots

was reported to raise medium pH (Morard et al. 1998). Since the amount of ammonium (0.134 g l^{-1} ammonium sulfate) is much less than that of nitrate (2.5 g l^{-1} potassium nitrate) in Gamborg's B5 medium (Gamborg et al. 1968), low initial concentration of minerals might lead to early depletion of ammonium and utilization of nitrate as main nitrogen source, and consequently a higher medium pH. In our previous study, medium pH above 7 was shown to be associated with a decrease in nicotine release into the hairy root culture medium (Zhao et al. 2013). Thus, the minerals affected the nicotine release by causing the changes in the medium pH.

4.3 Sucrose hydrolysis in tobacco hairy root culture medium

Sucrose is a common carbon source used in hairy root cultures, probably because it is the predominant form of carbon transported from source (leaves) to sink (roots, young leaves, flowers, fruits) tissues in higher plants (Giaquinta 1980). Sucrose at sink regions can be imported into cells via 1) symplastic phloem unloading, 2) uptake by sucrose-specific transporters, and/or 3) sucrose hydrolysis in apoplast and uptake of glucose and fructose by specific carriers (Giaquinta et al. 1983). The hydrolysis of sucrose in apoplast is mediated by invertase (EC 3.2.1.26), which catalyzes the irreversible hydrolysis of sucrose to glucose and fructose (Roitsch and Gonzalez 2004).

In the present study, hairy roots were cultivated with 20 g l^{-1} sucrose as initial sole carbon source. The medium with hairy roots was incubated for 10 days and the medium showed 8.9 g l^{-1} sucrose, 2.4 g l^{-1} glucose, and 2.5 g l^{-1} fructose. Then, the hairy roots were removed from the medium, and the medium without hairy roots was incubated for 4 more days, and the medium showed 9.1 g l^{-1} sucrose, 2.7 g l^{-1} glucose, and 2.5 g l^{-1}

fructose, which were close to the 10 days sugar concentrations. Thus, the sucrose hydrolysis was associated with the tobacco hairy roots, probably due to invertase bound to the cell wall (Roitsch and Gonzalez 2004).

In the present study, glucose and fructose accumulated in the culture medium and these monosaccharides were directly taken up by the tobacco hairy roots (data shown in the next section). These results imply that exogenous sucrose was possibly hydrolyzed in the apoplast prior to uptake into the hairy roots. In a previous investigation on corn roots, exogenous sucrose was hydrolyzed and monosaccharides were taken into the roots, whereas the uptake of endogenous sucrose was via symplastic phloem unloading (Giaquinta et al. 1983). What is unknown in the present study is whether sucrose can be directly utilized by the tobacco hairy roots. Since symplastic phloem unloading is not possible in hairy roots, sucrose utilization can only occur through exogenous sucrose uptake. Further investigations can be carried out using extracellular invertase inhibitors to show more direct evidence of whether exogenous sucrose can be directly transferred into the tobacco hairy roots.

4.4 Effects of various carbon sources on the growth of tobacco hairy roots

Since glucose and fructose accumulated in the tobacco hairy root culture medium, these monosaccharides might be directly used by the tobacco hairy roots without sucrose splitting. Glucose, fructose, and a mixture of these two sugars were investigated for their effects on tobacco hairy root growth. Since the 20 g l⁻¹ monosaccharides had higher osmolarity than the 20 g l⁻¹ sucrose, 40 g l⁻¹ sucrose was used as a control of osmotic stress. The 1 g l⁻¹ sucrose was used as a control of sugar starvation.

As shown in Table 3.6, the 40 g l⁻¹ sucrose (40S) resulted in the highest root dry weight of 77.2 mg, followed by 44.2 mg with 20 g l⁻¹ sucrose (20S). Although the tobacco hairy root tips were able to grow on glucose and/or fructose, the root biomass yield was reduced. The mixture of glucose and fructose (10G10F) showed 3.2 mg root dry weight, which was slightly higher than the 3.0 mg on fructose (20F), but lower than the 4.9 mg on glucose (20G). As shown in Figure 3.2, the hairy roots with sucrose were thick and strong, whereas the roots grown on glucose and fructose were thin and weak.

As shown in Figure 3.2, with 1 g l⁻¹ sucrose (1S), the inoculated five hairy root tips only grew by elongation, but the hairy roots grown on monosaccharides showed branches. The different growth patterns of 1 g l⁻¹ sucrose and monosaccharides imply that the lower root growth with monosaccharides was unlikely caused by carbon source starvation. The 40 g l⁻¹ sucrose which had similar osmolarity to the 20 g l⁻¹

Table 3.6 Effects of various carbon sources on tobacco hairy root growth

Carbon source	Sugar concentration (g l ⁻¹)			Root dry weight (mg)
	Sucrose	Glucose	Fructose	
1S	N.D.	N.D.	N.D.	2.2 ± 0.1
20S	8.9 ± 0.9	2.4 ± 0.3	2.5 ± 0.3	44.2 ± 6.9
40S	19.2 ± 0.2	3.2 ± 0.1	3.2 ± 0.2	77.2 ± 14.6
20G	N.D.	14.1 ± 0.2	N.D.	4.9 ± 0.7
20F	N.D.	N.D.	14.5 ± 0.1	3.0 ± 0.5
10G10F	N.D.	7.0 ± 0.1	7.2 ± 0.3	3.2 ± 0.1

1S: 1 g l⁻¹ sucrose (as initial carbon source); 20S: 20 g l⁻¹ sucrose; 40S: 40 g l⁻¹ sucrose;

20G: 20 g l⁻¹ glucose; 20F: 20 g l⁻¹ fructose; 10G10F: 10 g l⁻¹ glucose + 10 g l⁻¹ fructose;

N.D.: not detectable. Values are expressed in mean ± standard error (n = 3). Sugar

concentrations and root dry weight were measured after 10 days cultivation

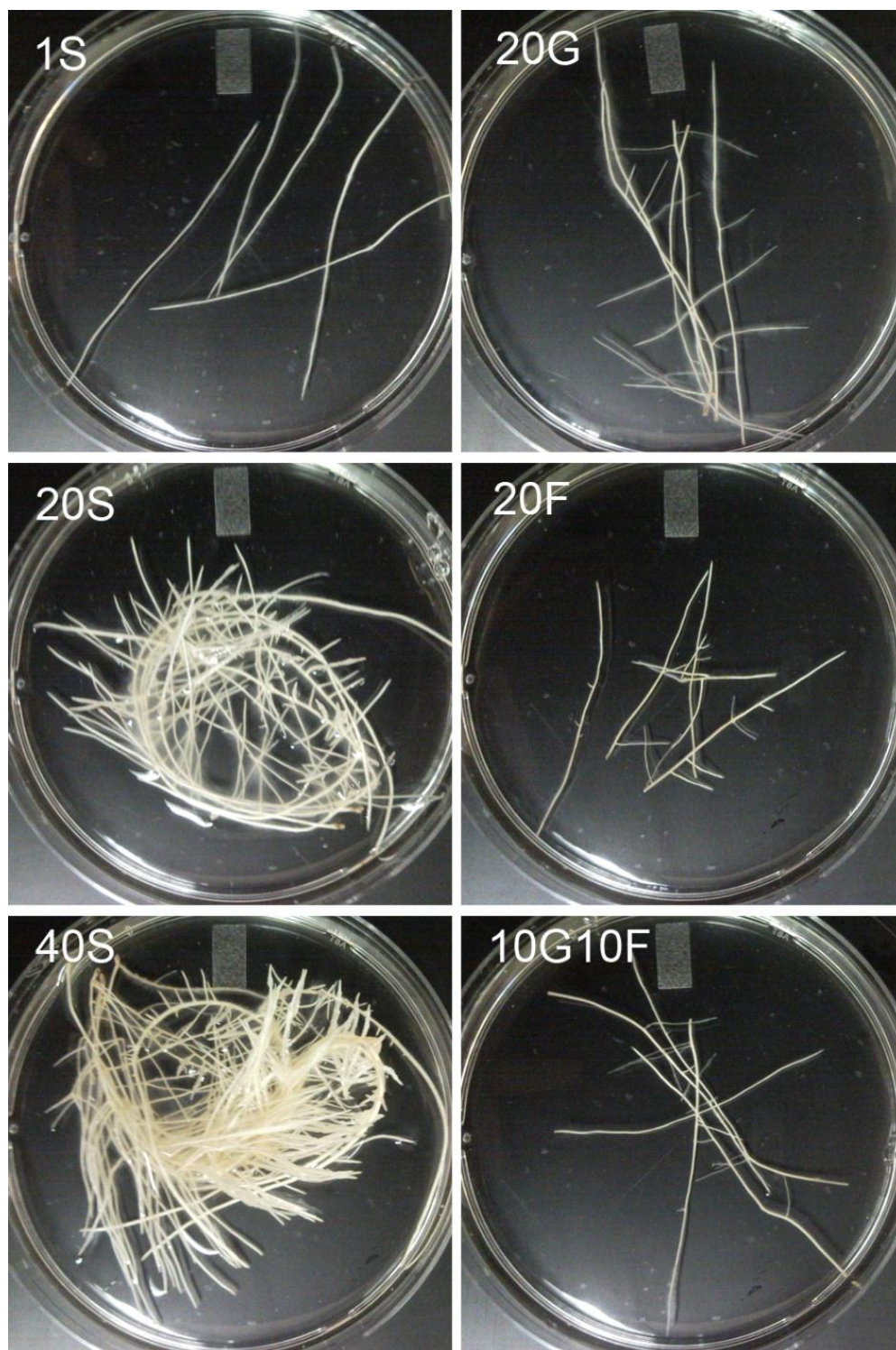


Figure 3.2 Effects of various carbon sources on the morphology of tobacco hairy roots.

1S: 1 g l⁻¹ sucrose; 20S: 20 g l⁻¹ sucrose; 40S: 40 g l⁻¹ sucrose; 20G: 20 g l⁻¹ glucose; 20F:

20 g l⁻¹ fructose; 10G10F: 10 g l⁻¹ glucose + 10 g l⁻¹ fructose

monosaccharides actually showed more hairy root growth. Thus, the lower root growth with glucose and fructose was unlikely a result of osmotic stress.

One possible explanation for the different morphology and reduced root growth on monosaccharides is that sucrose, in addition to a carbon and energy source, may also play a role as a source of signaling in the growth of tobacco hairy roots. In plants, sugars can be used as signaling and have regulatory functions like hormones, and sucrose transport and hydrolysis play key regulatory roles in sugar signal generation (Koch 2004; Rolland et al. 2002, 2006). In the growth of tobacco hairy roots, the possible signaling effect of sucrose might be different from that in plants and needs further investigations. This possible regulatory effect of sucrose might partly explain the stimulative effect of sucrose on tobacco hairy root growth (Table 3.2).

5. Conclusions

In the present study, Box-Behnken design was used to show the effects of nutrients on tobacco hairy roots. Although sucrose stimulated the growth of tobacco hairy roots, the total nicotine production was not significantly affected by the nutrients. The nicotine release was stimulated by low sucrose concentration. The culture medium with hairy roots showed the accumulation of glucose and fructose, which was caused by sucrose hydrolysis. The sucrose hydrolysis ceased when the hairy roots were removed from the culture medium. The tobacco hairy roots were able to grow with glucose and/or fructose as carbon sources, but the root growth was significantly lower than that with sucrose.

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CHAPTER 4

ENHANCED PRODUCTION OF THE ALKALOID NICOTINE IN HAIRY ROOT CULTURES OF *NICOTIANA TABACUM* L.**1. Abstract**

The utility of hairy root cultures to produce valuable phytochemicals could be improved by repartitioning more of the desired phytochemical into the spent culture media, thereby simplifying the bioprocess engineering associated with the purification of the desired phytochemical. The majority of nicotine produced by tobacco hairy root cultures is retained within roots, with lesser amounts exuded into the spent culture media. Reduced expression of the tobacco nicotine uptake permease (NUP1) results in significantly more nicotine accumulating in the media. Thus, *NUP1*-reduced expression lines provide a genetic means to repartition more nicotine into the culture media. The present study examined a wild type and a *NUP1*-reduced expression hairy root line during a variety of treatments to identify culture conditions that increased nicotine accumulation in the media. The *NUP1*-reduced expression line grew faster, used less oxygen, and exuded more nicotine into the media. Basification of the culture media associated with root growth resulted in a dramatic reduction in nicotine accumulation levels in the media, which was reversed by decreasing the pH of the media. Kinetic analysis of hairy root growth and nicotine accumulation in the media revealed a potential improvement in nicotine yields in the media by stimulating the branching of hairy roots.

Zhao B, Agblevor FA, K.C. R, Jelesko JG (2013) Enhanced production of the alkaloid nicotine in hairy root cultures of *Nicotiana tabacum* L. Plant Cell Tissue and Organ Culture 113:121-129

2. Introduction

Hairy root cultures provide novel opportunities for the production of valuable phytochemicals that are synthesized in roots. Hairy roots are developed by infecting plant leaf or stem tissue with *Agrobacterium rhizogenes* that transfers genes that encode hormone biosynthesis enzymes into the plants (Guillon et al. 2006; Willmitzer et al. 1982). Hairy root cultures have several advantages over undifferentiated plant suspension cell cultures (Flores et al. 1999). Hairy roots are genetically stable and grow in hormone-free culture media. Hairy roots show rapid growth and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types. Hairy root lines producing valuable phytochemicals have been developed from various plant species (Danphitsanuparn et al. 2012; Dehghan et al. 2012; Kim et al. 2013; Syklowska-Baranek et al. 2012).

The utility of producing a phytochemical in hairy root cultures depends upon both the yield of the desired phytochemical, and the expense associated with the bioprocessing required to isolate the chemical of choice. Phytochemicals accumulating primarily in the root tissue present a bioprocessing challenge because the root tissue must be homogenized and the phytochemical extracted and purified from a chemically complex solution. On the other hand, phytochemicals that accumulate mostly in the culture media are in a chemically less complex milieu than those which are retained within the hairy root tissue. Therefore, phytochemicals that accumulate in the culture media require substantially less bioprocessing effort to isolate and purify. To that end, a variety of treatments aimed at repartitioning phytochemicals from the root tissue into the media have been tried. These range from mild surfactants that do not dramatically effect hairy

root growth, to treatments such as electroporation or changes in pressure that disrupt membrane integrity resulting in loss of root viability (Cai et al. 2012). What is needed is a genetic-oriented approach to increase the accumulation of the desired phytochemical directly into the root culture media.

Plant roots exude a substantial amount of fixed carbon into the surrounding media/matrix. As much as 20% fixed carbon is exuded from roots (Walker et al. 2003). Exudation of secondary metabolites by hairy roots has been reviewed in detail by Cai et al. (2012). The chemical composition of root exudates ranges from polysaccharides to diverse specialized (secondary) metabolites. Relatively little is known about the biochemistry and molecular components responsible for root exudation. In the case of several phytoalexins, particular ABC (ATP-binding cassette) type transporters affect the levels of specific phytoalexins released from *Arabidopsis* roots, indicating that ABC-type transporters contribute to release of certain secondary metabolites into the media (Badri et al. 2012). It is likely that other categories of transporters also contribute to root exudation as well. Thus, plasma membrane-localized phytochemical transporters are desirable genetic targets for modulating phytochemical exudation into the media.

Plants belonging to the family of Solanaceae produce a variety of pyridine, tropane, and nortropane alkaloids. Within the Solanaceae, plants belonging to the genera *Nicotiana* predominantly produce the pyridine alkaloid nicotine (Saitoh et al. 1985). Tobacco produces nicotine exclusively in the roots (Dawson 1942a, b; Dawson and Solt 1959; Solt 1957). Nicotine biosynthesis is specifically localized to root tips (Dawson 1942b; Solt 1957). Moreover, root growth is an absolute requirement for net nicotine production (Baldwin 1988; Dawson 1942b). Nicotine is a potent natural insecticide

(Kircher and Lieberman 1967; Richardson and Busbey 1937; Smith and Goodhue 1943) and potential anti-inflammatory agent (Isman 2006; Mabley et al. 2011). *Nicotiana tabacum* hairy root lines with reduced expression levels of a nicotine uptake permease (*NUPI*) show increased nicotine levels in the culture media (Hildreth et al. 2011). *NUPI* is a plasma membrane-localized nicotine-specific uptake transporter that is mostly expressed in root tips, where nicotine synthesis is also localized. It is currently not known how reduced *NUPI* transcript levels results in more nicotine accumulation in the culture media. Nevertheless, as a model system for the genetic manipulation of phytochemical transport processes, *NUPI* is a target gene with demonstrated effects increasing the accumulation levels a valuable plant alkaloid in the media of hairy root cultures. Therefore, this study is focused on genetic (*NUPI*) and environmental (aeration levels and media pH) interactions that promote increased nicotine yields into the root culture medium. The kinetics of hairy root biomass and nicotine accumulation in the media brought new insights that could be valuable for developing an operation strategy to maximize the accumulation of nicotine in the culture media.

3. Materials and methods

3.1 Hairy root lines and culture conditions

A new wild-type (Xanthi-105) and a new T3 generation homozygous *NUPI*-reduced expression (T13-8-101) (Hildreth et al. 2011) hairy root line of *N. tabacum* were generated by inoculating sterile leaves, respectively, with *A. rhizogenes* ATCC15384. Xanthi-105 and T13-8-101 were maintained on solid media by subculturing every three

weeks on Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 20 g l⁻¹ sucrose and 3 g l⁻¹ phytigel.

Hairy roots were grown in screw cap sealed 300-ml Erlenmeyer flasks and Gamborg's B5 medium that lacked phytigel. As shown in Table 4.1, various volumes of air above the Gamborg's B5 media were used to investigate the effects of oxygen on hairy root growth and nicotine release into the culture media. For example, a flask designated A270 was comprised of 30 ml Gamborg's B5 liquid media inoculated with five 10-day-old root tips (about 0.015 g fresh weight) in a screw cap sealed 300 ml Erlenmeyer flask. Control flasks were similarly inoculated, except filter paper was used to cover the flask opening to allow continuous air exchange. Sample S270 were cultures with 270 ml air sealed in 300-ml screw cap flask that was not shaken. In samples N270, 270 ml of nitrogen gas was sealed in 300-ml screw cap flask and shaken. Three technical replicates were carried out for each treatment. The flasks were kept at 25°C in dark and harvested after 10 days incubation. Hairy roots were also grown in a 1-liter fermentor BIOSTAT Q (B. Braun Biotech International, Germany). The fermentor was filled with 0.7 liter of Gamborg's B5 medium and aerated at 1 liter min⁻¹. The fermentor was inoculated with 40 root tips (about 0.12 g fresh weight) of the *NUPI*-reduced expression line T13-8-101 and incubated at 25°C and ambient illumination (approximately on a 12-h light/12-h dark cycle). The medium pH in the fermentor was manipulated by adding 0.5 M H₂SO₄ and KOH.

Table 4.1 Aeration conditions for tobacco hairy roots grown in Erlenmeyer flasks

Code	Medium volume (ml)	Shaking speed (rpm)	Closure	Gas phase	Air volume (ml)
Control	30	100	filter paper	Air	270
A270	30	100	screw cap	Air	270
A200	100	100	screw cap	Air	200
A100	200	100	screw cap	Air	100
S270	30	0	screw cap	Air	270
N	30	100	screw cap	N ₂	0

3.2 Kinetics

Root growth rate was calculated by differentiating the smoothed curve of root growth in 1-liter fermentor (Figure 4.1). Extracellular nicotine production rate was calculated by differentiating the smoothed time course of extracellular nicotine production in the 1-liter fermentor (Figure 4.1). Specific rates were calculated by dividing the rates by root dry weight.

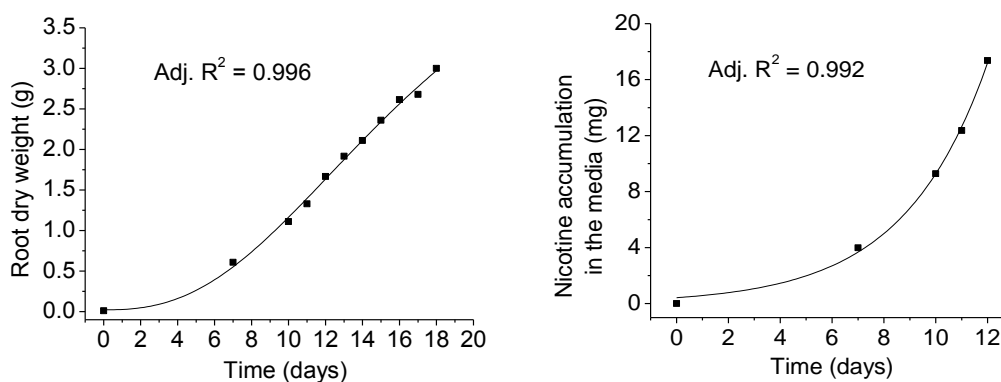


Figure 4.1 Smoothed root growth of T13-8-101 (left) and nicotine accumulation in medium (right) in 1-liter fermentor

3.3 Analytical methods

For dry weight measurement, fresh hairy roots were dried in an oven at 60°C for 12 h and then weighed. For hairy roots grown in the 1-liter fermentor, the dry weight was calculated from media conductivity. The relationship between conductivity (ms cm^{-1}) and root dry weight (g l^{-1}) of T13-8-101 was determined in shake flask cultures:

$$\Delta \text{ root dry weight} = -3.1717 \times \Delta \text{ conductivity}$$

Three shaker flasks were harvested on day 3, 7, 10, 14, and 21 for the measurements of root dry weight and media conductivity. The linear correlation between media conductivity and root dry weight is shown in Figure 4.2. Media conductivity was measured using a Traceable Digital Conductivity Meter (Control Company, Friendswood, TX, USA).

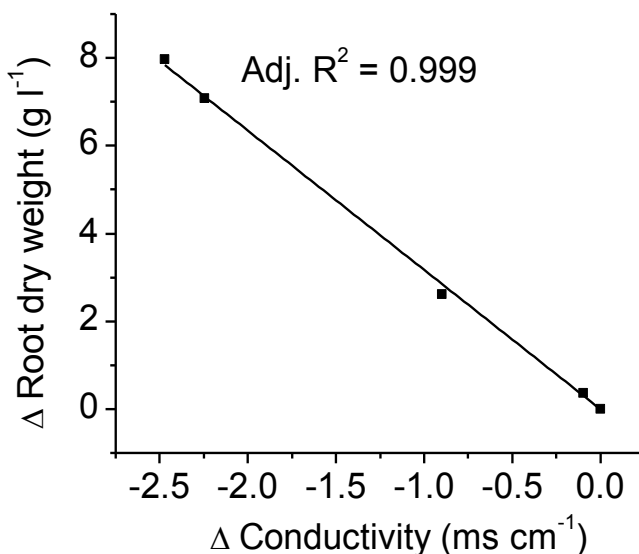


Figure 4.2 Correlation between medium conductivity and root dry weight of T13-8-101

The nicotine levels in the spent culture media were determined as follows: Ten ml of culture media was added to 1 ml 5 M KOH and extracted with 20 ml of CH₂Cl₂. The extract was concentrated by evaporation and analyzed on Agilent 7890A gas chromatography equipped with FID. Compounds were separated on a 30 m × 0.32 mm HP-5 capillary column (Agilent Technologies, Palo Alto, CA, USA) with helium as carrier gas. The split ratio was 100:1 and the injection volume was 1 µl. The injector temperature was set at 250°C. The initial oven temperature was 120°C, which was held for 1 min and programmed at 10°C min⁻¹ to 200°C. The detector temperature was set at 300°C. A calibration curve was established by using a nicotine standard (Sigma-Aldrich, St Louis, MO, USA). Sucrose, glucose, and fructose were measured on Shimadzu HPLC system (Shimadzu, Columbia, MD, USA) equipped with 802 BP-100 H⁺ carbohydrate column (Benson Polymeric Inc., USA) and an evaporative light scattering detector (Shimadzu, Columbia, MD, USA). Deionized water was used as mobile phase at a flow rate of 0.4 ml min⁻¹. In the 1-liter fermentor, pH value and dissolved oxygen were monitored online by electrodes.

4. Results and discussion

4.1 Effect of aeration on tobacco hairy root growth

Oxygen is usually a limiting factor in hairy root cultures because of the low solubility of oxygen in water (8.3 mg l⁻¹, 25°C, 1 atm, in water) and the mass transfer resistance imposed by the entangled root clump. Thus, one of the major concerns in large-scale hairy root cultures is oxygen supply. Bioprocess design for oxygen supply

depends on the amount of oxygen required by hairy roots. Quantification of oxygen demand, therefore, could be valuable for a rational bioprocess design.

As shown in Table 4.1, six aeration conditions were used to evaluate the effect of oxygen on the growth of tobacco hairy roots. The Control treatment was comprised of a shake flask capped with filter paper allowing continuous air exchange between the flask headspace and the surrounding environment. The amount of air available to five inoculated hairy root tips was controlled by adding different volumes of Gamborg's B5 media to screw-cap sealed 300-ml Erlenmeyer flasks. For example samples A270, A200, and A100 were filled with 30, 100, and 200 ml of Gamborg's media, respectively. In sample N270, 270 ml of air in the flask was replaced with N₂, effectively purging oxygen from the headspace. In addition to the amount of oxygen, mass transfer also influences the oxygen uptake by hairy roots, so a static culture (S270) was performed to demonstrate the effect of agitation on the oxygen available to the hairy roots.

The effect of aeration on T13-8-101 is shown in Table 4.2. The continuous air exchange (Control) produced 0.049 g dry root biomass after 10 days culture, which was the highest among the six aeration conditions. Root dry weight decreased from 0.044 to 0.022 g with decreasing air volumes sealed in the flasks. Root dry weight of 0.019 g was recorded for static culture S270, which was nearly 60% less than that of the aerated A270 culture. When the air in the flask was replaced with N₂ (sample N270), little root growth was observed. As a comparative study, identical aeration conditions were applied to the wild-type line. As demonstrated in Table 4.2, root growth of Xanthi-105 was lower than that of T13-8-101. Figure 4.3 shows the relationship between the volume of air sealed in the flask and the root dry weight. In general, the root biomass increased linearly with

increasing air volume. Figure 4.4 shows the images of T13-8-101 and Xanthi-105 hairy root cultures grown under various aeration conditions. The positive effect of oxygen on root growth was clearly demonstrated. The inoculated hairy root tips grown in the presence of more air both extended to greater length and branched to form more root tips, eventually forming an entangled root matrix. In contrast, hairy root tips cultured with less air showed less root biomass, less branching, and thus fewer root tips. These results are consistent with previous findings in which oxygen was found to be a necessary and growth-limiting nutrient for hairy root cultures (Kanokwaree and Doran 1997; Suresh et al. 2001).

Oxygen demand varies among hairy root species. A quantitative correlation between oxygen (air) and tobacco hairy root growth was not previously established in the literature. From the data in Figure 4.3, the air demand for of the tobacco hairy root cultures was calculated. The *NUPI*-reduced expression line T13-8-101 showed a slope of 1.52×10^{-4} (g ml⁻¹), indicating the growth of one gram dry weight of T13-8-101 biomass requires around 6.6 liters of air. According to the linear correlation for the wild type Xanthi-105, which showed a slope of 1.35×10^{-4} (g ml⁻¹), the growth of one gram dry weight of Xanthi-105 biomass requires about 7.4 liters of air. These kinetic calculations are only approximate because it is assumed that: the oxygen sealed in the flask was completely consumed at the end of culture, the amount of oxygen dissolved in the media was negligible, and oxygen was the limiting nutrient for growth. Nevertheless, these calculations provide a preliminary estimation of oxygen demand for tobacco hairy roots, which might be useful in bioprocess design for large-scale tobacco hairy root cultivations. The relative greater efficiency of oxygen utilization to root biomass by the *NUPI*-

Table 4.2 Effects of aeration on tobacco hairy root growth and nicotine accumulation in medium

Aeration conditions	Root dry weight (g)		Nicotine (mg)		Nicotine/root dry weight (mg/g)	
	T13-8-101	Xanthi-105	T13-8-101	Xanthi-105	T13-8-101	Xanthi-105
Control	0.049 ± 0.010	0.042 ± 0.007	1.77 ± 0.10	0.62 ± 0.24	36	15
A270	0.044 ± 0.006	0.038 ± 0.008	1.19 ± 0.01	0.49 ± 0.05	27	13
A200	0.034 ± 0.003	0.032 ± 0.004	0.80 ± 0.01	0.46 ± 0.04	24	14
A100	0.022 ± 0.002	0.018 ± 0.004	0.35 ± 0.03	0.08 ± 0.03	16	4
S270	0.019 ± 0.001	0.017 ± 0.003	0.28 ± 0.11	0.11 ± 0.04	15	6
N270	0.002 ± 0.000	0.002 ± 0.001	0.03 ± 0.03	0.01 ± 0.00	15	5

Aeration conditions, see Table 4.1. Values of root dry weight and nicotine are expressed as the mean ± standard deviation

reduced expression line T13-8-101 used in this study correlated with the previous observations that the roots of *NUPI*-reduced expression plants grow significantly faster than wild type roots (Hildreth et al. 2011), suggesting there is some metabolic efficiency conferred by lower *NUPI* expression levels in roots.

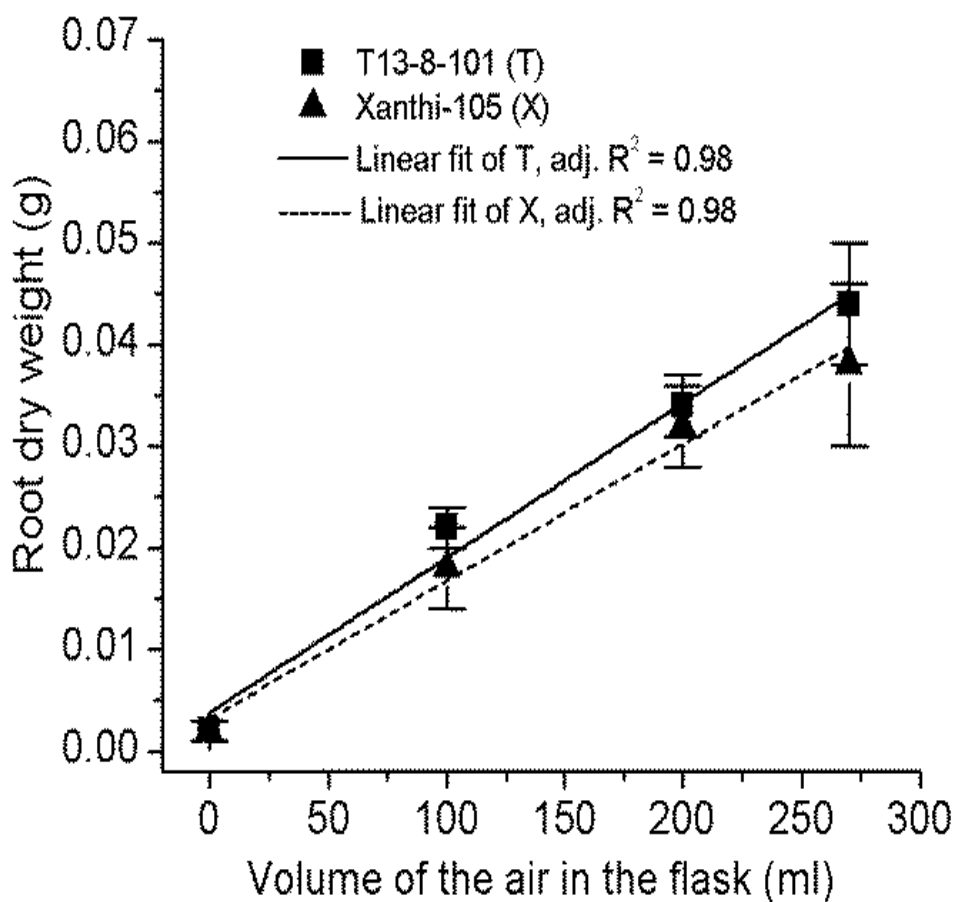


Figure 4.3 Correlation between volume of air sealed in flask and root dry weight. Values are expressed as the mean \pm standard deviation

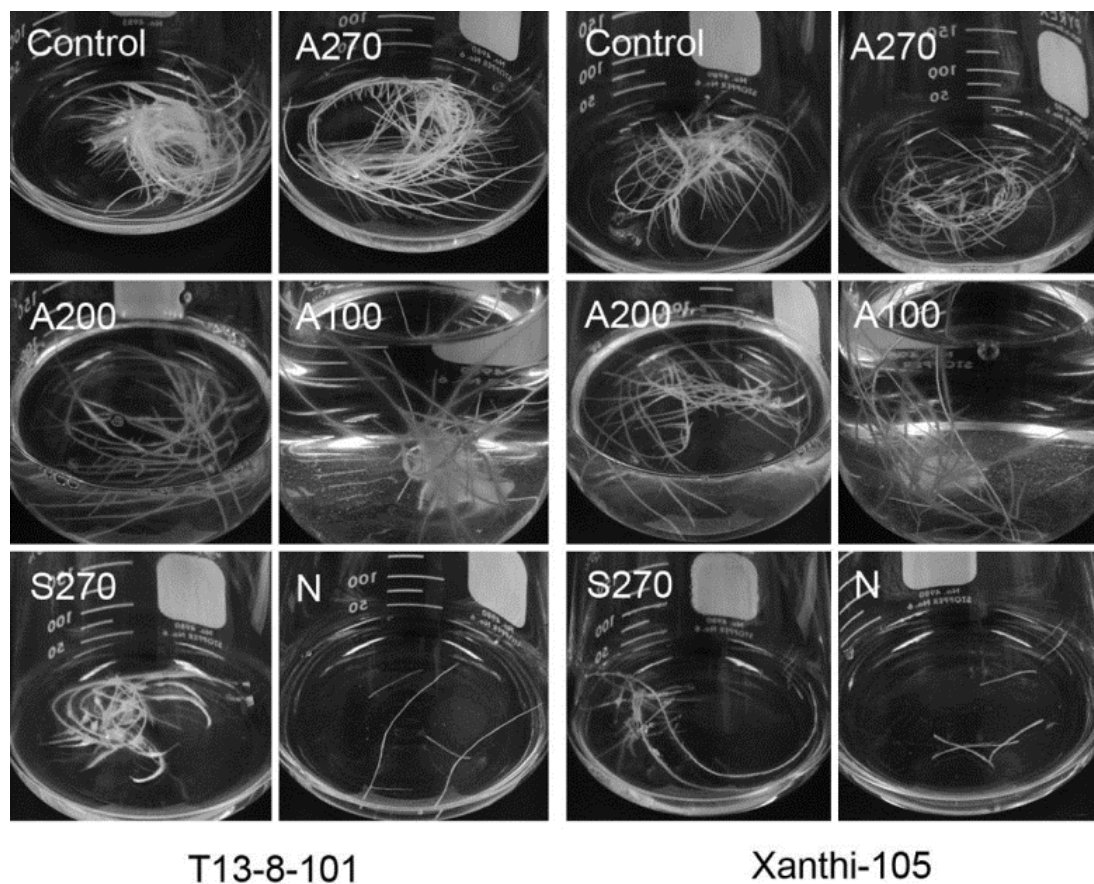


Figure 4.4 Tobacco hairy roots grown under various aeration conditions. In Control, filter paper was used as closure to allow continuous air exchange. In A270, A200, and A100, different volume of air was sealed in 300-ml screw cap flasks. S270 represents static cultures with 270 ml air sealed in 300-ml screw cap flask. In sample N270, 270 ml nitrogen gas was sealed in 300-ml screw cap flask

In liquid media, oxygen transfers from gas phase to liquid phase and finally to the solid surface of hairy roots. This process is influenced by convection and diffusion factors. In shake flask cultures, bulk flow of liquid media greatly reduces the oxygen concentration gradient in the medium, whereas diffusion is the primary force of oxygen transfer during static conditions. The comparison between S270 (static) and A270 (shake)

indicated that the bulk flow of the liquid medium (or convective oxygen transfer) played an important role supplying oxygen to the hairy roots. Since hairy roots develop into an entangled root matrix that resists the bulk flow of liquid media between entangled roots, improved media mixing between individual roots should greatly facilitate the oxygen supply to the hairy root cultures.

4.2 Effect of oxygen on extracellular nicotine production

The effects of various aeration conditions on nicotine accumulation in the media were demonstrated in Table 4.2. During continuous air exchange, a mean of 1.77 mg of nicotine was released by the *NUPI*-reduced expression line T13-8-101. Diminished air availability resulted in decreased nicotine accumulation levels in the media. The mean nicotine released into the media in S270 was 0.28 mg and sample N270 only produced an average of 0.03 mg nicotine in the 30 ml culture. The ratio of nicotine/root dry weight (mg/g) decreased gradually from 36 in continuous air exchange Control to 15 in sample N270 containing only nitrogen in the headspace. In comparison with the *NUPI*-reduced expression hairy root line, the wild type Xanthi-105 hairy root line produced significantly less nicotine in the media (Table 4.2). The control treatment resulted in an average of 0.62 mg nicotine in the 30 ml medium, which was only 35% of the 1.77 mg nicotine produced by T13-8-101. Likewise, the nicotine/root dry weight ratios of the wild type line were much lower than those of the *NUPI*-reduced expression line.

The nicotine levels in the media was plotted versus the root dry weight, as well as versus the volume of the air sealed in the flask (Figure 4.5). For T13-8-101 the nicotine yields in the culture media were greater on both a per root biomass (Figure 4.5a) and per available air basis (Figure 4.5b) relative to the Xanthi-105 wild type line. Interestingly,

the net root biomass for any particular aeration condition did not result in significant differences of final root biomass between the two lines. Thus, the increased nicotine yields from the *NUPI*-reduced expression line T13-8-101 was due to more nicotine released from this *NUPI*-reduced expression line, and not merely due to increased root biomass accumulation. It is not known whether the increased levels of nicotine released from *NUPI*-reduced expression lines was due to increased release from root tips or release from the open xylem vessels at the basal end of each hairy root section used to initially inoculate the cultures.

The effect of aeration on secondary metabolites stored in hairy roots has not been conclusively established (Suresh et al. 2001; Wilhelmson et al. 2006), and it is not known whether oxygen affects alkaloid accumulation in the medium. In the present study, increased aeration significantly enhanced the rate of hairy root growth and the accumulation of nicotine in the spent culture media. The nonlinear relationship between

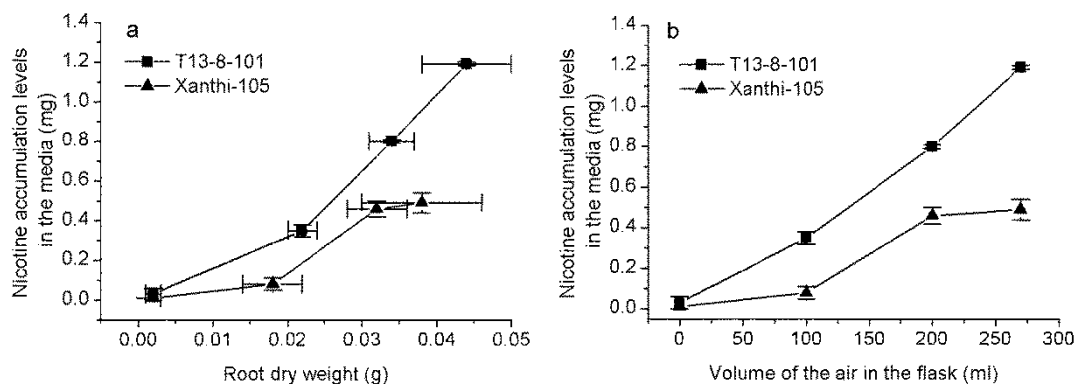


Figure 4.5 Nicotine accumulation levels in the media versus root dry weight (a) and volume of the air sealed in flask (b). Values are expressed as the mean \pm standard deviation

extracellular nicotine and root dry weight (Figure 4.5a) indicates that increased overall hairy root biomass can only partly explain the enhanced nicotine production. The increase in biomass (measured in terms of g dry weight) is the product of two distinct growth processes: 1) cell division and elongation of the five initial root tips initially inoculated in the flask, and 2) the formation of additional lateral root tips. Thus, as the hairy root cultures grow, there are increasing numbers of root tips in the culture. The number of root tips in a hairy root culture is an especially important parameter because nicotine biosynthesis is restricted to only growing root tips. This likely explains the observed non-linear relationship between extracellular nicotine levels and root biomass in Figure 4.5a. The initial *NUPI*-reduced expression lines in Hildreth et al. (2011) were hemizygous *NUPI-RNAi* transgenic lines resulting in seedlings with faster growing roots and hairy roots that release more nicotine to the culture media. The *NUPI*-reduced expression line used in this study was an independent homozygous *NUPI-RNAi* transgenic line that recapitulated both of these phenotypes. Having established that genetically reducing *NUPI* expression levels resulted in greater nicotine yields in the culture media of shake flasks, this hairy root line was grown in a fermentor that provides a more controlled culture environment with which to further improve nicotine yields in the culture media.

4.3 The effect of medium basification on nicotine accumulation levels in the media

Another environmental factor that might be important for optimal nicotine yield in the culture media is hydrogen ion concentration. In batch shake hairy root cultures, media pH is adjusted only when preparing the medium, whereas a fermentor can provide

continuous pH monitoring and control. Moreover, media basification usually occurs during hairy root propagation and the effects on alkaloid accumulation in the media have not been extensively studied.

The *NUPI*-reduced expression tobacco hairy root line T13-8-101 was grown in a 1-liter fermentor and various media conditions were monitored. The time course of nicotine accumulating in the culture media was plotted together with the pH, dissolved oxygen, and root dry weight, respectively. As shown in Figure 4.6a the culture media pH decreased beginning on the third day and reached a minimum value of 4.5 on the seventh day. Between day 12 and day 18, the medium pH increased rapidly from 5 to 8. In general, the dissolved oxygen decreased from around 95% to 85% air saturation (Figure 4.6b). Medium conductivity was not recorded after day 18 because acid and base were added after that day to adjust the pH of the media. As shown in Figure 4.6c, root dry weight increased continuously to 4.3 g l⁻¹ on day 18. As demonstrated in Figure 4.6d, the concentrations of glucose and fructose increased with the consumption of sucrose from the media. When sucrose was almost exhausted on day 18, glucose and fructose concentrations began to decrease.

As shown in Figure 4.6a, nicotine accumulated in the media slowly during the first week and increased rapidly in the next 5 days to a maximum value of 24.8 mg l⁻¹. Nicotine in the media then decreased from day 12 and almost completely disappeared on day 18. The concomitant decrease in media nicotine levels and increase in media pH between day 12 to day 18 suggests that these were mechanistically linked processes. At high pH nicotine is an uncharged free base (Lochmann et al. 2001) and this neutral base

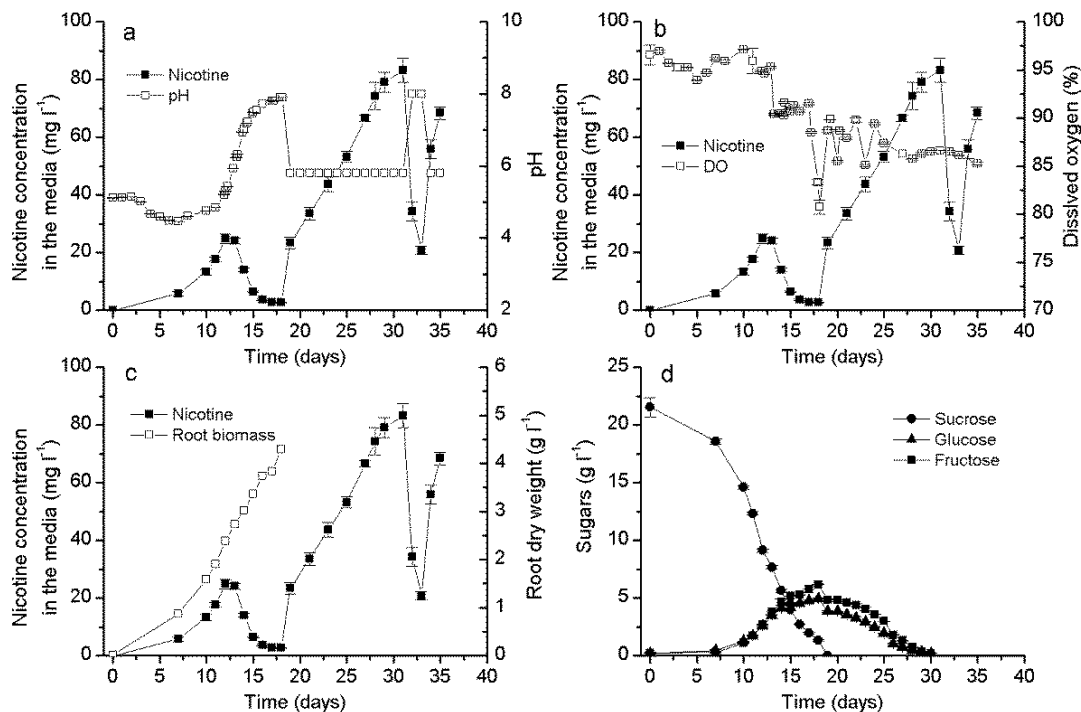


Figure 4.6 Time courses of T13-8-101 cultured in a 1-liter fermentor. a: nicotine concentration in the media versus media pH; b: nicotine concentration in the media versus dissolved oxygen (DO); c: nicotine concentration in the media versus root dry biomass; and d: sucrose, glucose, and fructose concentration in the media. Values are expressed as mean \pm standard deviation

can diffuse through plant membranes, and presumably becomes trapped in the acidic vacuole as a cationic quaternary amine (Larsen et al. 1993).

When the pH of culture broth was reduced from 8 to 5.8 on day 18, nicotine concentration in the media increased rapidly. Moreover, maintaining the pH at 5.8 increased the nicotine in the media to 83.3 mg l⁻¹. The effect of basified media on nicotine release was confirmed by deliberately raising the pH to 8 and then lowering it to

5.8. As expected, nicotine in the culture media decreased at pH 8, and then increased at pH 5.8. It is clear from these results that basic culture media caused the decline in nicotine accumulation in the culture media. What is not clear from the present study is whether the increase in nicotine concentration in acidified culture media was due to desorption, a diffusion process, or an active nicotine transport process across the plasma membrane. A tobacco nicotine MATE-type transporter called NtJAT1 was shown to localize to leaf protoplast vacuolar membranes. NtJAT1 was also proposed to localize to root plasma membranes where it was hypothesized to act as a proton antiporter of nicotine from the cytoplasm into the apoplast (Morita et al. 2009).

The release of nicotine from *Nicotiana* hairy root cultures into the media was previously reported (Green et al. 1992; Hamill et al. 1986; Larsen et al. 1993; Rhodes et al. 1986). Decline in extracellular nicotine was recorded for hairy roots of *N. glauca* and *N. rustica*, while the nicotine released from hairy roots of *N. tabacum* increased throughout the batch cultivation (Green et al. 1992; Hamill et al. 1986; Larsen et al. 1993). Although the mechanism of medium pH impact on nicotine release needs to be further clarified, an inverse correlation between medium basification and nicotine accumulation in the media was clearly demonstrated. Therefore, the decrease in nicotine concentration in the media, which is detrimental to the production efficiency, can be avoided simply by controlling the medium pH. From a practical point of view, this finding provides important insights for enhancing (or suppressing) the release of other secondary metabolites with similar protonation properties. Furthermore, the present study demonstrates that tobacco hairy roots have a significant impact on medium pH. In summary, the medium pH of tobacco hairy roots need to be closely monitored and

controlled throughout the cultivation to optimize alkaloid release into the spent culture media.

4.4 Kinetics of hairy root growth and nicotine accumulation in the media

Since nicotine levels in the media decreased after day 12, the relationship between root growth and nicotine accumulation in the media was kinetically analyzed for only the first 12 days. The nicotine accumulation in the media was coupled with the hairy root growth (Figure 4.7a). As shown in Figure 4.7b, the nicotine accumulation rate in the media lagged behind the root growth rate during the first week of hairy root culture, but increased rapidly from day seven to day 12. The specific root growth rate was around 0.8 day⁻¹ on day one and decreased to 0.16 day⁻¹ on day 12. The specific nicotine production rate decreased rapidly during the first four days to 2 mg g⁻¹ day⁻¹ but increased gradually from day 5 to over 3 mg g⁻¹ day⁻¹ on day 12 (Figure 4.7c).

It is noteworthy in Figure 4.7b that the nicotine accumulation rate in the media was low during the first week, but increased rapidly from day eight to day 12. The increase in root growth rate, however, was almost linear. These results suggest that the increase in root growth rate during the first week did not lead to a proportional increase in the nicotine accumulation rate. As discussed above, increase in root biomass is the product of both cell division at the root apical meristemic, cell elongation, and lateral root formation along the root maturation zone. Of these three sources of increased root biomass, only growing root tips are responsible for nicotine biosynthesis (Baldwin 1988; Dawson 1942a, b; Dawson and Solt 1959; Solt 1957). Therefore, a likely explanation for the low nicotine accumulation rate during the first week was that the observed

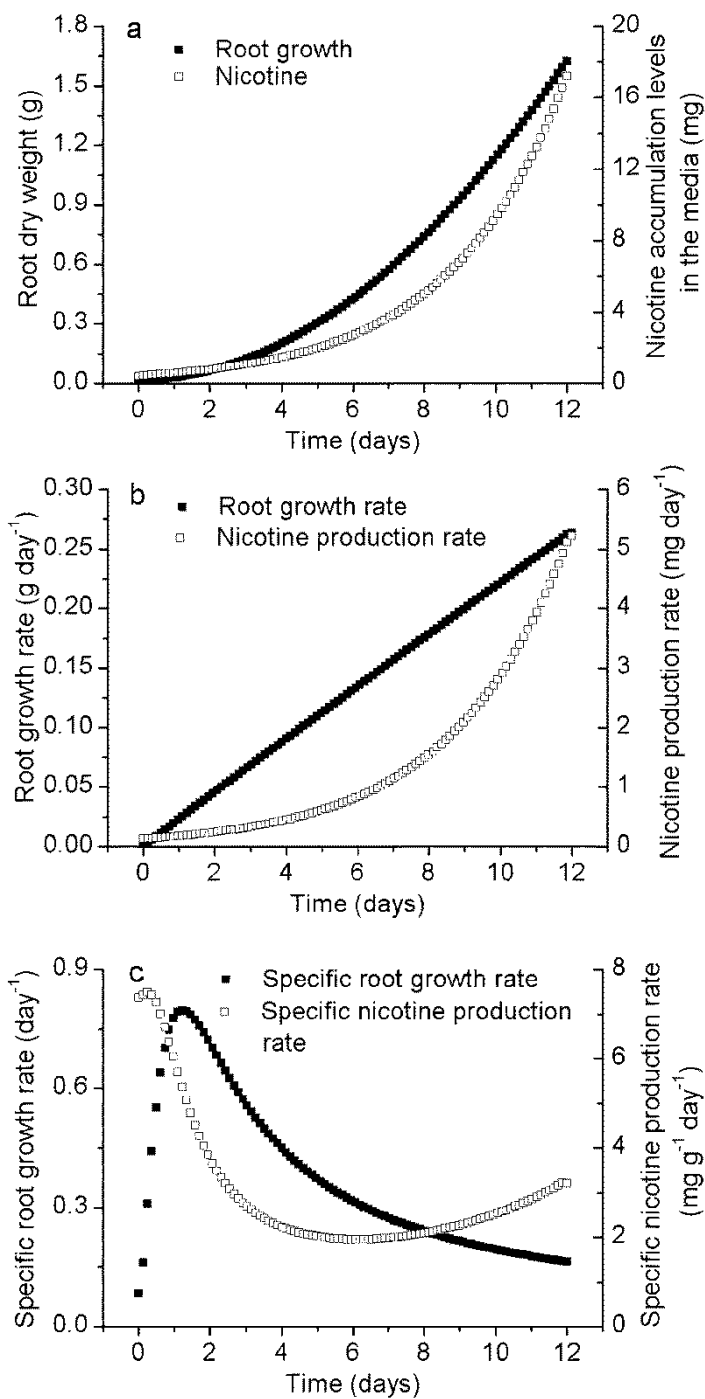


Figure 4.7 Kinetic analysis of transgenic hairy root growth and nicotine accumulation in media. a: root growth and nicotine accumulation in the media; b: root growth rate and nicotine production rate; and c: specific root growth rate and specific nicotine production rate

increase in root biomass was mostly due to the cell division and elongation of the inoculated root tips. Thus, nicotine production during that time was limited to only root tips. However, as the hairy roots elongated and matured, the net number of root tips increased because of the formation of lateral roots. This increase in the total number of root tips (primary and lateral root tips) enhanced the capacity to synthesize nicotine, which was then released into the media. The concentration of increased hairy root biomass correlating with more root tips was visually supported by Figure 4.4 in which hairy root cultures that showed greater biomass accumulation, also showed many more root tips than cultures with less biomass. This explanation is also supported by the specific nicotine accumulation rate in the media shown in Figure 4.7c. The increase in specific nicotine accumulation rate in the media after day seven was likely a result of increased lateral root formation. This finding implies that nicotine accumulation in the media of *NUP1*-reduced expression lines could be further improved by conditions that promote lateral root formation.

5. Conclusions

The growth of tobacco hairy roots and the production of nicotine in culture medium increased with increasing aeration. Basification of the culture media associated with root growth resulted in a dramatic reduction in nicotine accumulation levels in the media, which was reversed by decreasing the pH of the media. Kinetic analysis of hairy root growth and nicotine accumulation in the media revealed a potential improvement in nicotine yields in the media by stimulating the branching of tobacco hairy roots.

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CHAPTER 5

ENHANCED PRODUCTION OF HAIRY ROOT METABOLITES USING MICROBUBBLE GENERATOR

1. Abstract

Previously, increased partitioning of the natural product nicotine from tobacco hairy roots into the culture media was achieved by altering the expression of the nicotine uptake permease (*NUPI*) gene. The present study demonstrated that further increases in nicotine yield in the media were attained by using surfactant-stabilized microbubbles. Compared to other non-ionic surfactants (Tween 20 and Tween 80) and the ionic surfactant SDS, Triton X-100 both increased total nicotine production and exudation into the hairy root culture media. In comparison to surfactant-free medium, Triton X-100 (TX100) at 10, 25, and 50 mg l⁻¹ did not show strong inhibition of hairy root growth. At 4000 rpm shear speed, microbubbles stabilized by 10, 25, and 50 mg l⁻¹ TX100 had k_{La} of 22.3, 36.2, and 44.1 h⁻¹ in Gamborg's B5 medium, respectively, in comparison to 16.4 h⁻¹ with conventional air sparging. In a 1-liter fermentor, microbubbles stabilized by TX100 were applied to hairy roots after the inoculated root tips were self-immobilized by branching. With microbubble dispersion, dissolved oxygen rapidly increased from 60% to 85%, and hairy root growth rate increased. Nicotine accumulation in culture medium with microbubbles reached 146 mg l⁻¹ after 30 days cultivation. These results show that combining genetic modification with surfactant-stabilized microbubble dispersion can

Zhao B, Agblevor FA, Jelesko JG (2014) Enhanced production of hairy root metabolites using microbubble generator. *Plant Cell Tissue and Organ Culture* 117:157-165

substantially increase levels of nicotine in the media of hairy root cultures.

2. Introduction

Hairy roots are generated by infecting plant leaf or stem tissue with *Agrobacterium rhizogenes* (Chilton et al. 1982; Guillon et al. 2006; Willmitzer et al. 1982) resulting in the transfer of bacterial root inducing (Ri) DNA into the plant genome and the genetic transformation into perpetual hairy root cultures. As novel phytochemical producers, hairy roots show rapid growth in hormone-free media and promote the synthesis of phytochemicals whose biosynthesis requires differentiated root cell types (Flores et al. 1999; Sevon and Oksman-Caldentey 2002; Shanks and Morgan 1999). As previously reported, nicotine is synthesized by growing roots of tobacco plants (Dawson 1942a, b). In contrast, rapidly growing undifferentiated tobacco BY-2 cell cultures produce very little nicotine and instead accumulate high levels of anatabine, which is a minor alkaloid in wild type tobacco plants (Goossens et al. 2003).

Like the normal plant roots, hairy roots are heterotrophic and require oxygen as the electron acceptor in respiration. However, oxygen is usually a limiting nutrient in liquid culture media because of its low solubility in water and the mass transfer resistance caused by entangled hairy roots (Yu and Doran 1994). Microbubbles are bubbles with diameter on the order of 100 μm , compared to 3–5 mm for gas bubbles formed in conventional air sparging (Bredwell et al. 1995). Microbubble dispersion (MBD), which was shown to significantly increase the volumetric oxygen mass transfer coefficient (k_La) in microbial fermentation (Hensirisak et al. 2002; Weber and Agblevor 2005; Zhang et al. 2005), has not been evaluated for hairy root cultures.

Many desired phytochemicals are successfully produced by hairy root cultures (Dehghan et al. 2012; Kim et al. 2013; Syklovska-Baranek et al. 2012), and product exudation into the culture media is preferable for downstream purification processes (Brodelius and Pedersen 1993; Cai et al. 2012). Surfactants can facilitate metabolite accumulation into the hairy root culture media (Boitel-Conti et al. 1995, 1996; Sim et al. 1994; Zhang et al. 2011). Therefore, surfactant in the media can have positive effects both as a microbubble stabilizer in MBD and also promote phytochemical exudation into the media. On the other hand, prolonged exposure of hairy roots to high surfactant concentrations can have negative effects such as reduced root growth (Kanokwaree and Doran 1998; Thimmaraju et al. 2003). Facing this dilemma, it is necessary to determine appropriate surfactant levels that do not strongly inhibit hairy root growth, yet improve both metabolite accumulation in culture media and microbubble-mediated oxygen mass transfer.

Plants in the genus *Nicotiana* produce the pyridine alkaloid, nicotine (Saitoh et al. 1985). Nicotine biosynthesis in *Nicotiana tabacum* (tobacco) is exclusively located in root tips and root growth is an absolute requirement for net nicotine production (Baldwin 1988; Dawson 1942a, b; Dawson and Solt 1959; Solt 1957). Nicotine is a potent natural insecticide (Kircher and Lieberman 1967; Richardson and Busbey 1937; Smith and Goodhue 1943) and potential anti-inflammatory agent (Isman 2006; Mabley et al. 2011). Tobacco hairy root lines with reduced expression levels of a nicotine uptake permease (*NUPI*) gene result in increased levels of nicotine in the culture media (Hildreth et al. 2011). The repartitioning of nicotine from the *NUPI*-reduced expression hairy roots into the culture media provides new opportunities to isolate a high-value alkaloid directly

from the culture fluid (Hildreth et al. 2011; see also Chapter 4). The repartitioning of alkaloids into a medium with relatively simple chemical complexity affords considerable advantages for the economical extraction and purification of the alkaloid natural products from plant hairy root cultures. However, the potential increase in nicotine exudation imparted by reduced *NUPI* expression can be reversed by media pH > 8.0 causing an ion-trapping effect resulting in nicotine accumulation in hairy root tissues rather than the media (see Chapter 4). Therefore, realization of the maximal benefit of *NUPI*-oriented alkaloid accumulation in the culture media includes manipulation of the hairy root culture media as well.

The goal of the present study is to further improve nicotine accumulation in the culture media of *NUPI*-reduced expression hairy root line by using a surfactant in conjunction with MBD. Specifically, the surfactant Triton X-100 (TX100) served as a microbubble stabilizer enhancing oxygen supply, and increased nicotine accumulation levels in the media of a *NUPI*-expression hairy root line. The overall objective of this research is to develop a model system combining genetic and cultural technologies that produce high alkaloid concentrations in hairy root culture media as an efficient means for natural product/drug production.

3. Materials and methods

3.1 Hairy root line and culture media

T13-8-101 is a homozygous *NUPI-RNAi* reduced expression hairy root line of *N. tabacum*. This tobacco hairy root line was generated by inoculating sterile leaves of T13-8 plants with *A. rhizogenes* ATCC15384, as previously described (Hildreth et al. 2011),

resulting in hairy root line T13-8-101. T13-8-101 was maintained by subculturing every three weeks in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 20 g l⁻¹ sucrose and 3 g l⁻¹ phytigel. The basal Gamborg's B5 medium and sucrose were dissolved in distilled water and the pH was adjusted to 5.7 with 1 M KOH. After autoclaving, Gamborg's B5 vitamin mixture (Research Products International Corp., Mount Prospect, IL, USA) was added to the medium at 0.112 g l⁻¹. Gamborg's B5 medium supplemented with 20 g l⁻¹ sucrose and 0.112 g l⁻¹ Gamborg's vitamin mixture was used for hairy root cultivation in flasks and fermentors.

3.2 Effects of surfactants on hairy root growth and nicotine production

Experiments were carried out in 250-ml Erlenmeyer flasks filled with 30 ml medium. Surfactants were added to the medium at 10, 25, 50, and 100 mg l⁻¹. Medium without surfactant was used as control. Five 10-day-old root tips (about 1.5 cm in length) were inoculated in each flask and three replicate flasks were used for each treatment. Flasks were kept at 25°C in dark on a rotary shaker at 100 rpm for 10 days.

3.3 Microbubble generation and characterization

Microbubbles were generated in a microbubble generator, which was previously described by Zhang et al. (2005). The chamber of microbubble generator was filled with 300 ml Gamborg's B5 medium with TX100 at 10, 25, and 50 mg l⁻¹. Air bubbles were introduced into the chamber and sheared into microbubbles by a spinning disk. The generated microbubbles were stabilized by TX100 and dispersed throughout the medium.

The gas hold-up of the microbubble dispersion was determined using the following equation:

$$\text{Gas hold-up} = V_g/V_d = (V_d - V_s)/V_d \quad (1)$$

where V_g is the volume of gas, V_d is the volume of microbubble dispersion (the mixture of liquid medium and microbubbles), and V_s is the initial volume of liquid medium (Xu et al. 2008). Microbubbles were observed using an Olympus IX81 microscope (Olympus America Inc., Center Valley, PA, USA) and images of microbubbles were captured using a CCD camera connected to the microscope. The bubble diameter was calculated manually according to the scale bar shown on the image. Microbubble stability was measured as the time that the microbubble foam persisted.

Volumetric oxygen mass transfer coefficient (k_La) was determined using the unsteady-state method (Shuler and Kargi 2002). The chamber of microbubble generator was connected to a 1-liter fermentor BIOSTAT Q (B. Braun Biotech International, Germany), where dissolved oxygen was measured (Figure 5.1).

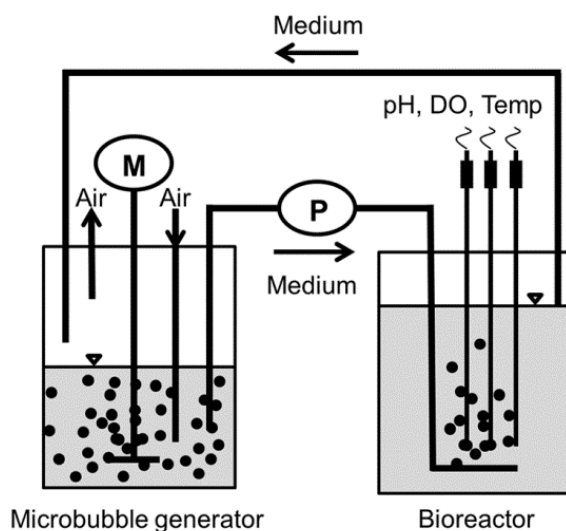


Figure 5.1 Schematic diagram of a microbubble generator connected to a fermentor. M: motor; P: peristaltic pump; DO: dissolved oxygen; and Temp: temperature

The MBD chamber and fermentor were filled with 300 and 700 ml of sterile Gamborg's B5 medium, respectively, and circulated at 300 ml min⁻¹ using a peristaltic pump. Oxygen was removed from medium by N₂ sparging. Then air was sparged into the MBD chamber and time course of dissolved oxygen was recorded. In this case, we have:

$$dC_L/dt = k_La(C^* - C_L) \text{ or} \quad (2)$$

$$\ln(C^* - C_L) = -k_Lat \quad (3)$$

where C^* is saturated dissolved oxygen concentration, C_L is actual concentration of dissolved oxygen in the medium, k_La is volumetric oxygen mass transfer coefficient, and t is time. From equation (3), k_La was estimated by plotting $\ln(C^* - C_L)$ versus t .

3.4 Hairy root cultivation with microbubble generator

Hairy root cultivation was carried out in a 1-liter fermentor BIOSTAT Q. The fermentor was filled with 600 ml sterile Gamborg's B5 medium and inoculated with 30 root tips of T13-8-101 (about 1.5 cm in length). Hairy roots were incubated with conventional air sparging at 0.1 vvm aeration rate and ambient illumination (approximately on a 12-h light/12-h dark cycle) at 25°C. After two weeks cultivation, elongation and branching of the hairy root tips led to a self-immobilized root matrix, and the microbubble generator was connected to the fermentor. The chamber of microbubble generator was filled with 400 ml sterile Gamborg's B5 medium and 50 mg TX100. Microbubbles were generated at 0.1 vvm aeration rate and shear speed of 4000 rpm. Instead of sparging air into the fermentor, medium dispersed with microbubbles was transferred with a peristaltic pump from the MBD chamber into the fermentor through the

air sparger at 100 ml min^{-1} . Medium was recycled back to the microbubble generator through a silicon tube mounted on the top of the fermentor to maintain a constant volume in the MBD and the fermentor (Figure 5.1). To avoid previously reported media pH-dependent nicotine ion trapping in the hairy roots, when the fermentor culture media reached a pH of 7.8 the media was adjusted to a lower pH as previously described in Chapter 4.

3.5 Analytical methods

Fresh hairy roots from Erlenmeyer shake flasks were rinsed with deionized water, blotted with tissue paper, and the fresh root weight was measured. For hairy root cultivation in 1-liter fermentor, root dry weight was estimated from medium conductivity (see Chapter 4). High performance liquid chromatography (Shimadzu Scientific, Columbia, MD, USA) was used for the quantification of nicotine in hairy root tissue and in culture medium. Fresh hairy roots were added to 15 ml methanol/water (40/60, v/v), ground with mortar and pestle, and extracted on a shaker at 100 rpm and 25°C for 12 h. Culture broth and extracted nicotine samples were filtered with $0.2 \mu\text{m}$ membrane and separated on a Waters, Resolve C18 column ($5 \mu\text{m}$, 90 \AA , $3.9 \times 150 \text{ mm}$) with a guard column (Waters, Resolve C18, $5 \mu\text{m}$, 90 \AA , $3.9 \times 20 \text{ mm}$). The isocratic mobile phase consisted of 40/60 (v/v) methanol/water and 2% (v/v) phosphoric acid. The pH of the mobile phase was adjusted to 7.25 with triethylamine. The flow rate of mobile phase was 0.5 ml min^{-1} and the injection volume was $20 \mu\text{l}$. Nicotine was measured with a UV detector at 254 nm. Nicotine standards were prepared in mobile phase at 1, 10, 25, 50, 75,

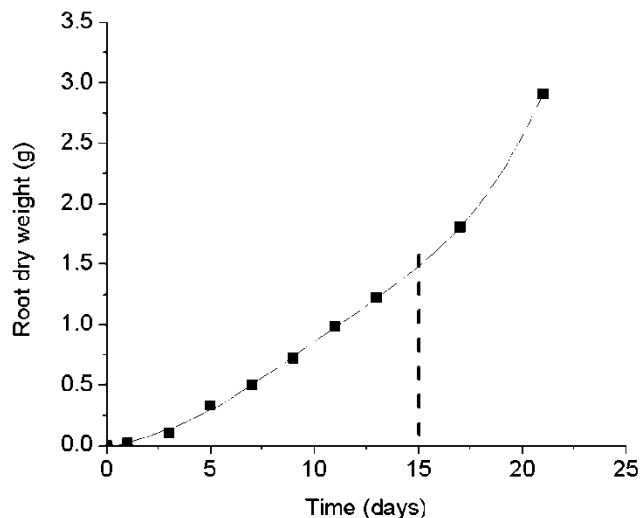


Figure 5.2 Time courses of hairy root growth in two-stage hairy root cultivation. Day 0 to day 15 was conventional air sparging at 0.1 vvm and day 15 to day 21 was microbubble dispersion at 0.1 vvm

and 100 mg l^{-1} . Root growth rate was calculated from smoothed time course of hairy root growth (Figure 5.2).

4. Results and discussion

4.1 Surfactant effects on hairy root growth and nicotine exudation

Thimmaraju et al. (2003) demonstrated that betalaine exudation into the media of *Beta vulgaris L.* hairy roots was improved by supplementing the culture media with surfactants. Table 5.1 shows that non-ionic detergents Tween 20 and TX100 increased the fraction of nicotine exuded into the hairy root culture media compared to non-surfactant controls. Tween 80 treatment did not appreciably improve nicotine exudation to the media. Increasing non-ionic surfactant concentrations on hairy root growth

resulted in modest reductions in hairy root growth up to 50 mg l^{-1} (Figure 5.3). However, 100 mg l^{-1} Tween 20 and TX100 treatments showed a more pronounced decline in hairy root biomass production, whereas Tween 80 did not. By comparison, the ionic surfactant SDS dramatically reduced hairy root growth beginning at 10 mg l^{-1} treatment resulting in essentially non-viable hairy roots at 25 mg l^{-1} SDS and higher. While Tween 20 and TX100 showed similar growth and fractional nicotine exudation to the media, TX100 treatments had a stimulatory effect on nicotine accumulation levels in both roots and media that was not observed with Tween 20 treatments. Therefore, the surfactant TX100 treatments became the focus of additional investigations to optimize nicotine exudation into the media of T13-8-101 hairy root cultures.

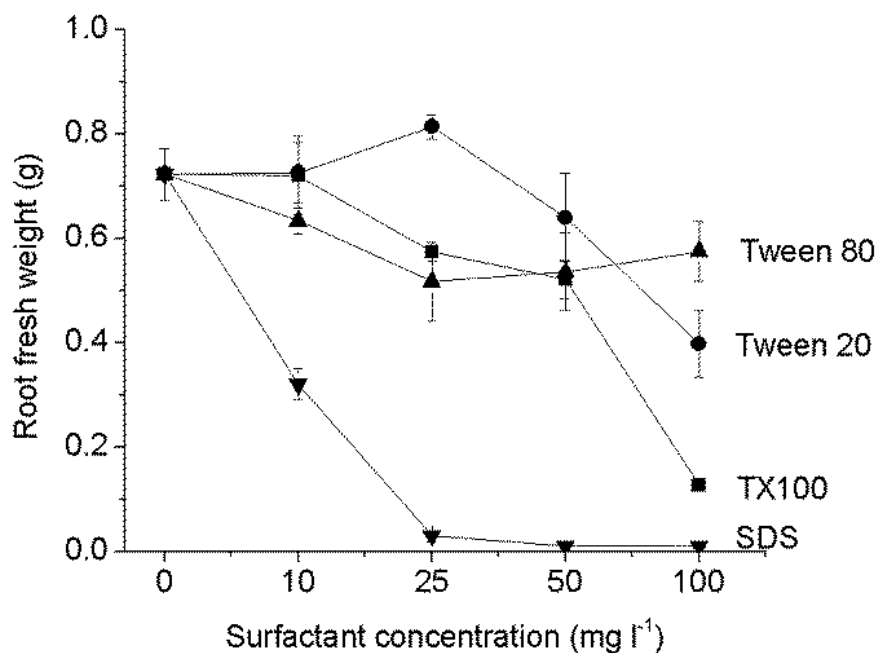


Figure 5.3 Hairy root biomass after 10 days cultivation with various surfactants

Table 5.1 Nicotine accumulation in T13-8-101 hairy roots and in culture medium

Surfactant (mg l ⁻¹)		Nicotine (mg g ⁻¹ FW)			
		In roots	In media	Total	Release ratio
Tween 20	10	0.83 ± 0.09	1.28 ± 0.14	2.11 ± 0.10	0.61
	25	0.71 ± 0.07	1.23 ± 0.08	1.94 ± 0.06	0.63
	50	0.68 ± 0.08	1.66 ± 0.29	2.34 ± 0.36	0.71
	100	0.58 ± 0.03	2.28 ± 0.15	2.86 ± 0.18	0.80
Tween 80	10	1.22 ± 0.05	1.36 ± 0.11	2.58 ± 0.14	0.53
	25	1.05 ± 0.04	1.39 ± 0.23	2.44 ± 0.26	0.57
	50	1.23 ± 0.32	1.60 ± 0.50	2.83 ± 0.81	0.56
	100	1.02 ± 0.08	1.26 ± 0.06	2.28 ± 0.11	0.55
TX100	10	0.92 ± 0.13	2.15 ± 0.20	3.07 ± 0.31	0.70
	25	0.98 ± 0.11	2.86 ± 0.36	3.84 ± 0.47	0.75
	50	1.04 ± 0.13	3.71 ± 0.08	4.75 ± 0.16	0.78
	100	0.60 ± 0.06	7.29 ± 0.89	7.89 ± 0.93	0.92
SDS	10	0.28 ± 0.11	2.02 ± 0.75	2.30 ± 0.86	0.88
	25	N.D.	2.19 ± 0.53	2.19 ± 0.53	N/A
	50	N.D.	0.51 ± 0.05	0.51 ± 0.05	N/A
	100	N.D.	N.D.	N/A	N/A
Control	0	0.69 ± 0.05	1.59 ± 0.12	2.28 ± 0.17	0.70

FW: fresh weight of hairy roots; N.D.: not detectable; N/A: not available. Nicotine concentrations were analyzed after 10 days cultivation. Release ratio = in medium/(in roots + in medium). Values are expressed as the mean ± standard error (n = 3)

4.2 Effects of TX100 on hairy root growth and nicotine production

Figure 5.4 shows 10 days hairy root growth after treatment with 0, 10, 25, 50, 100, and 300 mg l⁻¹ TX100. TX100 at 10, 25, and 50 mg l⁻¹ did not significantly affect the hairy root growth compared to the non-treated control (0 mg l⁻¹ TX100). On the other hand, hairy root growth was significantly inhibited by TX100 at 100 mg l⁻¹, and completely suppressed by 300 mg l⁻¹ TX100.

As shown in Figure 5.5a, with 10, 25, and 50 mg l⁻¹ TX100 treatments, the amount of nicotine (mg) in the culture medium generally increased in comparison to that

of the untreated control. TX100 above 50 mg l⁻¹ resulted in lower average nicotine content in culture medium, probably due to the significantly attenuated root growth. Likewise, the amount of nicotine (mg) in roots was dramatically reduced by TX100 above 50 mg l⁻¹, which may also be explained by the dramatically reduced hairy root growth. As shown in Figure 5.5b, the normalized nicotine levels in culture medium generally increased with TX100 treatment. The normalized nicotine levels within roots remained constant up to 50 mg l⁻¹ TX100, after which normalized nicotine levels decreased slightly.

Several lines of evidence suggest that moderate TX100 treatments promote overall nicotine production and release into the media. Up to and including 50 ml l⁻¹, TX100 treatment did not significantly reduce total root biomass accumulation. Likewise, during the same conditions the normalized nicotine levels in roots were not significantly different. Therefore, to the first approximation, the increasing normalized nicotine levels in the media were most likely due to increased nicotine production. However, there was clearly a threshold to this effect by TX100 treatment. At 100 mg l⁻¹ TX100, the hairy roots showed significantly reduced growth (Figure 5.3) and decreased normalized nicotine accumulation in roots (Figure 5.5b), most likely caused by impaired plasma membrane integrity. The apparent high normalized nicotine levels in the media in the 100 and 300 mg l⁻¹ TX100 treatments were artifacts of diminished root growth, as evidenced by the significant reduction in absolute root biomass (Figure 5.3) and reduced net nicotine levels in both the media and roots (Figure 5.5a). Between 10 to 50 mg l⁻¹ TX100 treatment, there was an increase in nicotine production. This was probably caused by a gentle permeabilization and nicotine release with 10 to 50 mg l⁻¹ TX100, and

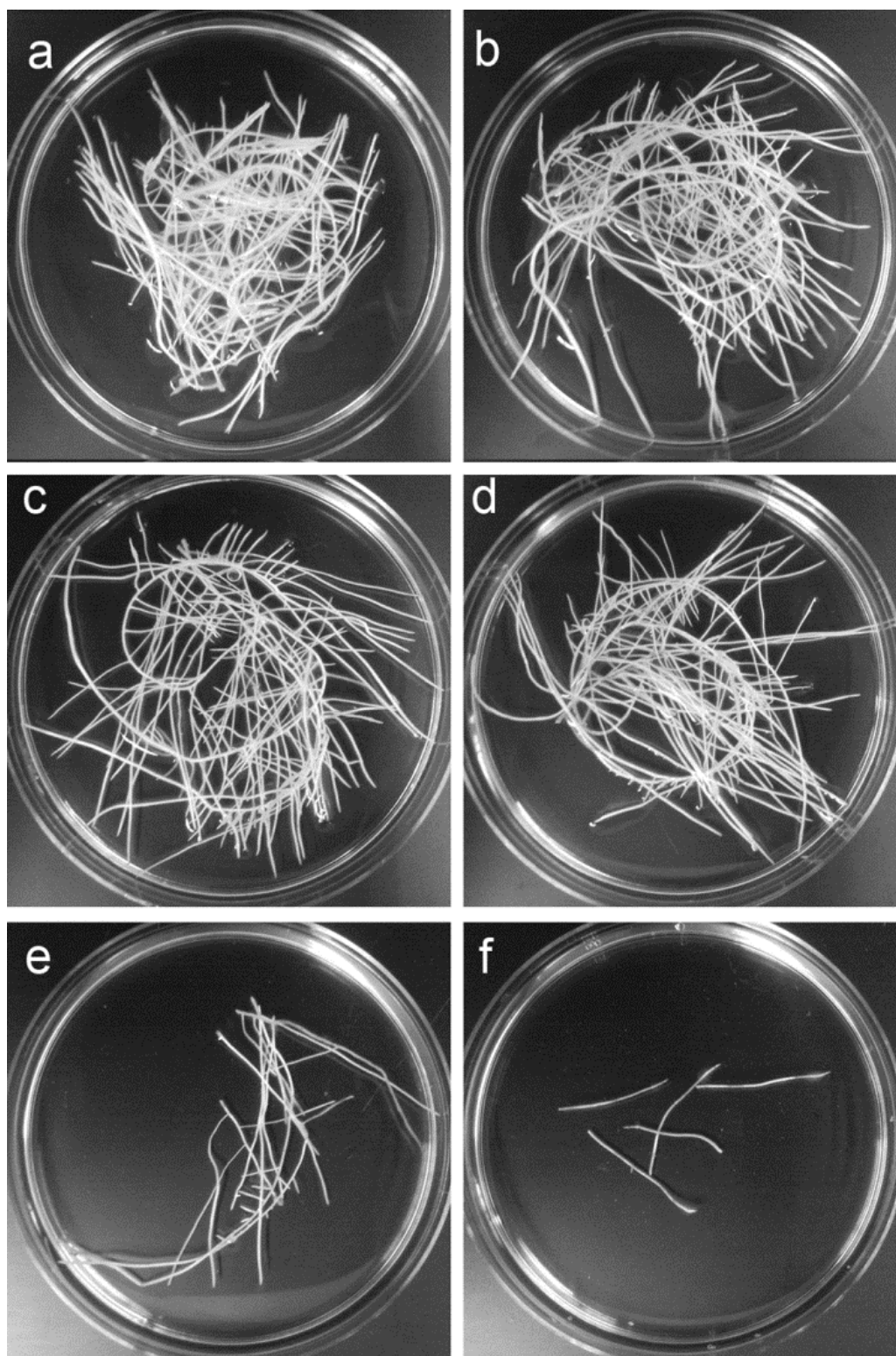


Figure 5.4 Pictures of hairy roots after 10 days cultivation with various Triton X-100 concentrations (mg l^{-1}). a: 0; b: 10; c: 25; d: 50; e: 100; and f: 300

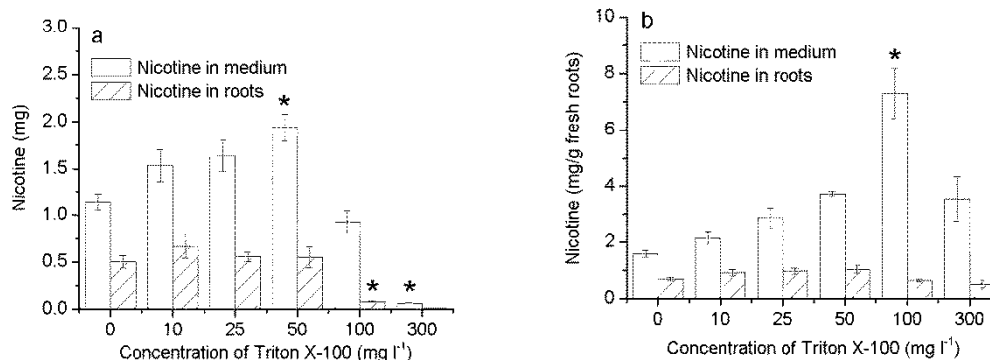


Figure 5.5 Effect of Triton X-100 on nicotine production. a: amount of nicotine (mg) in roots and medium; and b: normalized amount of nicotine (mg g^{-1} fresh roots) in roots and medium. Bars represent standard error ($n = 3$). ANOVA analysis was conducted between untreated control and each level of TX100, respectively. Statistical significance was shown by asterisk ($P < 0.01$, Tukey's test)

the new equilibrium of nicotine production in roots following the permeabilization. Above 50 mg l^{-1} TX100, there appeared to be detrimental permeabilization of the roots that decreased nicotine levels in the roots and increased nicotine in the medium (Figure 5.5b). Thus, TX100 can act as mild or detrimental permeabilizer depending on the TX100 concentration. Because of the obvious inhibition of hairy root growth, 100 and 300 mg l^{-1} TX100 treatments were not suitable for increasing net nicotine levels in the culture medium. Thus, 50 mg l^{-1} appeared to be the optimum concentration of TX100 resulting in increased nicotine accumulation levels in the culture medium.

In a previous investigation, 100 mg l^{-1} TX100 improved nicotine release into the hairy root culture medium (Larsen et al. 1993), which was consistent with the present study. The data showed that surfactant TX100 not only enhanced nicotine release ratio

but also stimulated the overall production of nicotine (Table 5.1). The increased total nicotine production also contributed to the enhancement of nicotine accumulation in the medium. The authors reported that 100 mg l^{-1} TX100 did not show negative effect on the root viability (Larsen et al. 1993), whereas 100 mg l^{-1} TX100 dramatically suppressed the root growth in the present study (Figure 5.4). This inconsistency might be explained by the fact that the viability was tested by treating the hairy roots with TX100 for 30 min (Larsen et al. 1993), whereas the surfactant was added from the beginning of the 10 days hairy root cultivation in the present study.

4.3 Effects of TX100 on k_{La} and microbubble properties

Cultured microorganisms often produce surfactants that stabilize microbubbles, and thus external surfactants are not required (Hensirisak et al. 2002; Weber and Agblevor 2005; Zhang et al. 2005). TX100 was used in the present study because microbubbles could not be stabilized in hairy root culture medium without adding surfactant. Because root growth was not substantially inhibited by 10, 25, and 50 mg l^{-1} TX100 treatments, the effects of these treatments to stabilize microbubbles and thereby improve the volumetric oxygen mass transfer coefficient (k_{La}) in Gamborg's B5 medium were investigated. TX100 concentration, shear speed, and aeration rate were examined for their effects on k_{La} . Table 5.2 shows that MBD at all TX100 levels achieved higher k_{La} than conventional air sparging. At 4000 rpm and 1 vvm aeration rate, k_{La} increased with increasing surfactant concentration. When the shear speed increased from 4000 to 5000 rpm, k_{La} increased from 22.3 to 30.4 h^{-1} . When the aeration rate decreased from 1 to 0.25 vvm, the k_{La} decreased slightly from 22.3 to 20.5 h^{-1} .

Table 5.2 Effects of microbubble generation conditions on volumetric oxygen mass transfer coefficient (k_{LA})

Conditions of microbubble generation				k_{LA} (h^{-1})
Code	TX100 (mg l^{-1})	Shear speed (rpm)	Aeration (vvm)	
C1	10	4000	0.25	20.5 ± 2.0
C2	10	4000	0.50	21.8 ± 1.6
C3	10	4000	1.00	22.3 ± 0.6
C4	10	4500	1.00	25.0 ± 1.4
C5	10	5000	1.00	30.4 ± 2.1
C6	25	4000	1.00	36.2 ± 0.9
C7	50	4000	1.00	44.1 ± 0.7
C8	Conventional air sparging		1.00	16.4 ± 0.3

Values of k_{LA} are expressed as the mean \pm standard error ($n = 3$)

Gas hold-up, bubble size, and bubble stability directly influence oxygen mass transfer. These properties, therefore, can be useful in explaining the effects of surfactant concentration and shear speed on k_{LA} . As shown in Figures 5.6 and 5.7, higher shear speed and surfactant concentration resulted in smaller average bubble diameter and higher gas hold-up. The microbubbles were generated in microbubble generator and pumped into the fermentor, hence the stability of microbubbles is important for effective mass transfer in the fermentor. Figure 5.6b shows that higher shear speed slightly increased the bubble stability, whereas higher surfactant level dramatically enhanced the stability of microbubbles. The comparison between C3 (10 mg l^{-1} , 4000 rpm, 1 vvm) and C5 (10 mg l^{-1} , 5000 rpm, 1 vvm) suggests that the improvement in k_{LA} at higher shear speed was because of larger gas-liquid interfacial area due to smaller bubbles generated. In comparison to C5 (10 mg l^{-1} , 5000 rpm, 1 vvm), C7 (50 mg l^{-1} , 4000 rpm, 1 vvm) showed similar gas hold-up and bubble size (Figure 5.6a) but higher bubble stability

(Figure 5.6b). This explains the increase in k_{LA} from 30.4 h^{-1} in C5 to 44.1 h^{-1} in C7. Therefore, increased shear speed improved k_{LA} by creating a larger gas-liquid interfacial area, whereas higher surfactant concentrations improved microbubble stability.

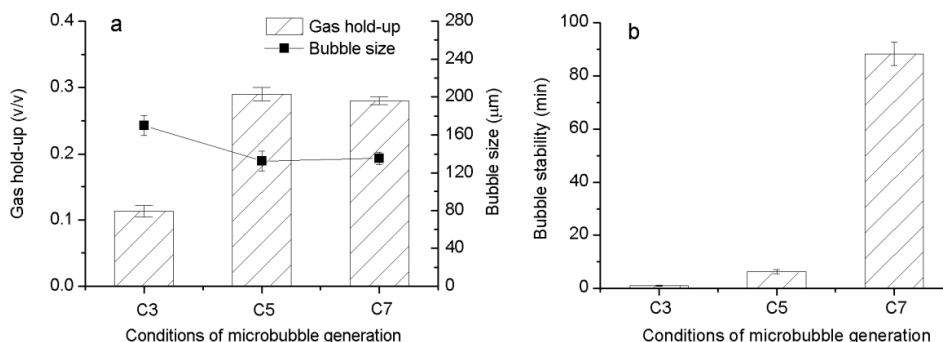


Figure 5.6 Gas hold-up, bubble size (a), and bubble stability (b) under various microbubble generation conditions. C3: 10 mg l^{-1} Triton X-100, 4000 rpm, 1 vvm; C5: 10 mg l^{-1} Triton X-100, 5000 rpm, 1 vvm; and C7: 50 mg l^{-1} Triton X-100, 4000 rpm, 1 vvm. Bars represent standard error ($n = 3$)

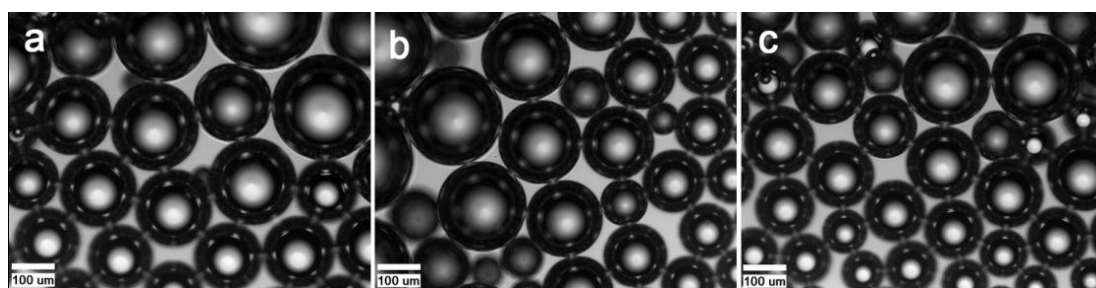


Figure 5.7 Microscope images of microbubbles generated at a: C3 (10 mg l^{-1} Triton X-100, 4000 rpm, 1 vvm); b: C5 (10 mg l^{-1} Triton X-100, 5000 rpm, 1 vvm); and c: C7 (50 mg l^{-1} Triton X-100, 4000 rpm, 1 vvm)

4.4 Hairy root cultivation with microbubble generator

Production of nicotine by hairy roots and release of nicotine into hairy root culture media were previously reported (Hamill et al. 1986; Rhodes et al. 1986). In later investigations, surfactant treatment, continuous product removal, and medium pH adjustment were shown to improve the extent of nicotine released into the culture media (Green et al. 1992; Larsen et al. 1993; see Chapter 4). As previously reported, surfactant-stabilized microbubbles improved oxygen mass transfer (Bredwell et al. 1995; Weber and Agblevor 2005). With surfactant-stabilized microbubbles, both hairy root growth (which is usually limited by oxygen supply) and nicotine release may be enhanced. In addition, microbubble dispersion could be integrated with in situ product removal because a column filled with absorbent could be combined with the circulating tubing transferring microbubbles.

The utility of MBD to promote hairy root growth depended on the organization of the hairy roots. At the early stages of fermentor cultivation, hairy roots were relatively small and existed as individually suspended root tips. When MBD was introduced with free flowing hairy root tips in the fermentor, the circulating culture medium carried the relatively small root tips out of the fermentor and obstructed media flow in the silicon tubing (Figure 5.1). Therefore, if MBD were to be used during early stages of hairy root growth in the fermentor, it would become necessary to immobilize the root tips within the fermentor. In contrast, during the early stage of hairy root cultivation using conventional air sparging, root biomass density was low and oxygen mass transfer efficiency was relatively high. This promoted hairy root elongation and branching within the fermentor, resulting in an entangled immobile root mass. This was advantageous because the hairy

roots were immobilized within the fermentor, but also disadvantageous because the densely packed hairy roots resulted in diminished access to limiting dissolved oxygen. Therefore, a two-step hairy root cultivation approach was implemented. Conventional air sparging was used from day 0 to day 15, whereas microbubbles were generated and introduced to the fermentor from day 15 to day 30. This two-stage hairy root cultivation avoided using a supporting frame for root tip immobilization during early hairy root cultures, and the MBD increased oxygen mass transfer when hairy root density was advantageous for alkaloid production.

As shown in Figure 5.8a, dissolved oxygen decreased to below 60% air saturation after 15 days cultivation. When microbubbles were transferred from the MBD chamber into the fermentor, the dissolved oxygen increased rapidly to 85%. With conventional air sparging at 0.1 vvm aeration rate in the first stage of cultivation, root growth rate reached a plateau at 0.12 g day^{-1} after 10 days incubation (Figure 5.8a). The plateau in hairy root growth rate during the first stage coincided with percent dissolved oxygen dropping from 100% to a plateau of about 60% (Figure 5.8a). These results showed that dissolved oxygen in conventional air sparging at 0.1 vvm was a limiting factor for the hairy root growth. However, this homeostasis changed when the MBD generator was activated at day 15, and oxygen levels increased to about 83% air saturation and hairy root growth rates steadily increased, suggesting that oxygen availability was no longer restricting hairy root growth rates. In addition to the removal of oxygen restriction, the increase in root growth rate with MBD might be partly due to the new medium introduced with microbubbles.

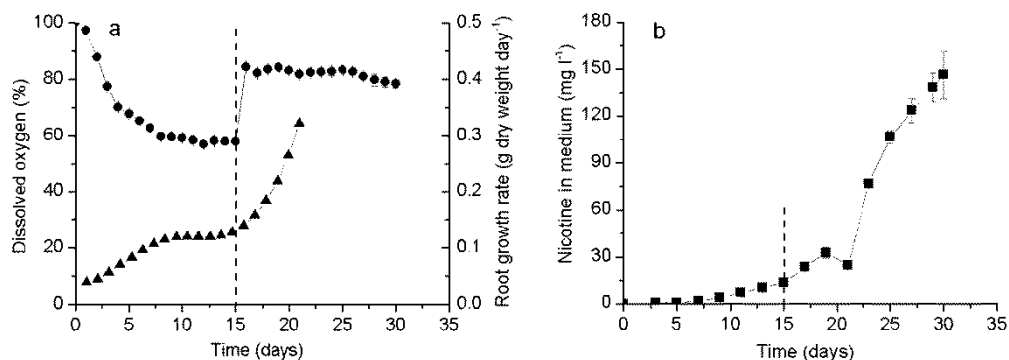


Figure 5.8 Two-stage hairy root cultivation. a: dissolved oxygen (●) and root growth rate (▲); b: concentration of nicotine in medium (■). Day 0 to day 15 was conventional air sparging at 0.1 vvm, and day 15 to day 30 was microbubble dispersion at 0.1 vvm

During the first week of hairy root cultivation, little nicotine accumulated in the culture medium (Figure 5.8b). From day 8 to day 15, nicotine accumulation in culture medium increased, most likely due to the branching of hairy roots with more root tips producing nicotine. Previously, we reported that increase in medium pH up to 8 resulted in less nicotine accumulation in the hairy root culture media (see Chapter 4). In the present study, a similar increase in medium pH was observed. The medium pH reached 7.8 by day 21 (data not shown), and resulted in decreased nicotine content in the culture medium. With adjusted medium pH and MBD at 0.1 vvm aeration rate, 146 mg l⁻¹ nicotine accumulated in culture medium after 30 days cultivation, which was higher than that with conventional air sparging (see Chapter 4). Therefore, TX100-stabilized microbubbles enhanced not only oxygen mass transfer and root growth rate, but also the accumulation of nicotine in the culture medium.

5. Conclusions

At 10, 25, and 50 mg l⁻¹, TX100 did not show strong inhibition of root growth, yet improved nicotine accumulation in culture medium and volumetric oxygen mass transfer coefficient (k_La). The improvement in k_La by microbubbles generated at higher shear speed was a result of larger gas-liquid interfacial area; while higher surfactant concentration led to increased bubble stability and concomitant improved oxygen mass transfer. In a 1-liter fermentor, the introduction of microbubbles to self-immobilized hairy roots increased k_La and root growth rate. In the present study, a combination of genetic improvement (*NUPI*) and microbubble dispersion resulted in substantial improvements in alkaloid accumulation in the hairy root culture medium. Currently, commercial nicotine production is achieved by extracting nicotine from tobacco leaf material. Nevertheless, nicotine biosynthesis in tobacco is the best characterized model system for valuable alkaloids produced in the roots of plants belonging to the family Solanaceae. Therefore, nicotine production using genetically modified hairy root cultures and cultural practices that increase alkaloid partitioning into root culture media should have broad applicability to the in vitro production of other high value alkaloids produced by other members of the Solanaceae. These methods can be useful to improve the efficiency of hairy roots as alkaloid producers, though more collaborative efforts are needed for the scale-up and practical utilization of hairy roots in industrial production of phytochemicals.

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CHAPTER 6

EFFECTS OF AERATION AND MICROBUBBLE GENERATOR ON *HYOSCYAMUS NIGER* HAIRY ROOTS

1. Abstract

Oxygen is widely reported as a limiting factor in hairy root cultures. In the present study, oxygen was found to affect the morphology of hairy roots. *Hyoscyamus niger* hairy roots under low oxygen conditions showed greater root diameter. Confocal microscope analysis showed the increase in root diameter was due to axial expansion of hairy root cells, rather than increase in cell number. In addition, higher aeration improved the hairy root growth and the conversion of hyoscyamine to scopolamine in *H. niger* hairy roots. The positive effect of oxygen on *H. niger* hairy root growth was confirmed in 1-liter fermentors. *H. niger* hairy roots were cultivated in a novel ground-joint column bioreactor connected to a microbubble generator. With microbubbles, the dissolved oxygen in hairy root culture media increased from 20% to 70%, and the hairy roots growth was improved.

2. Introduction

Hairy roots are developed by infecting plant tissue with *Agrobacterium rhizogenes* (Guillon et al. 2006; Willmitzer et al. 1982). As phytochemical producers, hairy roots show genetic stability, rapid growth, and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Flores et al. 1999).

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On the other hand, hairy roots are heterotrophic and require oxygen as the electron receptor in respiration. Hairy root growth in aqueous media is usually limited by insufficient oxygen supply due to the low solubility of oxygen in water and the entangled root matrix that reduces the convection flow of media (Yu and Doran 1994).

The efficiency of oxygen mass transfer can be measured by volumetric oxygen mass transfer coefficient (k_La), which is the product of mass transfer coefficient and volumetric gas-liquid interfacial area. At given k_L and volume, k_La increases with increasing gas-liquid interfacial area, which is inversely proportional to the size of gas bubbles. Microbubbles are bubbles with diameter on the order of 100 μm , which is less than one-tenth of the diameter of conventional air bubbles (3–5 mm) (Bredwell et al. 1995). Microbubble dispersion (MBD) significantly increases the oxygen mass transfer in microbial fermentations (Hensirisak et al. 2002; Weber and Agblevor 2005; Zhang et al. 2005). In our previous investigation on tobacco hairy roots, MBD was shown to improve k_La , hairy root growth, and nicotine accumulation in culture medium (see Chapter 5). However, MBD has not been widely studied for hairy root cultures, especially for *Hyoscyamus niger* hairy roots. In addition, a novel bioreactor with easy assembly and scale-up is needed to evaluate the suitability of MBD in hairy root cultivations.

H. niger is a Solanaceous and well documented plant that predominately produces scopolamine and hyoscyamine tropane alkaloids (Roberts and Wink 1998). Scopolamine and hyoscyamine are medically important as anticholinergic agents (Renner et al. 2005). Anticholinergics block the acetylcholine, which is the neurotransmitter in the central and peripheral nervous systems, and reduce the activities mediated by acetylcholine in

neurons through competitive inhibition (Gupta and Mehdi 2013). Anticholinergic drugs can be used in the treatment of motion sickness, which is caused by cholinergic stimulation of the vestibular apparatus (Tierney et al. 2010). In Solanaceous plants, tropane alkaloids are synthesized in the root and translocated to the shoots (Hashimoto et al. 1986; Sato et al. 2007). Differentiated root tissues accumulate more tropane alkaloids than undifferentiated plant cell suspension cultures (Tabata et al. 1972).

In the present study, the effects of aeration on *H. niger* hairy roots were investigated. Morphological adaptation of *H. niger* hairy roots to different aeration conditions was shown by light and confocal microscopy. The surfactants Tween 20, Tween 80, Triton X-100 (TX100), and sodium dodecyl sulfate (SDS) were investigated for their suitability as microbubble stabilizers for *H. niger* hairy roots. A novel column bioreactor with ground joint connection was designed and successfully used in combination with a microbubble generator to improve the growth of *H. niger* hairy roots.

3. Materials and methods

3.1 Hairy root line and culture media

H. niger hairy root line Hn2 was generated using *Agrobacterium rhizogenes* line ATCC 15834. Hn2 was subcultured every three weeks in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 20 g l⁻¹ sucrose and 3 g l⁻¹ phytagel. The basal Gamborg's B5 medium and sucrose were dissolved in distilled water and the pH was adjusted to 5.7 with 1 M KOH. After autoclaving, Gamborg's B5 vitamin mixture (Research Products International Corp., Mount Prospect, IL, USA) was added to the medium at 0.112 g l⁻¹. Gamborg's B5 medium supplemented with 20 g l⁻¹ sucrose and

0.112 g l⁻¹ Gamborg's vitamin mixture was used for hairy root cultivations in flasks and bioreactors.

3.2 Effects of aeration on hairy root growth

The effects of aeration on *H. niger* hairy roots were investigated in 250-ml Erlenmeyer flasks filled with 30, 100, and 200 ml medium, respectively. Five 10-day-old root tips (about 1.5 cm in length) were inoculated in each flask and three replicate flasks were used for each treatment. After inoculation, the flasks were sealed with screw caps. Flasks were kept at 25°C in continuous light on a rotary shaker at 100 rpm for 10 days.

The effects of aeration on *H. niger* hairy roots were also investigated in 1-liter fermentors (BIOSTAT Q, B. Braun Biotech International, Germany). The fermentor was filled with 0.7 liter of Gamborg's B5 medium and aerated at 0.2 and 1 vvm (volume of air per volume of medium per minute), respectively. The fermentor was inoculated with 40 hairy root tips (about 1.5 cm in length) and incubated at 25°C and ambient illumination (approximately on a 12-h light/12-h dark cycle). The medium pH in the fermentor was adjusted to 5.5–6.0 by adding 0.1 M H₂SO₄ and KOH.

3.3 Microscopy

Hairy root tips were suspended in deionized water on a slide. The hairy roots were observed over a distance of about 0.5 to 1 cm from the root tip under a Leica DM750 light microscope (Leica, Germany) and photographed with a Leica digital camera ICC50. Confocal microscope images were collected using a LSM 710 laser scanning microscope (Carl Zeiss, Germany). To reduce variations, all the hairy root tips in one set of experiments were processed at the same time.

3.4 Hairy root cultivation in a novel ground-joint column bioreactor

Hairy root cultivation was carried out in a 3-liter novel ground-joint column bioreactor (Figure 6.1). The column bioreactor was made of three parts: top column, middle column, and bottom column. The middle column was divided into two chambers for hairy root growth. The top, middle, and bottom columns were connected by two ground joints, which provided effective sealing, easy assembly, and convenient scale-up. The column bioreactor was filled with 3 liters sterile Gamborg's B5 medium and inoculated with one root clump in each chamber and kept at ambient illumination (approximately on a 12-h light/12-h dark cycle) and 25°C. The microbubble generator and the reservoir were filled with 300 and 700 ml sterile medium, respectively.

The column bioreactor, microbubble generator, and reservoir were connected by silicon tubes. The medium was moved by a peristaltic pump from the top column bioreactor to the reservoir, where the extra medium was automatically transferred to the microbubble generator by positive pressure. The medium in the microbubble generator flowed into the column bioreactor by negative pressure. The medium level in the column bioreactor remained constant because the medium was pumped out from the top and simultaneously absorbed into the column from the bottom. The medium level in the reservoir was constant because the vessel was sealed, except for the medium inlet and outlet. The medium outlet was a short silicon tube connected to the top of the reservoir (Figure 6.1). When the medium level was lower than the bottom of the outlet tube, air was pushed out as liquid medium was pumped into the reservoir. When the medium level reached the bottom of the outlet tube, liquid medium was pushed out and the medium level remained at the bottom of the outlet. The medium level in the microbubble

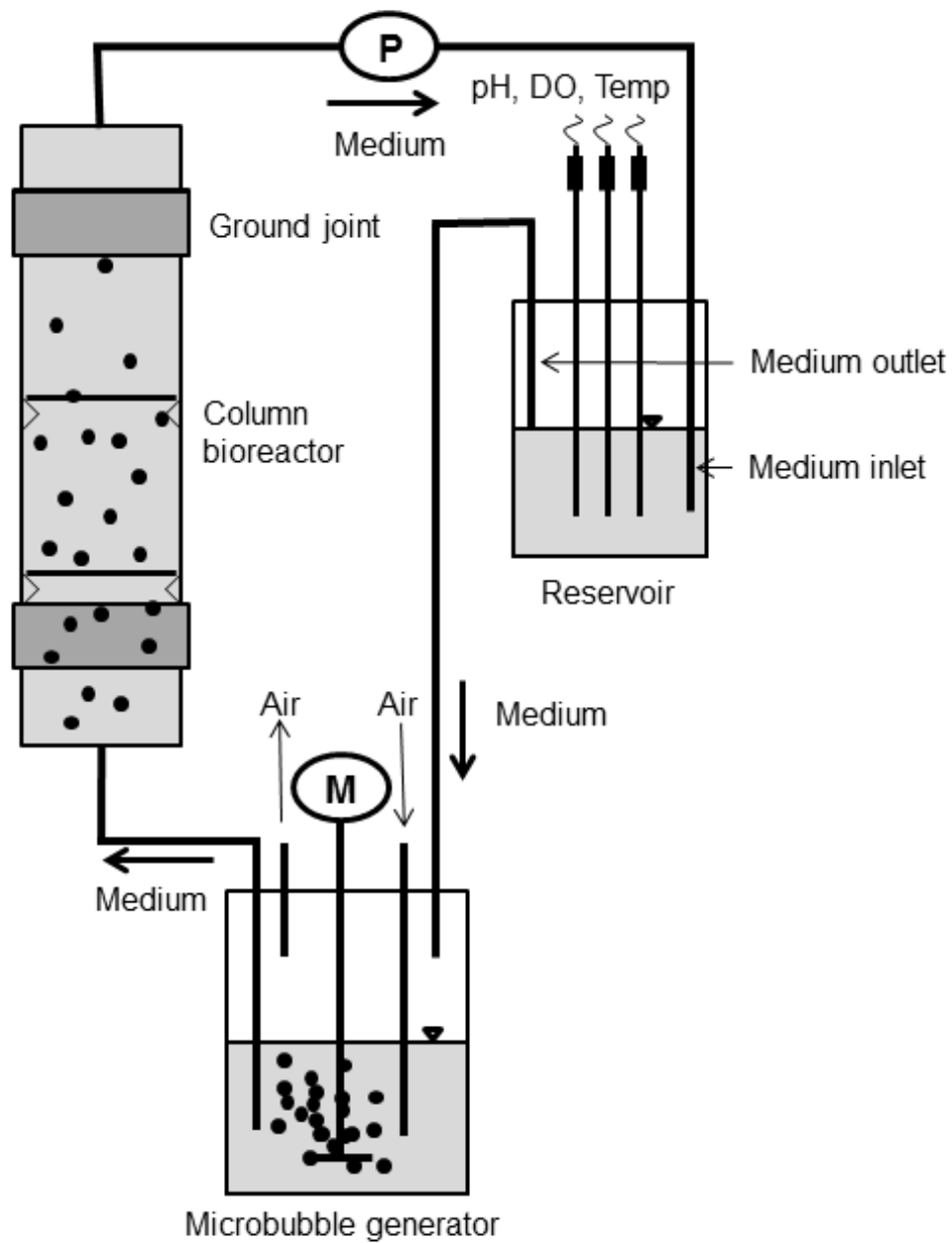


Figure 6.1 Schematic diagram of a novel ground-joint column bioreactor connected to a microbubble generator

generator was constant because the medium transferred into the generator was equivalent to the medium transferred out of the chamber (absorbed into the column bioreactor). For conventional air sparging, air was sparged into the chamber of microbubble generator at 0.1 liter per minute. For microbubble generation, 100 mg l⁻¹ Tween 80 was added to the medium. Air was sparged into the microbubble generator chamber at 0.1 liter per minute and sheared into microbubbles by a disk spinning at 3500 rpm. The medium pH was adjusted to 5.5–6.0 by adding 0.1 M H₂SO₄ and KOH.

3.5 Analytical methods

Fresh hairy roots from Erlenmeyer flasks were rinsed with deionized water, blotted with tissue paper, and the fresh root weight was measured. In bioreactors, root fresh weight was calculated from medium conductivity: Δ root fresh weight = $-50.3 \times \Delta$ conductivity. Media conductivity was measured using a Traceable Digital Conductivity Meter (Control Company, Friendswood, TX, USA). High performance liquid chromatography (Shimadzu Scientific, Columbia, MD, USA) was used for the quantification of hyoscyamine and scopolamine in the hairy root tissue and in the culture medium. Harvested fresh hairy roots were added to 15 ml methanol/water (40/60, v/v), ground with mortar and pestle, and extracted on a shaker at 100 rpm at 25°C for 12 h. Spent culture media and extracted samples were filtered with 0.2 μ m membrane and separated on a Waters, Resolve C18 column (5 μ m, 90 Å, 3.9 \times 150 mm) with a guard column (Waters, Resolve C18, 5 μ m, 90 Å, 3.9 \times 20 mm). The isocratic mobile phase consisted of 40/60 (v/v) methanol/water and 2% (v/v) phosphoric acid. The pH of the mobile phase was adjusted to 7.25 with triethylamine. The flow rate of mobile phase was

0.5 ml min⁻¹ and the injection volume was 20 µl. Scopolamine and hyoscyamine were measured at 220 nm with a UV detector. Standards of scopolamine and hyoscyamine (Sigma-Aldrich, St Louis, USA) were prepared in mobile phase at 0.1, 0.25, 0.5, 1, 5, and 10 mg l⁻¹.

4. Results and discussion

4.1 Morphology of H. niger hairy roots under various aeration conditions

The effects of aeration on *H. niger* hairy roots were investigated by sealing different volumes of air in screw-cap flasks. Figure 6.2 shows the microscope image and pictures of *H. niger* hairy roots cultivated with various volumes of air. For A270, in which 270 ml air was sealed in a 300-ml flask, the roots extended and branched much more than the less aerated A200 and A100 cultures. The roots were long and thin in A270, whereas most of the roots in A100 were short and thick. The microscope images show the hairy root diameter of A100 was almost twice that of A270. The confocal microscope images show root cell size variation between aeration conditions (Figure 6.3). Although the cell size varies in a single image, possibly due to different cell growth rate and view perspective, the measurement of a selected cell in each image shows a quantitative comparison between cell sizes in various aeration conditions. In A270 (Figure 6.3a), the selected cell was 158 µm long and 32 µm wide (as indicated by the arrow). In A200 (Figure 6.3b), the cell shown by the arrow was 184 × 47 µm (length × width). When the air sealed in flask was reduced to 100 ml (A100), the cells were wider and slightly shorter (132 × 55 µm, as is shown by the arrow in Figure 6.3c). It is

noteworthy that the roots in A100 also showed slight swelling sections where wider and shorter cells were observed ($79 \times 74 \mu\text{m}$, as is shown by the arrow in Figure 6.3d).

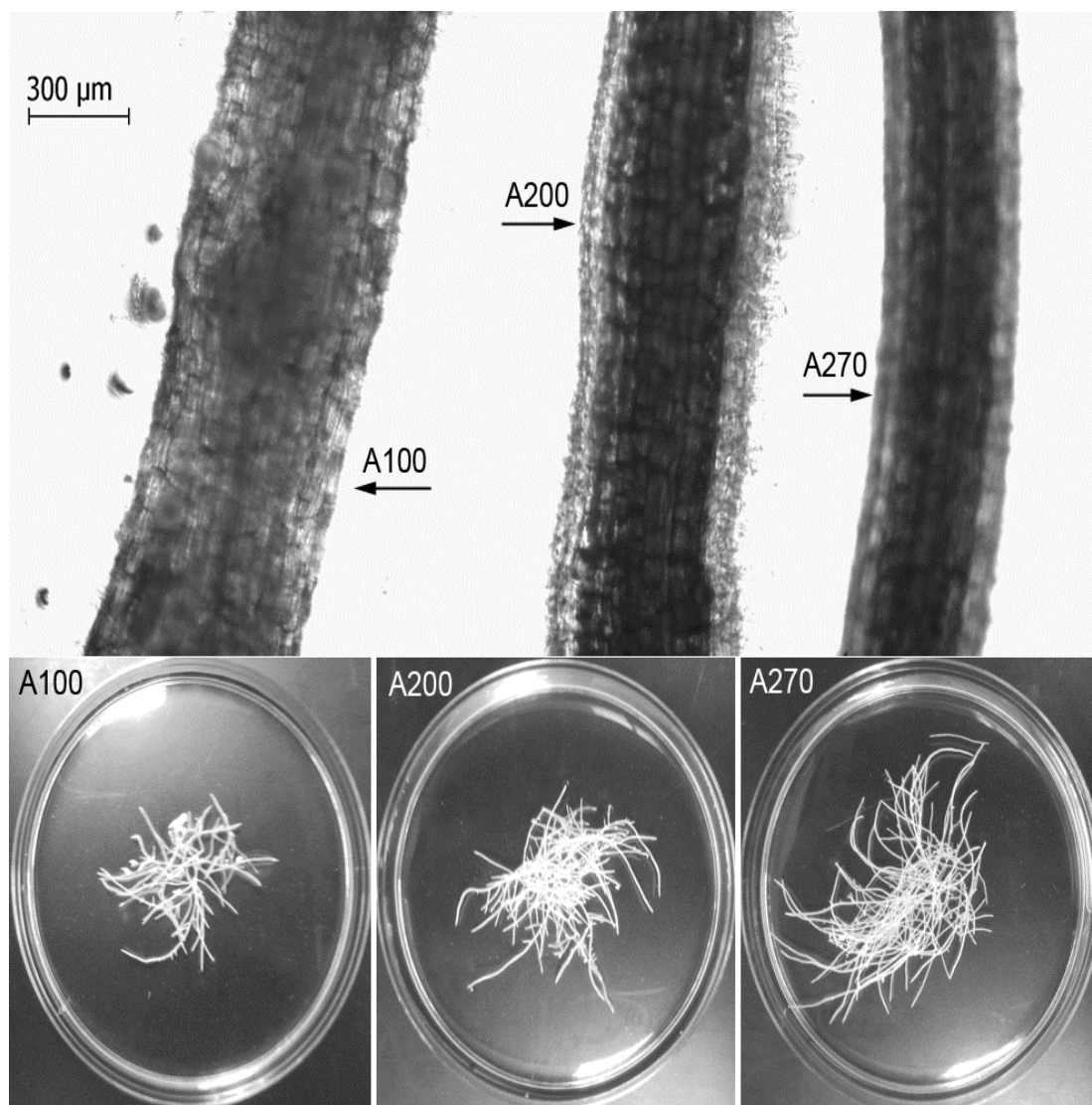


Figure 6.2 Light microscope images and growth of *H. niger* hairy roots with 270 (A270), 200 (A200), and 100 ml (A100) air sealed in 300-ml flasks. The microscope images were obtained with 10× magnification

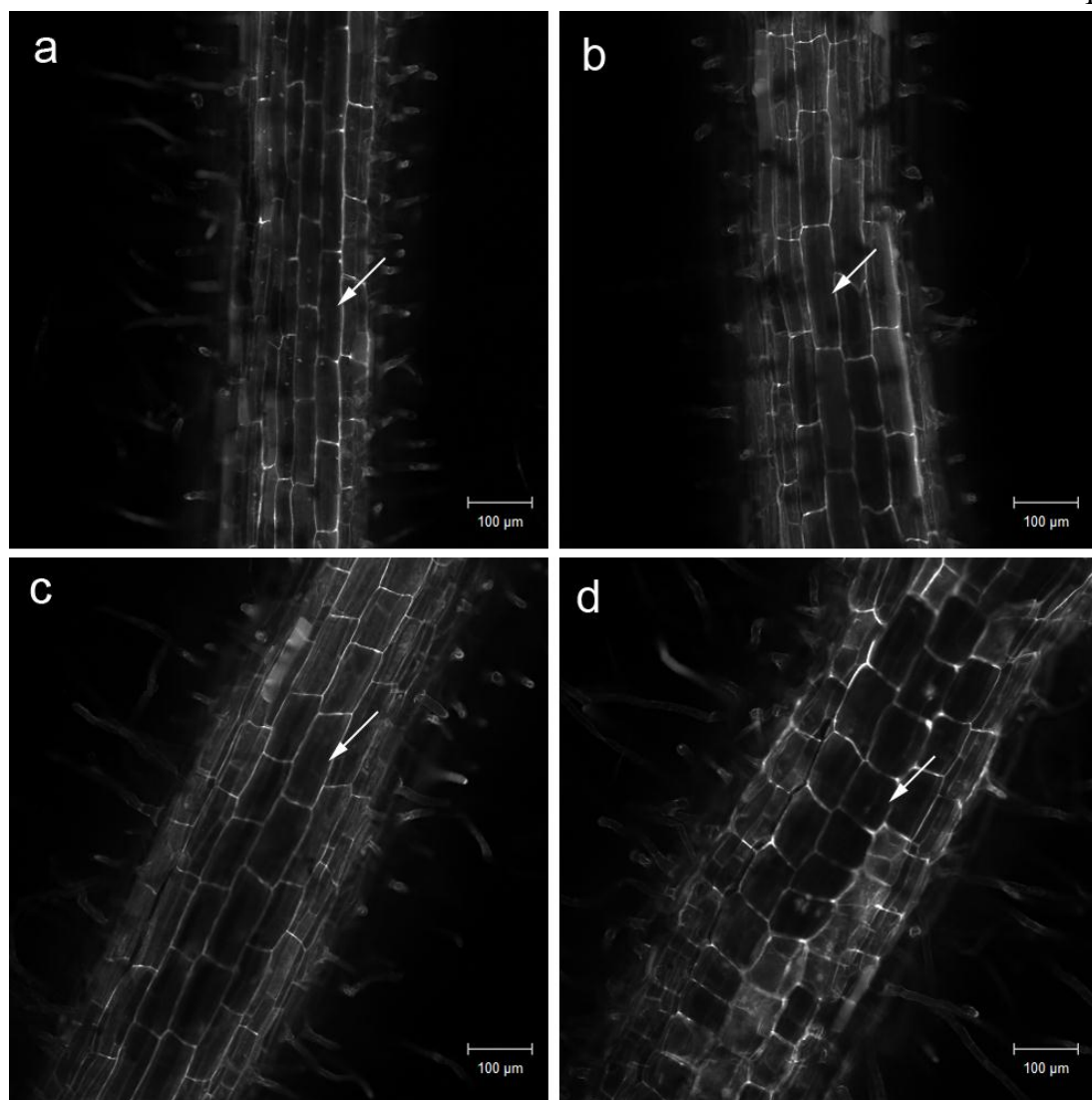


Figure 6.3 Confocal microscope images of *H. niger* hairy roots with 270 (a), 200 (b), and 100 ml (c and d) air sealed in 300-ml flasks. Arrows indicate selected cells for length and width measurement. The microscope images were obtained with 10× magnification

It is widely reported that the hairy root growth can be improved by higher aeration conditions (Kanokwaree and Doran 1997; Suresh et al. 2001; Yu and Doran 1994; see Chapter 4). The data show that aeration not only affected hairy root growth, but also the morphology of hairy roots and hairy root cells. In a previous study, non-aerated plant

roots were reported to have greater diameter than aerated roots, and this difference was caused by greater cell width rather than increased cell number (Pitman 1969). Likewise hypoxia results in increase in root diameter, which is correlated with an increase in cortical-cell diameter (Engelaar et al. 1993). In some wetland plants, low aeration treatment increases root diameter, which is associated with the formation of aerenchyma (Visser et al. 2000). The greater hairy root diameter in low aeration conditions was unlikely to be associated with aerenchyma, which provides an internal pathway for gas transport between shoots and roots (Colmer 2003). In addition, in one report aerenchyma are not found in hairy roots grown under limited oxygen conditions (Kanokwaree and Doran 1998). The confocal microscope images show that the greater diameter of hairy roots under less aerated conditions was possibly due to wider root cells, though detailed microscope observation is needed to get a whole picture of the hairy root structure under low oxygen conditions. In plants, cell size increase could be caused by 1) an increase in total cytoplasmic mass (cell growth), which accounts for the size increase of proliferating cells, or 2) vacuolation (cell expansion), which is responsible for the size increase of post-mitotic cells (Sugimoto-Shirasu and Roberts, 2003). Since the confocal microscope images were taken over 0.5 cm from the root tips, the imaged cells were unlikely undergoing cell division. Thus, the wider cells in the less aerated hairy roots were likely caused by increased radial cell expansion.

As shown in Figure 6.2, the cells in A200 were darker in comparison to the cells in A100. In a previous study, the density of cytoplasm in rice coleoptiles cells declined under anaerobic treatment (Polyakova and Vartapetian 2003), and the greater darkness in A270 and A200 might be explained by higher cytoplasmic density. The results suggest

that hairy roots show not only reduced growth but also dramatic changes in cell morphology during low aeration conditions. There is little data available on the anatomy of hairy roots, especially under low oxygen conditions, and further investigations are needed to clarify the mechanism and functions of such morphological response to low oxygen.

4.2 Effects of aeration on hairy root growth and the accumulation of hyoscyamine and scopolamine

Figure 6.4 shows the root fresh weight and the accumulation of hyoscyamine and scopolamine in *H. niger* hairy roots cultivated with various volumes of air. The root fresh weight increased with increasing air volume (Figure 6.4a). The correlation between root fresh weight and air volume was almost linear, which was similar to our previous report on the effect of air on tobacco hairy roots (see Chapter 4). Figure 6.4b shows the production of hyoscyamine, scopolamine, and the ratio of hyoscyamine to scopolamine. From A200 to A270, hyoscyamine accumulation decreased, whereas the accumulation of scopolamine increased from 34 to 49 $\mu\text{g g}^{-1}$ FW (Figure 6.4b). In addition, the H/S (hyoscyamine/scopolamine) ratio decreased from 4.6 in A100 to 2.4 in A270. These results show that higher aeration favored the accumulation of scopolamine.

Scopolamine is an epoxidized form of hyoscyamine (6,7 epoxide of hyoscyamine) (Matsuda et al. 1991). In *H. niger* plant roots, the epoxidization of hyoscyamine to scopolamine is catalyzed by hyoscyamine 6 β -hydroxylase (H6H) (Hashimoto and Yamada 1986). The activity of H6H requires molecular oxygen as a substrate (Hashimoto and Yamada 1986). Likewise, H6H activity in *Duboisia myoporoides* root

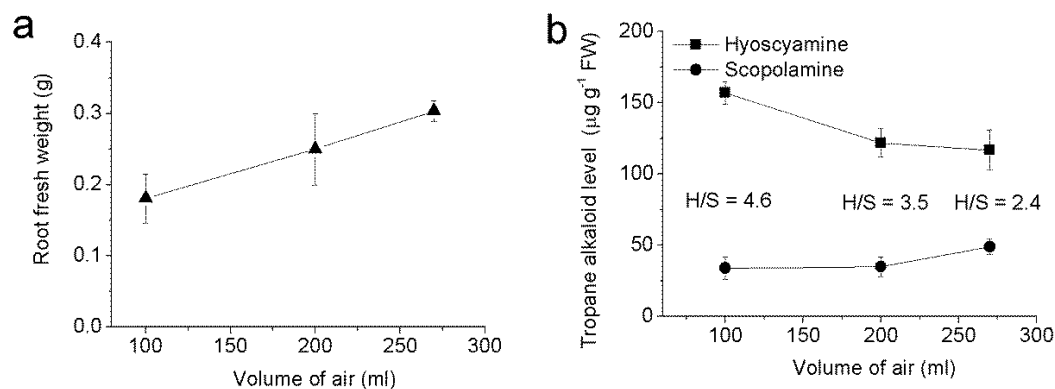


Figure 6.4 Hairy root fresh weight (a) and accumulation of hyoscyamine and scopolamine in roots (b) with 270, 200, and 100 ml of air sealed in 300-ml flasks. Samples were taken after 10 days cultivation

cultures increases with increasing oxygen supply (Yukimune et al. 1994). Thus, oxygen could improve the conversion of hyoscyamine to scopolamine, which possibly explains the enhanced scopolamine accumulation in A270 in the present study (Figure 6.4b).

4.3 Cultivation of *H. niger* hairy roots in 1-liter fermentors

The effects of aeration on *H. niger* hairy roots were also studied by using various aeration rates in 1-liter fermentors. As shown in Figure 6.5a, the dissolved oxygen at 1 vvm (volume of air per volume of medium per minute) aeration rate decreased slowly and remained above 80% air saturation, whereas the 0.2 vvm showed faster decrease in oxygen and the final dissolved oxygen was around 50% air saturation. In accordance to the dissolved oxygen, the hairy root growth was higher at 1 vvm aeration rate compared to 0.2 vvm aeration rate (Figure 6.5b). Figure 6.6 shows the concentrations of sucrose, glucose, and fructose in hairy root culture medium at 1 and 0.2 vvm aeration rates. The glucose and fructose accumulated more rapidly and to greater levels at 1 vvm compared

to those at 0.2 vvm. The glucose concentration was lower than fructose concentration at 1 vvm after day 13, whereas the concentrations of these monosaccharides were similar at 0.2 vvm. In addition, sucrose was depleted on day 15 at 1 vvm, while the sucrose in medium was completely consumed on day 25 at 0.2 vvm.

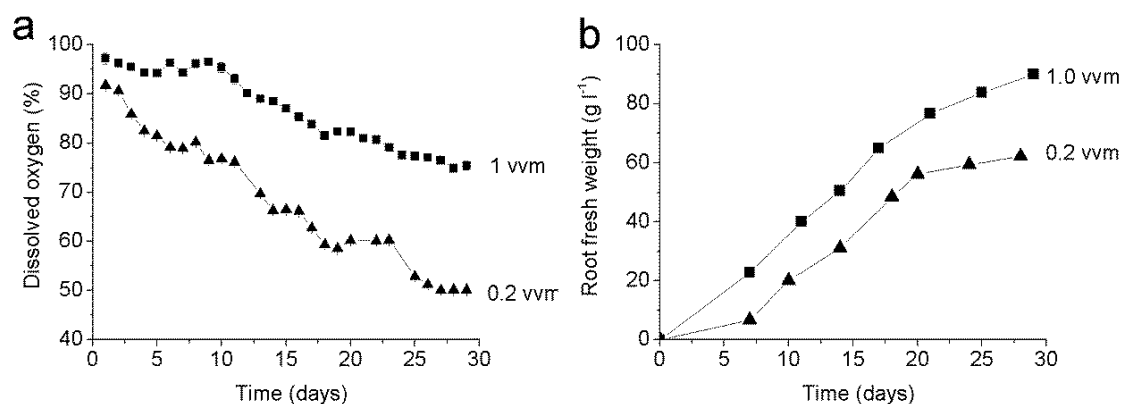


Figure 6.5 Dissolved oxygen (a) and root fresh weight (b) of *H. niger* hairy roots cultivated in a 1-liter fermentor at 0.2 and 1 vvm aeration rates

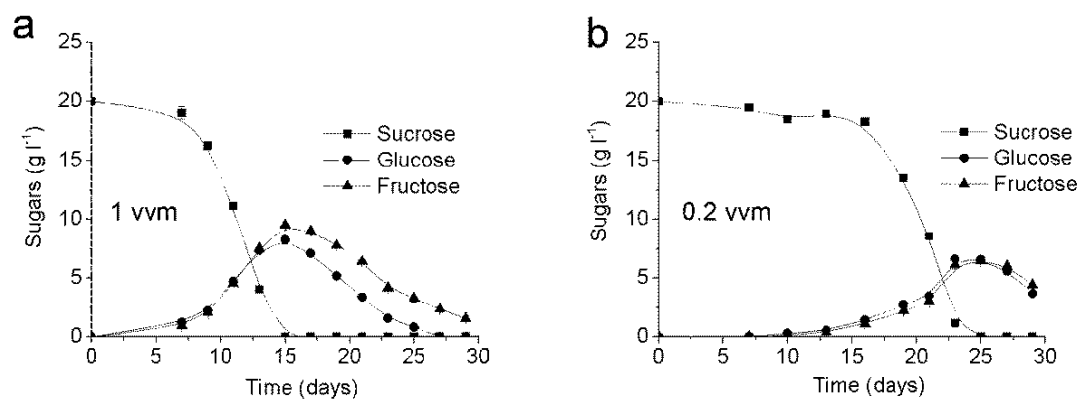


Figure 6.6 Sugar concentrations in the medium of *H. niger* hairy roots cultivated in a 1-liter fermentor at 0.2 and 1 vvm aeration rates

Sucrose is a commonly used carbon source for plant tissue cultures because it is the predominant form of carbon transported from source (leaves) to sink (roots, young leaves, flowers, fruits) tissues (Giaquinta 1980). In plants, sucrose at sink regions can be transferred into cells via 1) symplastic phloem unloading, 2) uptake by sucrose-specific transporters, and/or 3) sucrose hydrolysis in apoplast and uptake of glucose and fructose by specific carriers (Giaquinta et al. 1983). For hairy root cultures, the uptake of sugars has not been intensively investigated. As shown in Figure 6.6, glucose and fructose reached the maximum concentrations when sucrose completely disappeared in the media. After the sucrose depletion, glucose and fructose concentrations decreased gradually. These results suggest that sucrose might be hydrolyzed before uptake, but we are not sure if sucrose can be directly used by the *H. niger* hairy roots.

The decrease in sucrose concentration in the medium at 0.2 vvm lagged behind that at 1 vvm (Figure 6.6). It is noteworthy that the hairy roots grew gradually but the decrease in sucrose concentration in the medium was during a period of 7–10 days (Figure 6.6). In other words, the decline in sucrose was not closely associated with the growth of hairy roots. One possibility is that the sucrose hydrolysis mainly occurred when the hairy root biomass accumulated to a certain level. Sucrose began to decrease sharply on day 7 at 1 vvm, while a rapid decline in sucrose started on day 13 at 0.2 vvm. The root fresh weight at 1 vvm on day 7 was about 25 g l⁻¹, and the root fresh weight at 0.2 vvm on day 13 was about 28 g l⁻¹.

The lower glucose concentration than fructose concentration in hairy root culture medium is previously reported, but its explanation remains elusive (Kim et al. 2003; see Chapter 4). In sugar beet roots, extracellular glucose inhibited the uptake of fructose

(Wyse 1979). Likewise, strong competing effect of glucose over fructose uptake was reported for maize root tips (Xia and Saglio 1988). The lower concentration of glucose than fructose in hairy root culture medium might be caused by similar competing effect. In contrast to the preferential uptake of glucose at 1 vvm after sucrose depletion, the consumption of glucose and fructose at 0.2 vvm was similar (Figure 6.6b). Thus, aeration level appears to affect the uptake of glucose and fructose by the *H. niger* hairy roots.

4.4 Cultivation of H. niger hairy roots in a novel ground-joint column bioreactor connected to a microbubble generator

In our previous investigation, *Nicotiana tabacum* hairy root growth was improved by using microbubbles (see Chapter 5). Since the *H. niger* hairy root growth was enhanced at 1 vvm (Figure 6.5b), microbubbles might also be used to improve dissolved oxygen and the growth of *H. niger* hairy roots. Similar to the *N. tabacum* hairy roots, *H. niger* hairy roots culture medium did not stabilize microbubbles, and surfactants needed to be added for microbubble stabilization. In the present study, several surfactants were investigated for their effects on the hairy root growth and the production of hyoscyamine and scopolamine. As shown in Figure 6.7, SDS showed the highest inhibitory effect on the hairy root growth at concentrations above 25 mg l⁻¹. Likewise, at concentrations above 50 mg l⁻¹, TX100 produced less than 50% of root fresh weight in comparison to the untreated control. In general, Tween 80 did not show inhibitory effect on the growth of *H. niger* hairy roots, while Tween 20 showed decrease in root fresh weight at 50 and 100 mg l⁻¹ concentrations (Figure 6.7). In general, the effects of surfactants on *H. niger* hairy root growth were similar to our previous study on tobacco hairy roots (see Chapter 5).

As shown in Table 6.1, the addition of surfactants generally stimulated the production of hyoscyamine, whereas the accumulation of scopolamine in roots was improved only by Tween 20 and 80. In our previous study, TX100 treatment improved the nicotine release from tobacco hairy roots (see Chapter 5). In the present study, all concentrations of TX100 showed higher release of scopolamine into the media compared to the untreated control. Hyoscyamine was not detectable in the hairy root culture medium (Table 6.1).

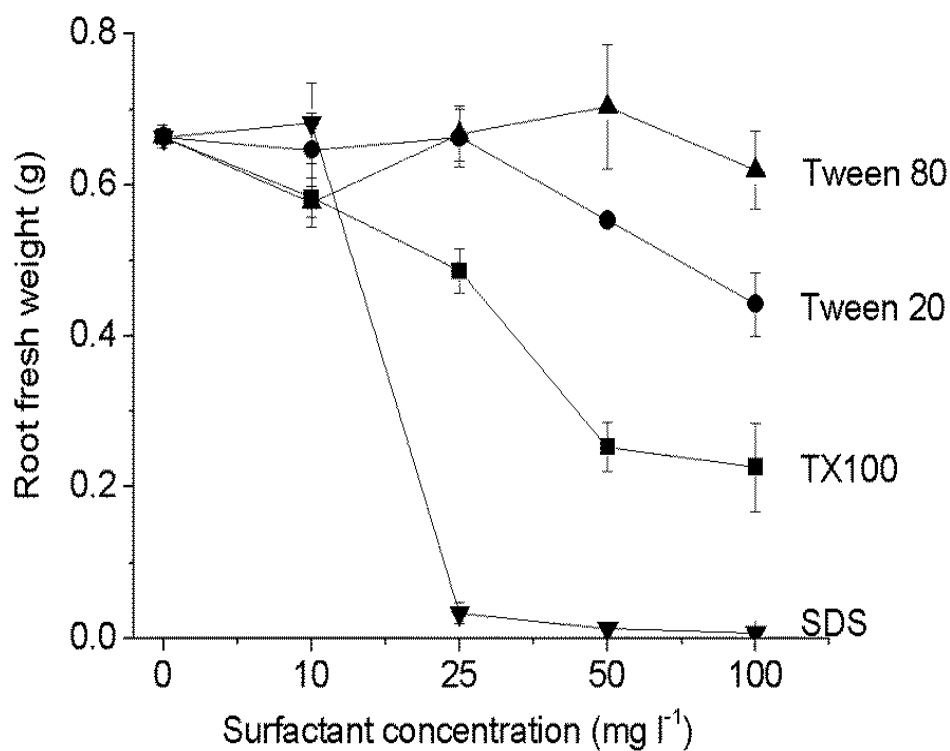


Figure 6.7 Effects of Tween 80, Tween 20, Triton X-100 (TX100), and sodium dodecyl sulfate (SDS) on the fresh weight of *H. niger* hairy roots

Table 6.1 Production of hyoscyamine and scopolamine by *H. niger* hairy roots

Surfactant (mg l ⁻¹)		Scopolamine (µg g ⁻¹ FW)		Hyoscyamine (µg g ⁻¹ FW)	
		In roots	In medium	In roots	In medium
Control	0	29.0 ± 0.8	3.3 ± 0.3	68.6 ± 4.0	N. D.
Tween 20	10	49.0 ± 1.5	N. D.	147.0 ± 4.9	N. D.
	25	41.6 ± 0.1	N. D.	119.8 ± 4.6	N. D.
	50	34.7 ± 0.1	N. D.	143.0 ± 9.6	N. D.
	100	91.1 ± 3.8	N. D.	232.8 ± 1.6	N. D.
Tween 80	10	148.6 ± 9.8	7.2 ± 0.1	386.9 ± 30.8	N. D.
	25	66.2 ± 12.0	N. D.	115.3 ± 17.3	N. D.
	50	57.3 ± 7.0	N. D.	116.5 ± 17.9	N. D.
	100	60.0 ± 3.2	N. D.	94.9 ± 15.7	N. D.
TX100	10	25.6 ± 0.2	23.5 ± 2.8	43.0 ± 7.6	N. D.
	25	33.0 ± 0.1	21.5 ± 2.4	110.7 ± 1.2	N. D.
	50	17.9 ± 0.1	19.7 ± 2.5	112.7 ± 2.1	N. D.
	100	18.7 ± 0.2	23.9 ± 4.1	187.0 ± 2.4	N. D.
SDS	10	9.8 ± 1.1	N. D.	204.8 ± 5.0	N. D.
	25	N. D.	N. D.	N. D.	N. D.
	50	N. D.	N. D.	N. D.	N. D.
	100	N. D.	N. D.	N. D.	N. D.

FW: fresh weight of hairy roots; N.D.: not detectable. Values are expressed as the mean ± standard error (n = 3). Samples were taken after 10 days cultivation

In our previous study, TX100 stimulated the nicotine steady state levels in tobacco hairy roots (see Chapter 5). The production of nicotine and hyoscyamine share a pathway from ornithine to 1-methyl-pyrroinium cation (Hashimoto and Yamada 1994; Lee et al. 2005). It is possible that the stimulative effect of TX100 targeted enzymes in this shared metabolic pathway. Since the steady state levels of hyoscyamine and scopolamine were detected, a possible degradation of these two tropane alkaloids might also be involved in the effect of TX100 treatment.

As shown in Table 6.1, the TX100 treatment improved the release of scopolamine but not hyoscyamine into the culture medium. In a previous study, dimethyl sulfoxide

(DMSO) treatment of *H. niger* hairy roots showed various proportions of scopolamine, hyoscyamine, and anisodamine released into the media (Jaremicz et al. 2014). If the improved alkaloid repartition into medium was merely due to cell permeabilization, the release of these alkaloids would likely be similar. Thus, it is likely that altered biological active transport and/or subcellular localization processes were involved in the net release of alkaloids in the *H. niger* hairy roots.

Since Tween 80 showed the least inhibitory effect on the *H. niger* hairy root growth, 100 mg l⁻¹ Tween 80 was used for stabilizing microbubbles in the microbubble generator. At 4000 rpm and 1vvm aeration rate, 100 mg l⁻¹ Tween 80 stabilized microbubbles and showed k_{La} of 55 h⁻¹, this was much higher than the k_{La} of 16.4 h⁻¹ in conventional air sparging (see Chapter 5). As shown in Figure 6.8, the addition of microbubbles improved the growth of *H. niger* hairy roots. The root fresh weight reached about 100 g after 18 days cultivation with microbubbles, whereas the hairy roots only grew slightly with conventional air sparging. The dissolved oxygen decreased rapidly to below 20% with conventional air sparging, while the oxygen in the medium was about 70% after 18 days cultivation with microbubbles at the same aeration rate. In our previous study on tobacco hairy roots, 50 mg l⁻¹ TX100 was used as microbubble stabilizer because it stabilized the microbubbles, improved the release of nicotine into the medium, and showed less inhibition on root growth (see Chapter 5). In the present study, however, the 50 mg l⁻¹ TX100 dramatically inhibited the *H. niger* hairy root growth (Figure 6.7). Thus, microbubble generator is generally useful for improving the oxygen mass transfer in hairy root cultures, though surfactants need to be selected for not only stabilizing microbubbles but also maintaining the growth of hairy roots.

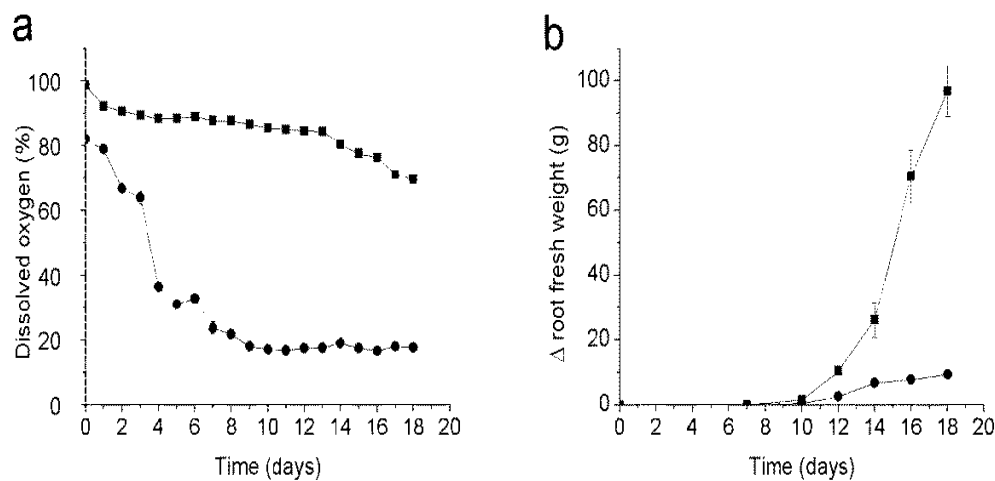


Figure 6.8 Dissolved oxygen (a) and root fresh weight (b) of *H. niger* hairy roots in a novel ground-joint column bioreactor with (filled square) and without (filled circle) microbubbles

5. Conclusions

In the present study, the morphology of hairy roots was affected by aeration conditions. Hairy roots cultivated under low aeration were shorter and considerably wider in comparison to well aerated hairy roots. Confocal microscope observation showed that the hairy root cells with less aeration were much wider than the cells with sufficient aeration. The growth of *H. niger* hairy roots in 1-liter fermentors increased with increasing aeration rate. The combination of microbubble generator and a novel ground-joint column bioreactor improved the dissolved oxygen and consequently the growth of *H. niger* hairy roots.

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CHAPTER 7

TROPANE ALKALOID PRODUCTION IN CULTURE MEDIUM BY TRANSGENIC *HYOSCYAMUS NIGER* HAIRY ROOTS

1. Abstract

The release of tropane alkaloids from hairy roots into culture medium provides opportunities to develop low-cost purification processes. In the present study, the release of hyoscyamine and scopolamine from transgenic *H. niger* hairy roots was significantly improved by adjusting the medium pH from 6 to 3. Medium pH 3 inhibited *H. niger* hairy root growth, though the root branches appeared to survive the low pH treatment. A preliminary study on tropane alkaloid release by wild-type and transgenic *H. niger* hairy roots showed that the transgenic line released more tropane alkaloids, especially hyoscyamine, in comparison to the wild-type line.

2. Introduction

Hairy roots are developed by infecting plant tissue with *Agrobacterium rhizogenes* (Guillon et al. 2006; Willmitzer et al. 1982). As phytochemical producers, hairy roots show genetic stability, rapid growth, and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types (Flores et al. 1999).

Hyoscyamus niger is a Solanaceous and produces scopolamine and hyoscyamine (Roberts and Wink 1998). Scopolamine and hyoscyamine belong to tropane alkaloids and are medically important as anticholinergic agents (Renner et al. 2005). Anticholinergics block the acetylcholine, which is the neurotransmitter in the central and

the peripheral nervous systems, and reduce the activities mediated by acetylcholine in neurons through competitive inhibition (Gupta and Mehdi 2013). Anticholinergic drugs can be used in the treatment of motion sickness, which is caused by cholinergic stimulation of the vestibular apparatus (Tierney et al. 2010). In plants, tropane alkaloids are synthesized in the root and translocated to the aerial parts (Hashimoto et al. 1986; Sato et al. 2007). Differentiated root tissues were reported to accumulate more tropane alkaloids than undifferentiated plant cell suspension cultures (Tabata et al. 1972).

Tropane alkaloids produced by hairy roots remain mainly inside the roots (Boitel-Conti et al. 1995; Pitta-Alvarez and Giulietti 1999). Tropane alkaloids accumulating primarily in the root tissue present a bioprocessing challenge because the root tissue must be homogenized and the tropane alkaloids extracted and purified from a chemically complex solution. On the other hand, tropane alkaloids that accumulate mostly in the culture media are in a chemically less complex milieu than those which are retained within the hairy root tissue. Therefore, tropane alkaloids that accumulate in the culture media require substantially less bioprocessing effort to isolate and purify.

In the present study, the effects of medium pH on tropane alkaloid release by *H. niger* hairy roots were investigated. The effects of medium pH 3 on hairy root growth and viability were also investigated. In addition, a preliminary investigation was conducted on the comparison between the transgenic and wild-type *H. niger* hairy root lines for tropane alkaloid release.

3. Materials and methods

3.1 Hairy root lines and culture media

Transgenic *H. niger* hairy root line HnRKC and wild-type *H. niger* hairy root line Hn2 were developed at Virginia Tech. *H. niger* hairy roots were subcultured every three weeks in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 20 g l⁻¹ sucrose and 3 g l⁻¹ phytigel. The basal Gamborg's B5 medium and sucrose were dissolved in distilled water and the pH was adjusted to 5.7 with 1 M KOH. After autoclaving, Gamborg's B5 vitamin mixture (Research Products International Corp., Mount Prospect, IL, USA) was added to the medium at 0.112 g l⁻¹. Gamborg's B5 medium supplemented with 20 g l⁻¹ sucrose and 0.112 g l⁻¹ Gamborg's vitamin mixture was used for hairy root cultivations in flasks and bioreactors.

3.2 Cultivation of *H. niger* hairy root RKC in 1-liter fermentors with medium pH adjustment

The *H. niger* hairy roots were cultivated in 1-liter fermentors (BIOSTAT Q, B. Braun Biotech International, Germany). The fermentor was filled with 0.7 liter of Gamborg's B5 medium and aerated at 1 vvm (volume of air per volume of medium per minute). The fermentor was inoculated with 40 hairy root tips (about 1.5 cm in length) and incubated at 25°C and ambient illumination (approximately on a 12-h light/12-h dark cycle). The medium pH in the fermentor was adjusted by adding 0.1 M H₂SO₄ and KOH.

3.3 Preliminary investigation on tropane alkaloid release by wild-type and transgenic *H. niger* hairy roots

The wild-type line Hn2 and transgenic line HnRKC *H. niger* hairy roots were cultivated in 1-liter fermentors. The fermentor was filled with 0.7 liter of Gamborg's B5

medium and aerated at 1 vvm. The fermentor was inoculated with 40 hairy root tips with branches and incubated at 25°C and ambient illumination (approximately on a 12-h light/12-h dark cycle). For each of the two hairy root lines, the medium pH in one fermentor was maintained at around 6 from day 0 to day 21. The medium pH in another fermentor was maintained at around 6 from day 0 to day 14, and the medium pH was adjusted and kept at around 3 from day 15 to day 21. The medium pH in the fermentor was adjusted by adding 0.1 M H₂SO₄ and KOH.

3.4 Analytical methods

High performance liquid chromatography (Shimadzu Scientific, Columbia, MD, USA) was used for the quantification of hyoscyamine and scopolamine in the hairy root tissue and in the culture medium. Fresh hairy roots were added to 10 ml methanol/water (40/60, v/v), ground with mortar and pestle, and extracted on a shaker at 100 rpm and 25°C for 12 h. Culture broth and extracted samples were filtered with 0.2 µm membrane and separated on a Waters, Resolve C18 column (5 µm, 90 Å, 3.9 × 150 mm) with a guard column (Waters, Resolve C18, 5 µm, 90 Å, 3.9 × 20 mm). The isocratic mobile phase consisted of 40/60 (v/v) methanol/water and 2% (v/v) phosphoric acid. The pH of the mobile phase was adjusted to 7.25 with triethylamine. The flow rate of mobile phase was 0.5 ml min⁻¹ and the injection volume was 20 µl. Scopolamine and hyoscyamine were measured at 220 nm with a UV detector. Standards of scopolamine and hyoscyamine (Sigma-Aldrich, St Louis, USA) were prepared in mobile phase at 0.1, 0.25, 0.5, 1, 5, 10, 25, and 50 mg l⁻¹.

Samples were sent to the Center for Integrated BioSystems, Utah State University, for high performance liquid chromatography and mass spectrometry (HPLC-MS) analysis of scopolamine and hyoscyamine in the culture medium.

4. Results and discussion

4.1 Effects of medium pH on the release of hyoscyamine and scopolamine

The HnRKC hairy roots were cultivated in 1-liter fermentors. Figure 7.1 shows the correlation between the medium pH and the accumulation of hyoscyamine in the hairy root culture medium. From day 0 to 18, the medium pH was maintained at about 6.0, and hyoscyamine was not detectable in the culture medium. From day 19 to 21, the medium pH was adjusted to 3 and hyoscyamine release was detected. This result showed that the release of hyoscyamine could be triggered by acidic culture medium. In order to find the critical medium pH for hyoscyamine release, the medium pH was increased from 3 to 4. As shown in Figure 7.1, hyoscyamine concentration in the medium decreased at pH 4 (days 22 and 23), which implies that the critical medium pH for hyoscyamine release could be between 3 and 4. When the medium pH was adjusted from 4 to 3 (on day 24), hyoscyamine concentration in the medium increased from 0.5 to 5 mg l⁻¹ in one day (Figure 7.1). When the medium pH was maintained at 3 after day 24, the accumulation of hyoscyamine increased dramatically from 5 to 41 mg l⁻¹ (Figure 7.1). As shown in Figure 7.2, the accumulation of scopolamine in medium was also stimulated at pH 3 and showed a similar correlation to the medium pH.

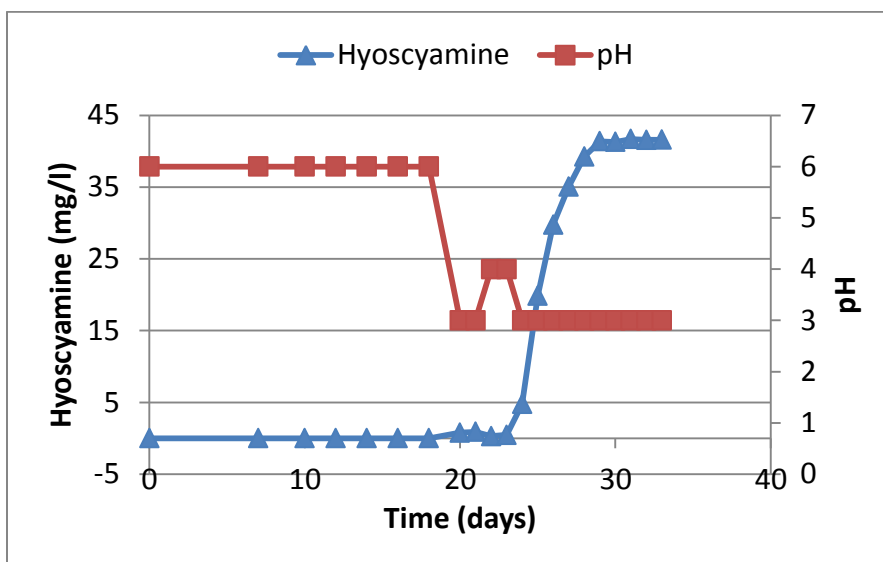


Figure 7.1 Effect of medium pH on the accumulation of hyoscyamine in hairy root culture medium

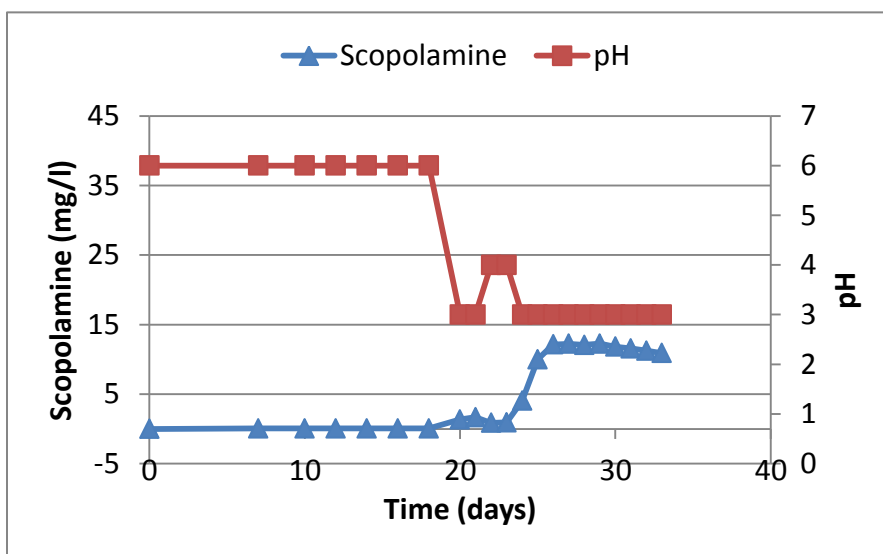


Figure 7.2 Effect of medium pH on the accumulation of scopolamine in hairy root culture medium

In our previous investigation on nicotine release from tobacco hairy roots, the accumulation of nicotine in the culture medium decreased with medium pH increasing from 6 to 8 (see Chapter 4). In the present study, the medium pH was initially maintained around 6. However, hyoscyamine was not detectable and about 0.1 mg l⁻¹ of scopolamine accumulated in the medium, which was low in comparison to the 20 to 90 mg l⁻¹ of nicotine in medium at pH 6 (see Chapter 4). The increase in hyoscyamine and scopolamine release at pH 3 implies that the optimal medium pH for alkaloid release varies among alkaloid types and/or hairy root species. However, the medium pH adjustment appears to be generally useful for manipulating alkaloid release from hairy roots. It was reported that the release of hyoscyamine and scopolamine from *Datura stramonium* hairy roots increased when the medium pH (4.8 to 7.0) was reduced to 3.5 (Saenz-Carbonell et al. 1993). For the two *D. stramonium* hairy root lines investigated, the maximum increase in hyoscyamine and scopolamine release was 22-fold (from 0.2 to 4.7 mg l⁻¹) and 30-fold (from 0.5 to 15 mg l⁻¹), respectively (Saenz-Carbonell et al. 1993). For the release of tropane alkaloids, the transgenic *H. niger* hairy roots appear to be more sensitive to low pH treatment, especially for hyoscyamine, whose concentration in the medium increased from zero to 41 mg l⁻¹ (Figure 7.1).

A possible explanation for the release of tropane alkaloids at medium pH 3 is that tropane alkaloid molecules were positively charged at medium pH 3 and blocked in the medium by plant cell membranes (ion-trapping). The ion-trapping theory was previously proposed for nicotine alkaloid release (Larsen et al. 1993). According to the ion-trapping theory, nicotine is an uncharged free base at high pH (Lochmann et al. 2001) and this neutral base can diffuse through plant membranes, and presumably becomes trapped in

the acidic vacuole as a cationic quaternary amine (Larsen et al. 1993). Similar to the nicotine alkaloid, the free base tropane alkaloids might diffuse through plant membranes, but charged tropane alkaloid molecules were blocked. The charged tropane alkaloid molecules increase with decreasing pH. Thus, tropane alkaloids could accumulate inside or outside the plant membranes where the pH was lower. Another possible explanation for the release of tropane alkaloids is absorption and desorption on the hairy root surface at various medium pH. In order to show whether the tropane alkaloids were taken inside the root cells or absorbed on the surface of the hairy roots, the root cell wall might be isolated from the cell protoplast, and the absorption of tropane alkaloids on the cell wall could be investigated.

Figure 7.3 shows the comparison between hyoscyamine and scopolamine accumulation in the hairy root culture medium. It shows a similar pattern of hyoscyamine and scopolamine release as a response to the medium pH adjustment, whereas the maximum concentration of hyoscyamine (42 mg l^{-1}) was 3.8-fold of scopolamine (11 mg l^{-1}). The hyoscyamine content in roots ($70 \text{ } \mu\text{g g}^{-1} \text{ FW}$) was considerably higher than that of scopolamine ($18 \text{ } \mu\text{g g}^{-1} \text{ FW}$) at the end of cultivation. In addition, the release ratio of hyoscyamine and scopolamine at the end of cultivation was 89.0% and 89.5%, respectively. It appears that the effect of pH 3 on tropane alkaloid release did not distinguish between hyoscyamine and scopolamine, and thus the similar release ratio at the end of cultivation.

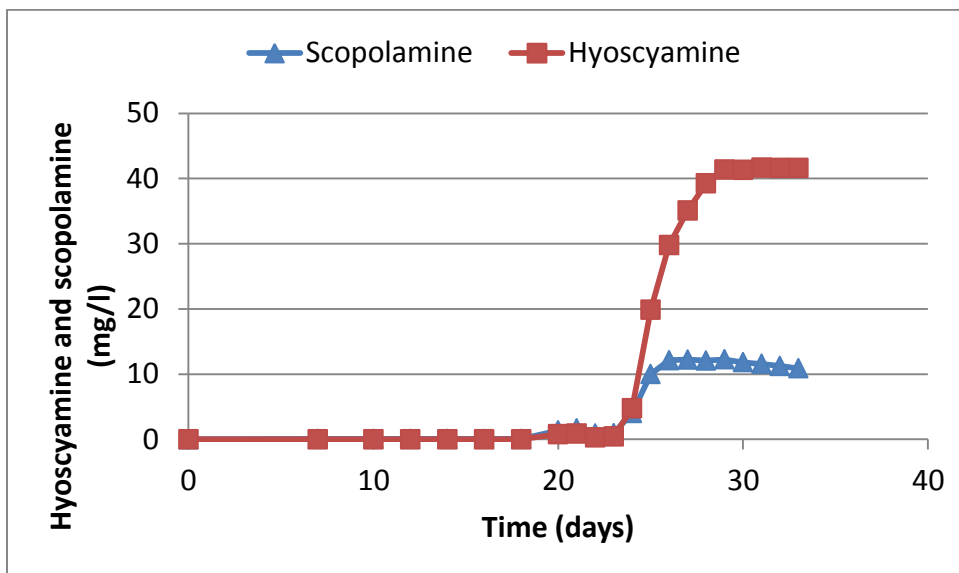


Figure 7.3 Comparison of hyoscyamine and scopolamine accumulation in hairy root culture medium (day 0 to day 33)

4.2 Effects of medium pH on the growth and viability of HnRKC hairy roots

Figure 7.4 shows the correlation between medium pH and dissolved oxygen during the cultivation of HnRKC. At pH 6, the dissolved oxygen decreased gradually to below 40%. When the medium pH was adjusted to 3, the dissolved oxygen increased rapidly to above 90%. Although the dissolved oxygen slightly decreased at pH 4, it remained above 95 % when the medium pH was maintained at 3. The increase in dissolved oxygen at pH 3 implies a decrease in the activity of the HnRKC hairy roots.

To investigate the effects of low medium pH on hairy root growth, five hairy root tips of HnRKC were incubated with initial medium pH 4 and pH 3 for 7 days. As shown in Figure 7.5, the HnRKC hairy roots grew rapidly at pH 4, whereas the root growth was completely suppressed at pH 3. At pH 3, the root tips did not grow but showed small branches (protrusions), as indicated by the arrows in Figure 7.5b.

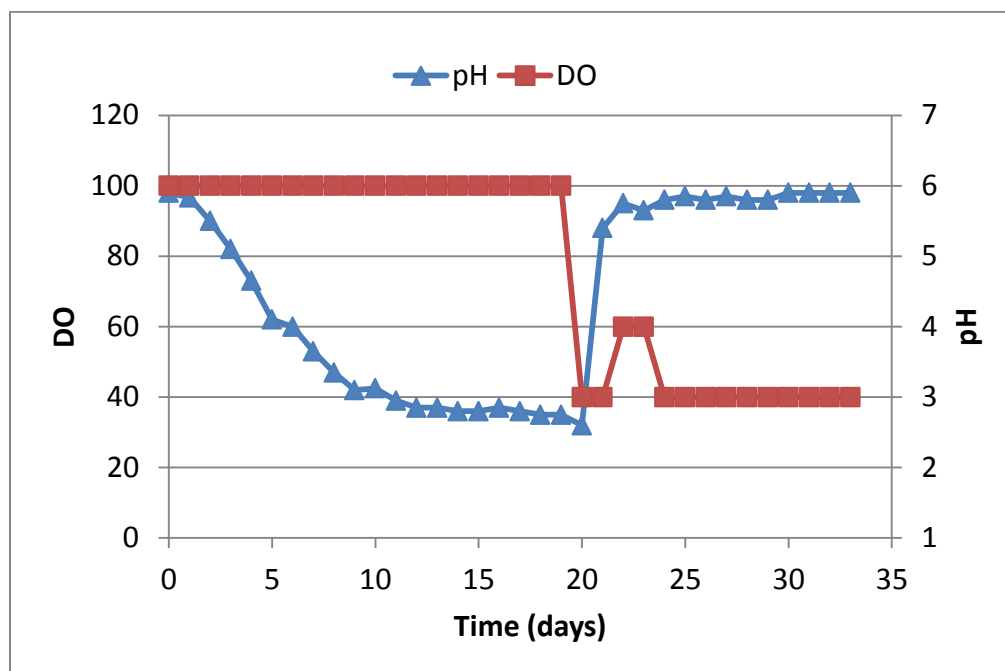


Figure 7.4 Dissolved oxygen and medium pH

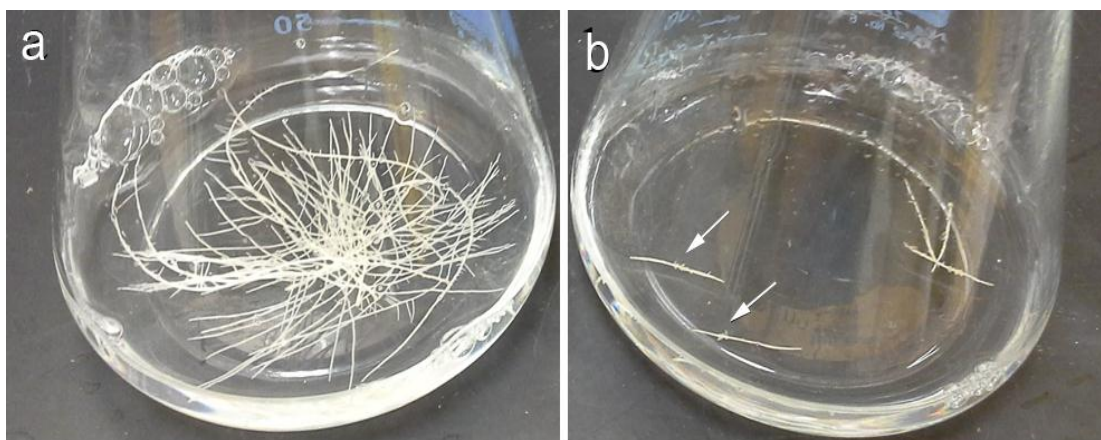


Figure 7.5 Effects of initial medium pH 4 (a) and initial medium pH 3 (b) on the growth of hairy root line HnRKC. Photos were taken after 7 days cultivation

The effects of low medium pH on HnRKC hairy roots were also investigated by treating the root matrix at medium pH 3 in Erlenmeyer flask. After 24 h treatment, the root tips along with the tiny branches were cut off, washed in deionized water, and incubated on Gamborg's B5 plate at pH 5.8. Figure 7.6a shows the hairy root branches cut from the hairy root matrix treated at pH 3 for 24 h. The root tips turned black, whereas the tiny branches generally remained unaffected. Figure 7.6b shows the same hairy root branches incubated on the plate for 24 h. It shows a rapid growth of the tiny branches at pH 5.8. Thus, medium pH 3 damaged the hairy root tips, but the root branches were not affected by pH and were still viable. In addition to the branches, the hairy roots appear to extend slightly, which implies the growth of the hairy roots might not be completely suppressed after 24 h treatment at medium pH 3 in Erlenmeyer flask.

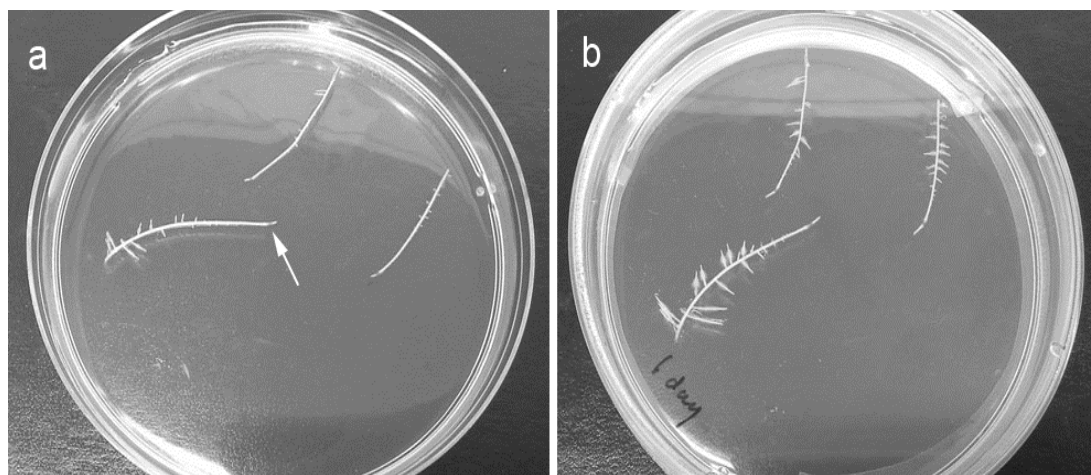


Figure 7.6 HnRKC hairy root branches cut from hairy root matrix treated at medium pH 3 in Erlenmeyer flask for 24 h (a), and the same hairy root branches grew on pH 5.8 Gamborg's B5 plate for 24 h (b)

4.3 Comparison between wild-type and transgenic H. niger hairy roots for tropane alkaloid release

The wild-type line Hn2 and transgenic line HnRKC were investigated for tropane alkaloid release in 1-liter fermentors. Figures 7.7 and 7.8 show the HPLC spectrum on culture medium and hairy root extract, respectively. As shown in Figures 7.7b and d, the wild-type line released scopolamine and small amount of hyoscyamine into the medium at pH 3, whereas the transgenic line showed higher tropane alkaloid accumulation in the medium at pH3. The wild-type line showed 0.016 mg g⁻¹ FW hyoscyamine and 0.038 mg g⁻¹ FW scopolamine in medium pH 3, whereas the transgenic line showed 0.47 mg g⁻¹ FW hyoscyamine and 0.066 mg g⁻¹ FW scopolamine in medium pH 3. Thus, the transgenic line showed about 10-fold increase in tropane alkaloid release in comparison to the wild-type line. It is noteworthy that the transgenic line HnRKC showed considerably higher hyoscyamine accumulation in medium at pH 3 in comparison to the wild-type line. For tropane alkaloid accumulation within the wild-type hairy roots, medium pH 3 showed lower scopolamine but slightly higher hyoscyamine in comparison to medium pH 6 (Figures 7.8a and b). For the transgenic line, medium pH 3 showed dramatically lower accumulation of both scopolamine and hyoscyamine within the hairy roots in comparison to medium pH 6 (Figures 7.8c and d).

In order to confirm the release of tropane alkaloids into the medium by the transgenic line at medium pH 3, the hairy root culture medium at the end of cultivation was analyzed by high performance liquid chromatography and mass spectrometry (HPLC-MS). Figure 7.9 shows the chromatograms for scopolamine standard, and the medium of the transgenic line cultivated at pH 6 and pH 3. Figure 7.10 shows the mass

spectrometry for the scopolamine standard, and the medium of the transgenic line cultivated at pH 6 and pH 3. The medium pH 3 showed the same molecular ion peak (304.1 in Figure 7.10c) and similar fragmentation pattern as the scopolamine standard (Figure 7.10a), which confirms the presence of scopolamine in the medium. The medium pH 6 also showed the molecular ion peak of 304.1 (Figure 7.10b), which implies that the transgenic line also released scopolamine at pH 6. However, the medium pH 6 did not show the peak of scopolamine in the HPLC chromatogram (Figure 7.9a), whereas the medium pH 3 clearly showed the peak of scopolamine at 5.07 min (Figure 7.9c). Thus, the release of scopolamine by the transgenic line at medium pH 6 was extremely low in comparison to the release at medium pH 3.

Figures 7.11 and 7.12 show the HPLC-MS analysis for hyoscyamine in the medium of transgenic *H. niger* hairy roots cultivated at medium pH 3 and pH 6. The medium pH 6 did not show the peak of hyoscyamine in the HPLC chromatogram, whereas the medium pH 3 showed a prominent peak of hyoscyamine at 6.33 min (Figure 7.11). As demonstrated in the mass spectrometry analysis, medium pH 3 showed a major molecular ion peak of hyoscyamine (Figure 7.12c), whereas the medium pH 6 showed a very small molecular ion peak of hyoscyamine (Figure 7.12a). Similar to the scopolamine, the concentration of hyoscyamine in medium pH 6 could be extremely low (Figure 7.11a).

These results confirmed the positive effect of medium pH 3 on the release of tropane alkaloids. In addition, the wild-type line appeared to release less tropane alkaloids, especially hyoscyamine, in comparison to the transgenic line at medium pH 3.

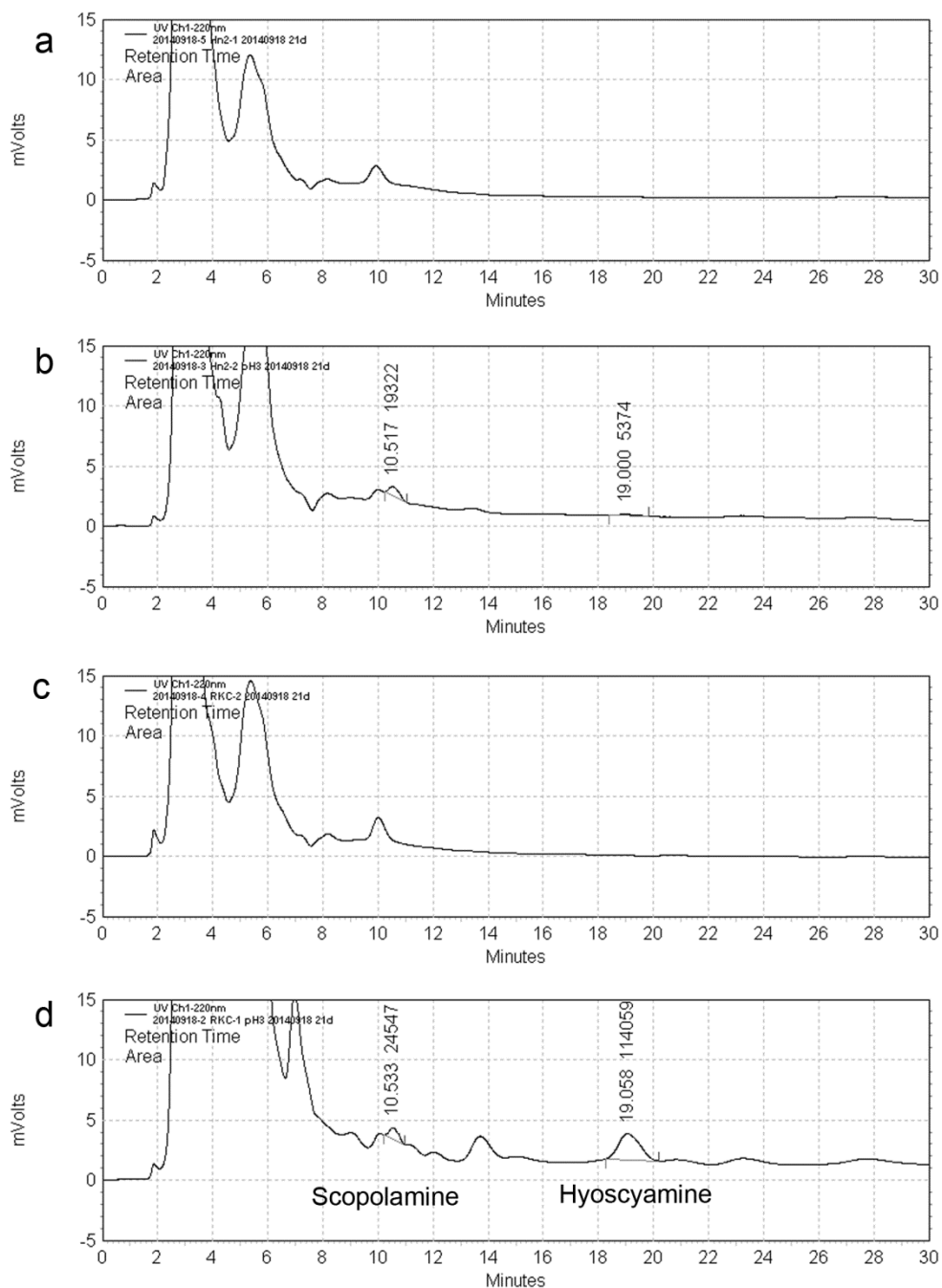


Figure 7.7 HPLC spectrum on *H. niger* hairy root culture medium at the end of cultivation. a: wild-type line and medium pH 6; b: wild-type line and medium pH 3; c: transgenic line and medium pH 6; and d: transgenic line and medium pH 3

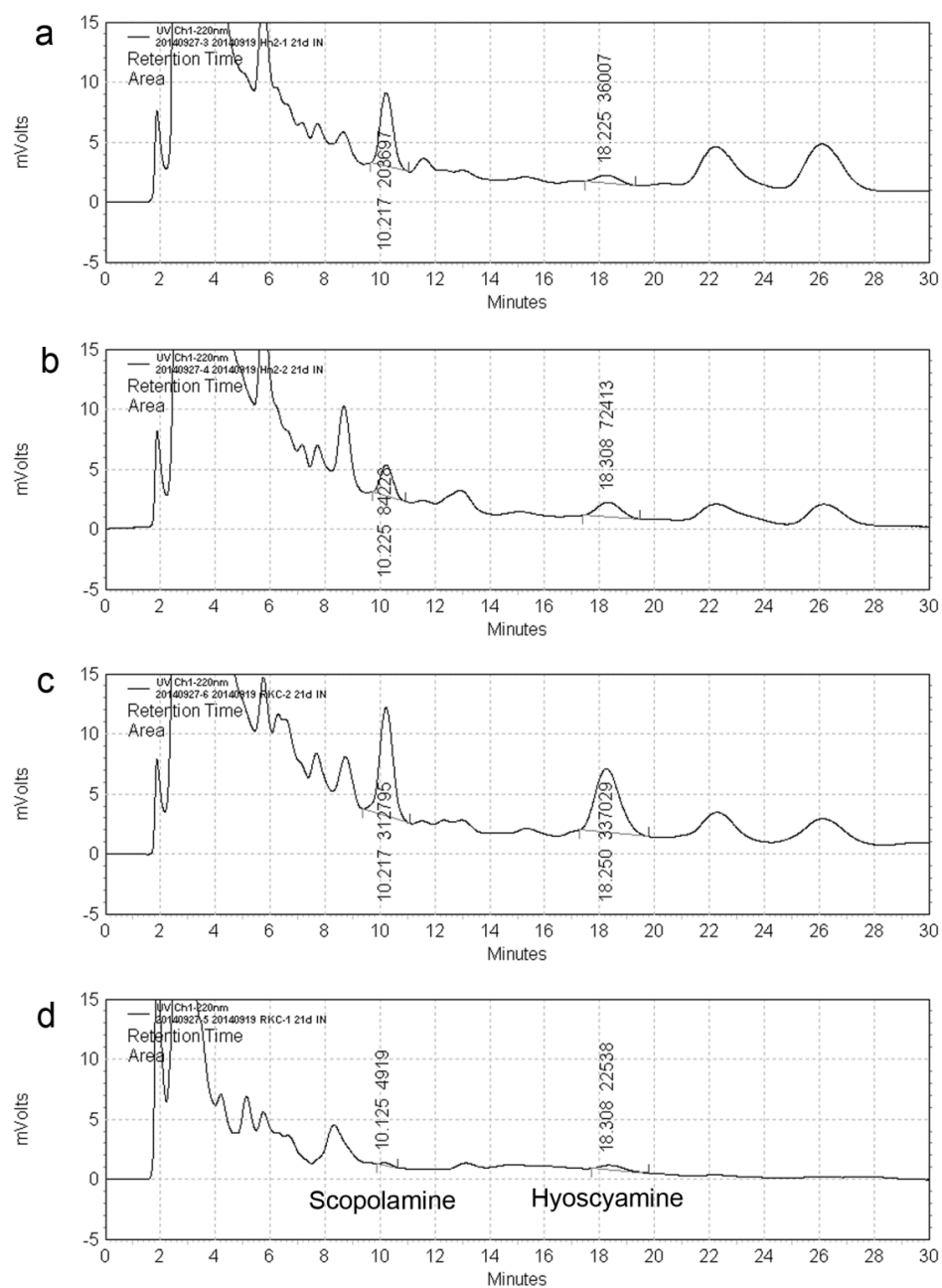


Figure 7.8 HPLC spectrum on *H. niger* hairy root extract at the end of cultivation. a: wild-type line and medium pH 6 (1.366 g fresh roots, 10 ml extract); b: wild-type line and medium pH 3 (1.243 g fresh roots, 10 ml extract); c: transgenic line and medium pH 6 (1.036 g fresh roots, 10 ml extract); and d: transgenic line and medium pH 3 (1.052 g fresh roots, 10 ml extract)

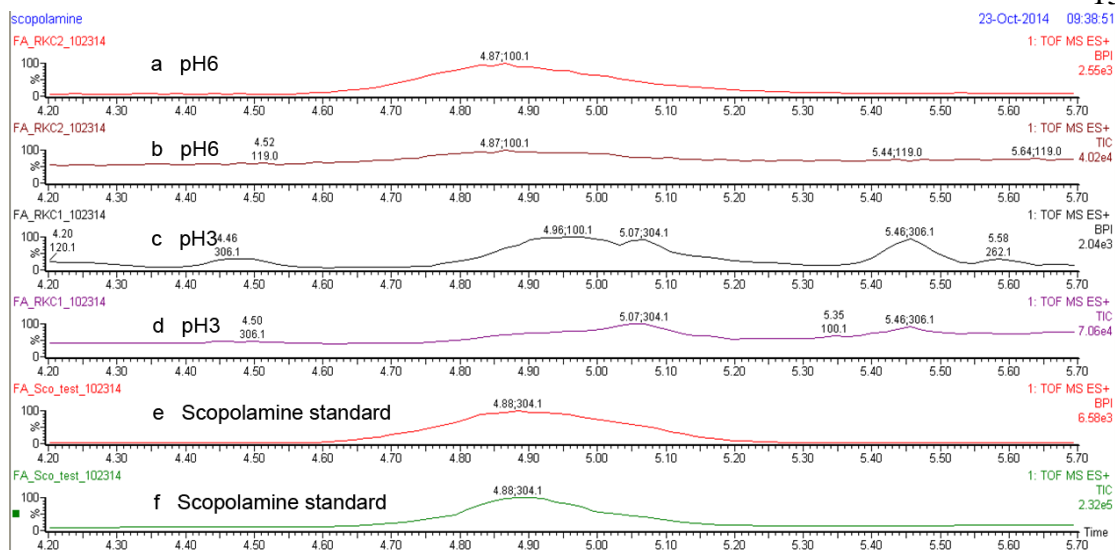


Figure 7.9 Zoomed HPLC chromatograms for the medium of the transgenic *H. niger* hairy roots cultivated at medium pH 6 (a and b), medium pH 3 (c and d), and the scopolamine standard (e and f)

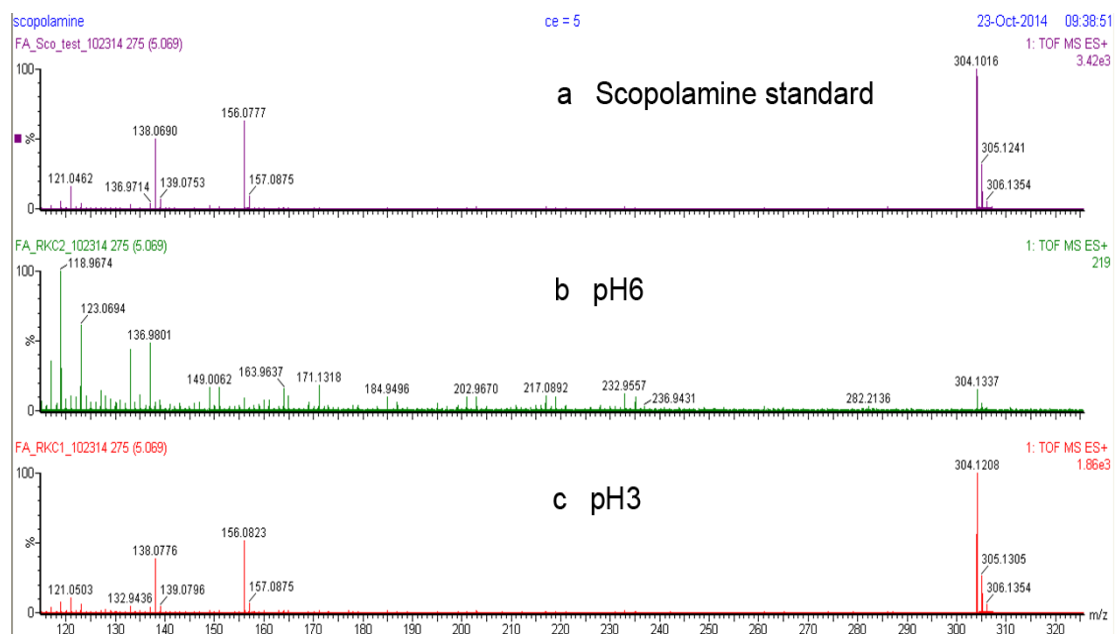


Figure 7.10 Mass spectrometry for the scopolamine standard (a), the medium of the transgenic *H. niger* hairy roots cultivated at medium pH 6 (b), and medium pH 3 (c)

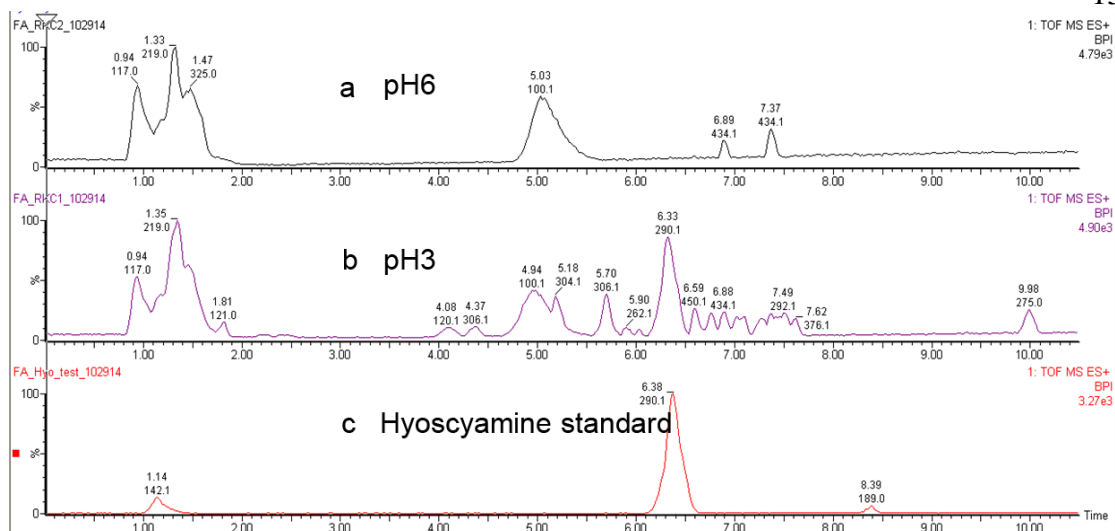


Figure 7.11 HPLC chromatograms for the medium of the transgenic *H. niger* hairy roots cultivated at medium pH 6 (a), medium pH 3 (b), and the hyoscyamine standard (c)

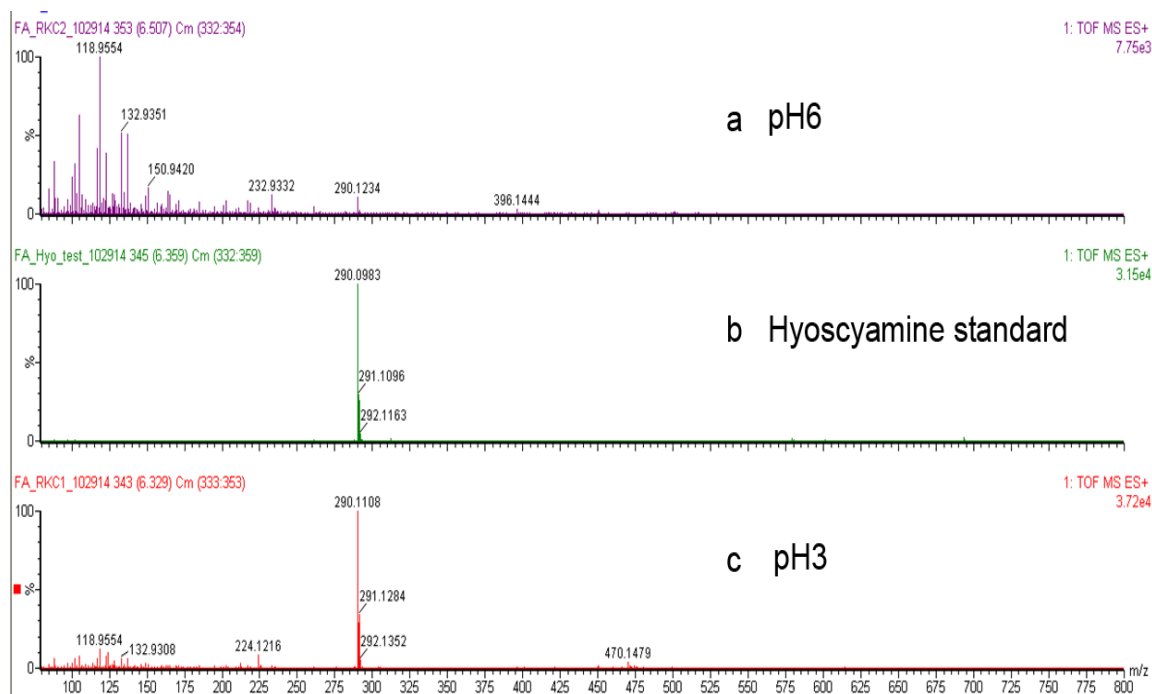


Figure 7.12 Mass spectrometry for the medium of the transgenic *H. niger* hairy roots cultivated at medium pH 6 (a), the hyoscyamine standard (b), and medium pH 3 (c)

5. Conclusions

In the present study, a transgenic *H. niger* hairy root line HnRKC was investigated for the release of tropane alkaloids into the culture medium. The release of hyoscyamine and scopolamine were significantly improved at medium pH 3 in comparison to medium pH 6. Medium pH 3 inhibited *H. niger* hairy root growth. In comparison to the wild-type *H. niger* hairy root line, the transgenic line showed about 10-fold increase in tropane alkaloid release, especially hyoscyamine, at medium pH 3.

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CHAPTER 8

SUMMARY

Alkaloid nicotine, hyoscyamine, and scopolamine were produced by *Nicotiana tabacum* and *Hyoscyamus niger* hairy roots. The positive effects of oxygen on hairy root growth and alkaloid production were demonstrated. Microbubble generator and novel ground-joint column bioreactor were developed and utilized for improving the oxygen mass transfer in hairy root cultures. The release of alkaloids from hairy roots into the culture medium was enhanced by manipulating the medium pH.

As a carbon and energy source, sucrose stimulated the growth of tobacco hairy roots. The tobacco hairy roots were able to grow with glucose and/or fructose as carbon sources, but the root growth was lower than that with sucrose.

Oxygen was a limiting nutrient in tobacco hairy root growth and nicotine production. Basification of the culture medium associated with tobacco hairy root growth resulted in a dramatic reduction in nicotine accumulation levels in the medium, which was reversed by decreasing the medium pH. Kinetic analysis of hairy root growth and nicotine accumulation in the medium revealed a potential improvement in nicotine yields in the media by stimulating the branching of tobacco hairy roots.

At 10, 25, and 50 mg l⁻¹, TX100 did not show strong inhibition of tobacco hairy root growth, yet improved nicotine accumulation in culture medium and volumetric oxygen mass transfer coefficient (k_{La}). The improvement in k_{La} by microbubbles generated at higher shear speed was a result of larger gas-liquid interfacial area; while higher surfactant concentration led to increased bubble stability and concomitant

improved oxygen mass transfer. In a 1-liter fermentor, the introduction of microbubbles to self-immobilized hairy roots increased k_{La} and the growth of tobacco hairy roots.

The morphology of wild-type *H. niger* hairy roots was affected by aeration conditions. Light microscope observation showed that hairy roots cultivated under low aeration were considerably wider in comparison to well aerated hairy roots. Confocal microscope analysis showed that the hairy root cells with less aeration were wider than the cells with sufficient aeration. The combination of microbubble generator and a novel ground-joint column bioreactor improved the dissolved oxygen and the growth of wild-type *H. niger* hairy roots.

A transgenic *H. niger* hairy root line was investigated for the release of tropane alkaloids into the culture medium. The release of hyoscyamine and scopolamine were significantly improved at medium pH 3 in comparison to medium pH 6. Medium pH 3 inhibited the *H. niger* hairy root growth. In comparison to the wild-type *H. niger* hairy root line, the transgenic line released more tropane alkaloids, especially hyoscyamine, at the medium pH 3.

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This paper was submitted to *Applied Biochemistry and Biotechnology* in 2014.

Best regards, -JJ

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November 18, 2014

To whom it may concern,

My name is Ritesh KC, currently in the human genetics department at the University of Michigan . Bo Zhao and I wrote the manuscripts titled:

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Bo Zhao was the first author for these two manuscripts. I give him permission to reprint the manuscripts in their entirety in his PhD dissertation.

Sincerely,
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VITA

Bo Zhao
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