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Medium optimization for the rhizosecretion of three monoclonal antibodies from *Nicotiana tabacum* hydroponic culture

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Spécialité Biologie Intégrative de l'Arbre et des plantes cultivées

Medium optimization for the rhizosecretion of three
monoclonal antibodies from *Nicotiana tabacum*
hydroponic culture

DAPHNÉ BOCCIARELLI

Mémoire de stage, soutenu à Nancy le 3 septembre 2014

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1. Introduction

1.1 Plant Molecular Pharming

Plant molecular pharming (PMP) is a branch of plant biotechnology in which plants or their tissues are genetically engineered to produce various recombinant proteins (e.g., pharmaceuticals, industrial proteins/enzymes and biopolymers) in large quantities (Xu, 2012).

1.1.1 A brief history

In 1986, human growth hormone was the first recombinant protein successfully produced in a plant (tobacco) (Barta, 1986). Three years later the first functional antibody was expressed in tobacco and demonstrated that plants could assemble complex glycoproteins with several subunits (Hiatt, 1989). This capability was taken a step further in 1992 with the first experimental vaccine produced in plants: the hepatitis B virus (HBV) surface antigen (Mason, 1992). Since then, PMP has been widely studied and led in 2012 to the first commercialization by PROTALIX of a plant cell-produced recombinant protein: taliglucerase alfa (ELELYSO™) (Nature news blog, 2012). This protein is involved in the treatment of the rare genetic condition Gaucher's disease. Many other applications can be found for plant-produced pharmaceuticals such as for anti-cancer activity, prevention and treatment of infectious diseases, enzyme replacement therapy, diagnostics, *etc* (Davies, 2010).

1.1.2 Recombinant protein production platform

Many different production platforms can be used to produce recombinant proteins (Table 1), and plants are a very promising alternative to the currently favoured mammalian cell systems. It is estimated that recombinant proteins could be produced in plants at 2-10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cells culture (Giddings 2001). Apart from cost benefits, plants also offer ease of scalability and enhanced safety profile because plants do not support human pathogens (e.g., viruses and prions). Several pharmaceuticals produced using plants are presently being tested in clinical trials such as an H5N1 vaccine candidate (end of phase II; Medicago US) and recombinant human lactoferrin, for the treatment of diarrhea currently in phase II (Ventria Bioscience), both from the tobacco plant, *Nicotiana benthamiana*.

System	Overall cost	Production timescale	Scale-up capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Mammalian cell culture	High	Long	Very low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor differences	Low risk	Moderate
Transgenic plants	Very low	Long	Very high	High	Minor differences	Low risk	Inexpensive

Table 1: A comparison of platforms used for recombinant protein production (Ma, 2003)

1.1.3 Plant transformation strategies

Host plants

The versatility of plant molecular farming resides in the numerous plant platforms that can be used such as 1) transgenic plants: duckweed (*Lemna* sp.); microalgae (*Chlamydomonas reinhardtii*); and 2) *in vitro* culture systems: cell suspensions (tobacco BY-2, carrot, rice); hairy roots (*Nicotiana* sp.) and moss protonema (*Physcomitrella patens*) (Xu, 2011). The choice of plant and tissue depends on many different factors such as space available, containment levels required, protein processing considerations, protein stability, growth and harvest timeline and the economics of production systems (Egelkrout, 2012).

The LEX platform has been recently used to produce interferon-alpha for the treatment of Hepatitis C (Locteron®), a therapeutic enzyme for thrombosis prophylaxis and an anti-CD20 antibody for the treatment of non-Hodgkin's lymphomas (Biolex Therapeutics).

The tobacco species *Nicotiana* has emerged as the leading plant platform for leaf-based recombinant protein expression (Tremblay, 2010). The major advantages of tobacco include the well-established technology for gene transfer and expression, high biomass yield, prolific seed production and the existence of a large-scale processing infrastructure (Twyman, 2003). However, extracted leaves have to be frozen soon after harvesting to avoid protein degradation, which can be an issue for large scale production. Furthermore tobacco is an unpopular crop due to the cigarette industry. However, as smoking has nowadays been banned from most public places, PMP could represent an interesting conversion opportunity for tobacco farmers.

Other plants species like cereals or legumes can also be used. Seed-targeted transformation of a cereal provides a stable and abundant source of recombinant protein which can be stored for more than 3 years at room temperature without degradation (Horn, 2004). For example; ISOkine and DERMOkine are human growth factors produced in barley seeds and commercialized by ORFGenetics (<http://orfgenetics.com/>)

Plant cell-suspension culture is an alternative production platform to mammalian cells for the production of pharmaceuticals. It combines sterile *in vitro* condition and cheaper downstream processing (Kim, 2008). Moreover, it is a rapid system as productive cell lines can be generated within a few months (Shaaltiel, 2007). The current standard for plant cell-suspension is the now well characterized tobacco Bright Yellow-2 (BY-2) cell line because of its high growth rate (Nagata, 1992).

Stable transformation

Stable nuclear transformation involves the insertion of a transgene of interest into the nuclear genome of the plant (Figure 1). The resulting plant or plant cell-suspension acquires inheritable ability to express the target gene. This technique has been used to produce many recombinant proteins, and allows the accumulation of the protein in the dry seeds of cereals (Horn, 2004), which can be produced at large scale. Transplastomic plants have the transgene insertion carried out in the chloroplast genome. Usually transformed by particle

bombardment, such plants present high levels of expression partly due to the absence of silencing effects, but lack glycosylation activity. This type of stable transformation strategy can be used to avoid transgene transmission as pollen does not contain chloroplast-derived DNA (Cardi, 2010).

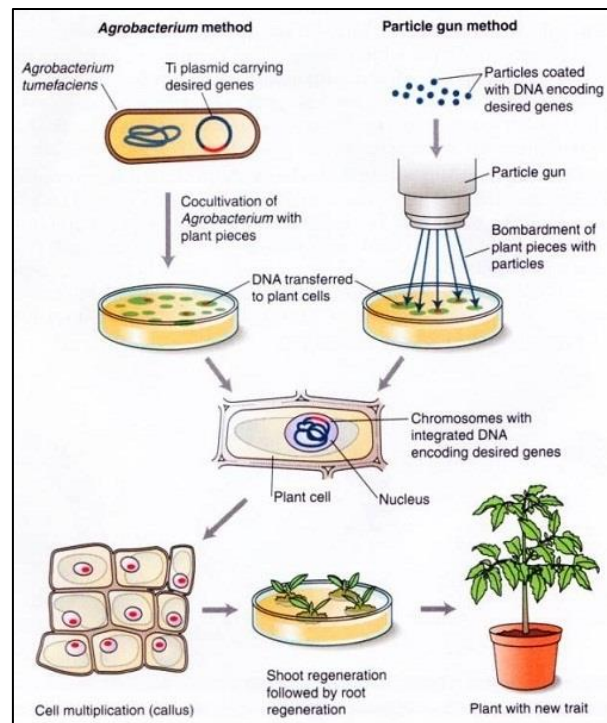


Figure 1: How to make a stable transgenic plant (from Mirkov, 2003)

Transient transformation

The generation of a high expressing stable transgenic plant is a long process and can take years. For this reason transient transformation is useful alternative. Agroinfiltration is most commonly used and involves infiltration of a suspension of *Agrobacterium tumefaciens* (transformed with the transgene) into the plant cells. This is generally conducted on *Nicotiana benthamiana*. The bacterium's natural ability to transfer T-DNA (containing the transgene) to the plant's genome is exploited to make the plant express the protein of interest. Another transformation strategy relies on plant viruses such as tobacco mosaic virus (TMV) and their ability to be used as vectors to deliver foreign gene into a plant, without integration (Porta and Lomonosoff, 2002). Physical techniques can also be used such as particle bombardment of the cells (particles coated with the recombinant plasmid vector) and electroporation where a brief electric shock allows the recombinant plasmid into the cell. Though not permanent, these transformations lead to quick protein production (typically within a week) and can thereby be used to produce emergency therapeutics such as vaccines in case of an epidemic (Whaley, 2011). A current example of this advantage is the experimental antibody cocktail ZMapp composed of three anti-Ebola virus mouse/human chimeric monoclonal antibodies (c13C6, h-13F6, and c6D8) and Zmab, by Mapp Biopharmaceuticals and Leaf Biopharmaceuticals, respectively (Olinger, 2012; <http://www.mappbio.com/>), which has been produced urgently to fight the current Ebola epidemic in West Africa.

1.1.4 Advantages and current limitations of molecular farming

Plant molecular farming is a promising technology, optimizing a cheaper, easier (low technology) way of producing pharmaceuticals, especially aimed for developing countries (Table 2).

Advantages	Limitations
✓ Post translational processing	✗ Low yields
✓ Free from animal pathogens	✗ Non-mammalian glycosylation
✓ Ease of combining transgenes by crossing	✗ Host plant management
✓ Rapid scale up	✗ Downstream processing
✓ Storage of the protein (cereals)	✗ Biosafety and regulatory issues (food contaminations and gene spreading)
✓ Possible oral consumption without purification(food hosts)	

Table 2: Main advantages and limitations of PMF

However, many pharmaceutical companies remain reluctant to take up plant molecular farming. A consistent number of companies that bet on this technology have filed bankruptcy (e.g. SemBioSys in 2012; Large Scale Biology in 2006; ProdiGene in 2012; Biolex Therapeutics in 2012). Innovative PMF industries have to compete with the well settled pharmaceutical companies in terms of product quality (purity, efficiency) and legislation. Yet, while mammalian cell-based production platforms benefit from years of technological improvement and therefore an established route to commercialization, plant-based production technology is hardly accepted and legal restrictions to the marketplace are very constraining (Paul and Ma, 2011).

Regarding the limitations of molecular farming, low yields and downstream processing are major draw backs, as well as non-mammalian glycosylation. Professor Julian Ma's team at St George's University of London is working on overcoming some of these factors. The adoption of the use of hydroponic cultures (which may be more socially acceptable than transgenic crops) for the rhizosecretion of antibodies (see Section 1.3.) in the lab has gone some way to addressing cost and time issues associated with downstream processing. I will also describe recent efforts to improve the yields of three monoclonal antibodies: M12; VRC01 (anti-HIV); and χ E559 (anti-rabies).

1.2 Monoclonal antibodies

1.2.1 Monoclonal antibody

Antibodies are naturally produced proteins of the immune system belonging to the immunoglobulin (Ig) family of proteins. They recognise and bind specific epitopes of antigens and are produced by B cells as a part of the humoral response to an infection. They act on pathogens either by direct neutralisation or by signaling the recruitment of other components of the immune system to fight the infection.

Monoclonal antibodies (mAbs) can be described as antibodies of the same specificity (i.e., targeting the same epitope) produced by clonally expanded B cells. The pharmaceutical industry has developed methods to produce mAbs for the treatment of specific diseases. Two years ago, 28 branded mAbs were approved in Europe and the US (Reichert, 2012). They are used for disease prophylaxis and diagnostics as well as therapeutic treatments (cardiovascular diseases, autoimmune disorders, malignancies, tumours, arthritis, Crohn's disease and plaque psoriasis among others) (Obembe, 2011; Elbakri, 2010).

MAbs currently available on the market are mainly produced from mammalian cells such as CHO (Chinese hamster ovaries) and hybridomas, as they allow a very high product quality and mammalian-type glycosylation (Hossler, 2009). Advances over 30 years in bioreactor technology and media composition have resulted in highly productive clones. However, costs associated with bioreactor establishment, complex media and serum requirement (risking contamination with human pathogens) remain major concerns of this production platform (Li, 2010; Kelley, 2009). High amounts of mAbs are needed annually worldwide and supply struggles to match demand. Therefore, a cheaper, scalable way of production remains a main challenge for mAb manufacturing. As they offer such possibilities, transgenic plants could provide a solution (Shukla and Thömmes, 2010).

1.2.2 M12 monoclonal antibody

The M12 monoclonal antibody was first identified as an anti-breast cancer antibody (Wong, 2001). However, it was found later of no therapeutic value, as it in fact targets vitronectin, a host extracellular matrix protein (Dr. Nicole Raven, unpublished data). Recently though, it was shown that vitronectin could serve as a promising serum marker for the detection of primary breast cancer (Kadowaki, 2011). In addition, vitronectin was found to be a potential prognosticator following heart attacks (Aslan, 2014). Therefore, M12 antibody might be of use in the future in a diagnostic capacity. Currently it is used in our lab as a model antibody for transgenic plant root secretion (rhizosecretion) because of its relatively high expression and recoverable yields.

Two other mAbs used in my studies on rhizosecretion, VRC01 (anti-HIV) and χ E559 (anti-rabies), will be described in section 1.3.

1.3 Plant biotechnology disease targets: HIV/AIDS and Rabies

1.3.1 HIV/AIDS

The worldwide spread of the acquired immunodeficiency syndrome (AIDS) epidemic is a consequence of an infection by the human immunodeficiency virus (HIV) that infects and depletes vital cells of the human immune system in particular CD4 T-cells. In 2013 it was estimated by the World Health Organisation (WHO) that 35 million people worldwide were living with HIV and that 1.5 million died from AIDS-related causes (Figure 3; WHO, 2013). There is currently no cure or vaccine for this disease, and only an estimated 12.9 million people had access to antiretroviral therapy (ART) that reduces viral load by slowing down replication.

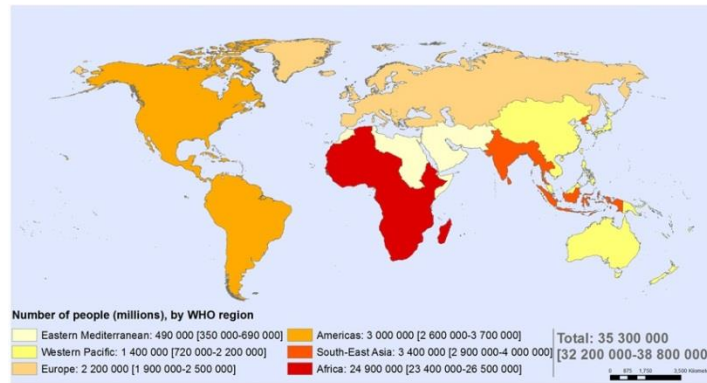


Figure 2: WHO estimated numbers of people living with HIV worldwide, 2013.

Transmission occurs via body fluids from infected individuals (e.g., blood, breast milk, semen and vaginal secretions) and unprotected heterosexual intercourse is responsible for the vast majority of new cases. As diagnostics is only possible from 2 to 6 weeks post-infection, the main prevention remains the use of male and female condoms.

VRC01 is one of a new generation of broadly neutralising antibodies (bNab) capable of neutralizing 91% of known HIV-1 isolates *in vitro* (Wu, 2010). It has been demonstrated to be protective against HIV infection in animal challenge models (Veselinovic, 2012; Ko, 2014). VRC01 binds to the HIV envelope glycoprotein gp120, blocking its interaction with CD4 to inhibit viral entry into the host cells. Its promising properties have made it attractive to researchers globally and a good candidate for my project of antibody production platform development.

1.3.2 Rabies

Rabies virus (RABV) infection is a zoonotic disease chiefly transmitted by domesticated dogs, and wild animals (e.g., bats) that targets the central nervous system. Every year rabies is responsible for an estimated 55,000 deaths around the world, mainly in developing countries in Asia and Africa (Figure 3, WHO).

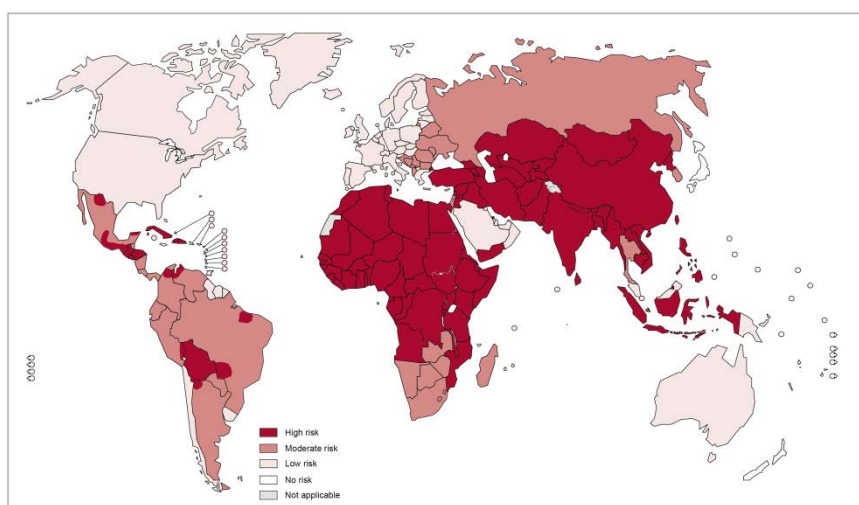


Figure 3: Distribution of risk levels for contracting rabies worldwide, 2011 (WHO)

Transmission is caused by contact with infected saliva from bites or scratches from a rabid animal. There is currently no test available for diagnosis before clinical disease onset, which invariably leads to death. In suspected cases of exposure/infection, WHO recommends thorough wound cleansing followed by immediate post-exposure prophylaxis (PEP). PEP is composed of two components – a RABV vaccine and neutralising rabies immunoglobulins (RIGs), which are injected at the site of injury. Early administration of PEP is extremely effective in preventing disease. Currently RIGs are derived from immunised humans (hRIGs) or equine (eRIGs). In developing countries, where rabies is the most prevalent, access to RIGs is expensive and limited. Therefore, there is a requirement to develop cheaper alternatives. We are investigating the production of a chimeric human-mouse monoclonal antibody, χ E559, which has potent neutralising activity against RABV and conferred protection in a hamster challenge model (van Dollenweerd, 2014).

1.4 Rhizosecretion

1.4.1 Rhizosecretion as a platform for recombinant protein production

Rhizosecretion, the secretion of biomolecules and chemicals via the root, is a natural process used by plants to encourage symbiotic interactions (e.g., mycorrhizal fungi for nitrogen fixation), as a defence against parasites and pathogens, and to compete with neighbouring plants for resources in the rhizosphere (Coats, 2014). Rhizosecretion has been exploited by researchers for recombinant, heterologous protein production (Gleba, 1999). The first rhizosecreted proteins were from tobacco grown hydroponically and included green fluorescent protein (GFP), human placental alkaline phosphatase (SEAP), and bacterial xylanase (Borisjuk, 1999). Since then, several strategies have been tried to boost yields. Increasing tobacco root tissue mass by infecting with *Agrobacterium rhizogenes* to cause hairy roots, increased SEAP secretion 5—7 times (Gaume, 2006). The co-secretion of a protease inhibitor (BBI) to reduce degradation also increased production yields (Komarnytsky, 2006). Using these and other strategies, it is now possible to accumulate pharmaceutically important molecules such as monoclonal antibodies to significant yields in defined culture medium.

Rhizosecretion has many advantages compared to transient Agro-infiltration of leaves for the production of recombinant proteins. It is a non-destructive technique that allows extraction throughout the life of the plant, it is easily scalable, and because it is grown in a defined medium simplifies and reduces the cost of downstream processing (Drake, 2003). However, rhizosecretion remains an un-exploited promising production method held back by relatively low yields compared to mammalian expression systems adopted and favoured by the pharmaceutical companies.

Low yields can be the result of many different factors such as low expression in the plant (epigenetic factors), poor secretion, or protein degradation *in planta* or in the medium. As many of the *in vivo* mechanisms are still obscure and complex, we are trying in this project to increase the final yields by optimising the culture medium *in vitro*.

1.4.2 Hydroponics + Nutrient Film Technique

Hydroponics is a system where plants take their water and nutrients from a wholly liquid medium. The main advantage of using hydroponics for the production of recombinant proteins by rhizosecretion is the ease of downstream processing (protein purification) thanks to a simple, defined culture medium.

The hydroponic system for the production of recombinant protein (chiefly monoclonal antibodies) that has been developed in Professor Julian Ma's lab is to grow individual transgenic tobacco plants in glass jars under a sterile environment (see Materials and Methods for more details). This technique allows good control of the medium composition. Collection (harvesting) of the medium containing the monoclonal antibody involves manual pipetting and this can result in contamination and therefore, reduced final protein yield. Therefore, harvest automation is highly desired to scale up this time consuming and laborious process.

Nutrient film technique (NFT) is a non-sterile hydroponic culture system used in agriculture for the large scale production of vegetables such as lettuces, tomatoes and potatoes (Morgan, 1983; Cooper, 1977; Wheeler, 1990). Water containing nutrients flows over and through the bare roots along a trough and is continuously circulated via a pump in a closed circuit. This way of growing plants has many advantages such as water saving, oxygen aeration for roots and constant nutrient availability without toxic accumulation. It is envisaged that NFT can also be used for the secretion of valuable pharmaceuticals by genetically engineered plants. However, technical issues have so far limited the recoverable yields (pers. comm. Tim Szeto). It was one of my project aims to investigate factors such as media composition which might contribute to this observation.

2. Material and methods

2.1 Material

2.1.1 Host organisms

Transgenic *Nicotiana tabaccum* seeds from M12, VRC01 and χ E559 antibody secreting plant lines were kindly provided by Dr. Audrey Teh and Dr. Luisa Madeira from Julian Ma's Laboratory.

Competent Library Efficient DH5 α *E.coli* cells from Invitrogen were used for all cloning experiments.

2.1.2 Media

Two principal media were used – a Murashige Skoog (MS) based medium and a proprietary Nutrient Film Technique (NFT) based medium. The formulations are shown below. All media was autoclaved before use.

For 1L (pH 5.8)	MSN	MSNg
MS basal medium (g)	4.4	4.4
Sucrose (g)	30	30
70% HNO ₃ (mL)	4.4	4.4
NaOH (g)	2.5	2.5
Gelatin powder (g)		8

For 1L (pH 5.8)	NFT + Sucrose	NFT + Sucrose + Nitrates
Solufeed H (g)	0.7	0.7
KNO ₃ (g)	0.7	1.9
Ca(NO ₃) ₂ (g)	0.25	3.4
Sucrose (g)	30	30
69% HNO ₃ (mL)		4.4
NaOH (g)		2.5

Table 3: Hydroponic culture medium compositions.

2.2 Methods

2.2.1 Hydroponic plant culture settlement

The seeds were surface sterilised in a microwave on high power for 10mn, allowed to cool down then heated for 5 more minutes under the same power setting. The sterilised seeds were sown onto MS medium solidified with 0.7% w/v agar. Two-three week old seedlings (four leaf stage) were transferred to liquid culture into jars under sterile conditions. Plants were supported on a small Petri dish turned upside down and previously pierced in the middle, so that the roots only were immersed in liquid medium (Figure 4). Plants were maintained at approximately 25°C with a 16h photoperiod.



Figure 4: Tobacco plant in sterile hydroponic culture, week 5.

The plants were grown for 4 weeks in the different media before the first medium sampling, then harvested on a weekly basis (see timeline in Figure 5). From week 4 onwards, the medium was supplemented weekly with 1µg/mL of the plant growth regulator NAA (α -naphthalene acetic acid). NAA was previously demonstrated to enhance recombinant protein rhizosecretion (Drake, 2009; Madeira, 2012). At the end of the experiment, the remaining plant roots were collected and left to dry for 48h on absorbent tissue before being weighed.

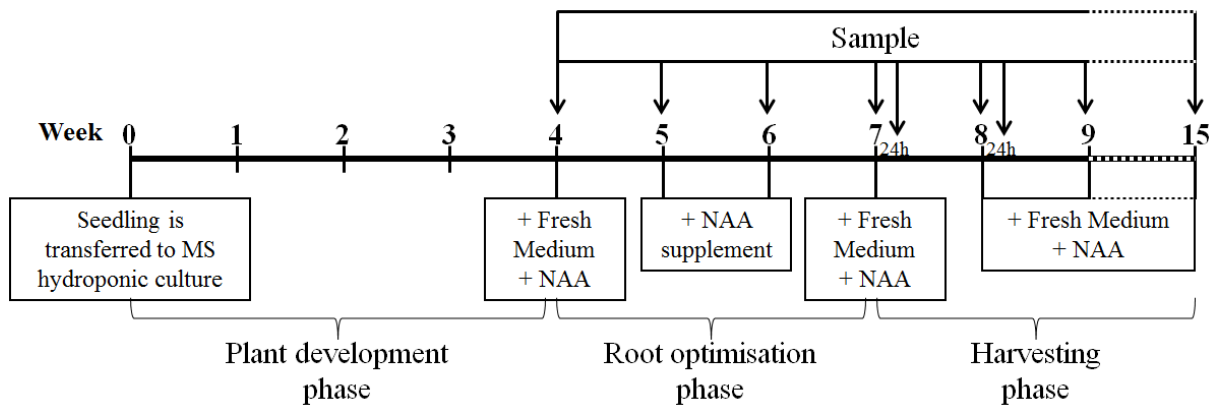


Figure 5: Standard experiment timeline

2.2.2 ELISA quantification

A capture ELISA (also known as ‘sandwich ELISA’) was operated for the antibody yields quantification. For M12 antibody detection, a purified M12 standard was kindly provided by Dr. Luisa Madeira. Human IgG1 κ chain was used as VRCO1 and χ E559 positive control. The plates were coated with sheep anti-human IgG and blocked with 2.5% bovine serum albumin (BSA). The samples and standard were titrated 1:2 or 1:3 and detected with anti-human λ (for M12) or κ (for VRCO1 and χ E559) chain bound to horseradish peroxidase. TMB tablets were used for developing and the reaction was stopped with 2M sulphuric acid before reading on TECAN Sunrise™ plate reader.

2.2.3 Experimental design and statistical analysis

The raw data obtained from ELISAs were processed using Excel and GraphPad Prism 6.0 software. The significance of the results were analysed by unpaired parametric t-test with Welch’s correction (equal standard deviation non-assumed), using GraphPad Prism software.

2.2.4 Northern blot and DNA labelling

EF1- α gene fragment (179-1065) was generated by PCR and inserted into PGEM®-T easy plasmid using Promega PGEM®-T Easy vector systems Kit. DH5 α E.coli were thermally transformed with the recombinant plasmid and screened for blue-white colonies on Xgal + Kanamycin + IPTG plates. Presence of the insert was further verified by colony PCR and double digestion with Nco1 HF and Spe1 and confirmed by sequencing.

Random prime digoxigenin (DIG) labelled EF1- α probes were generated using DIG High Prime DNA Labelling and Detection Starter Kit II from Roche. In random prime labelling, Klenow enzyme extends DNA templates in the presence of hexameric primers and DIG-dUTP (Figure 6). Probes are detected by alkaline phosphatase conjugated anti-DIG antibody.

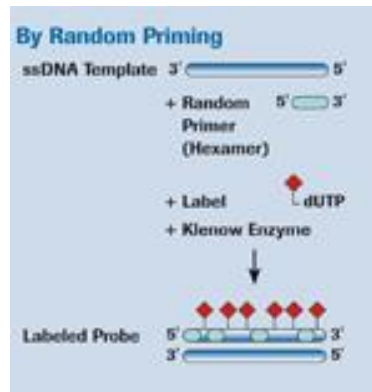


Figure 6: Random primer labelling principle (Roche)

Northern blotting was realised using NorthernMax® Kit from Life Technologies. Downward transfer assembly was set for Northern blotting (as shown in Figure 7). The mRNAs migrate from the agarose gel to the membrane by capillarity. Pre-hybridization and hybridization with EF1- α probes was conducted at 42°C (DNA) followed by a low stringency washing.

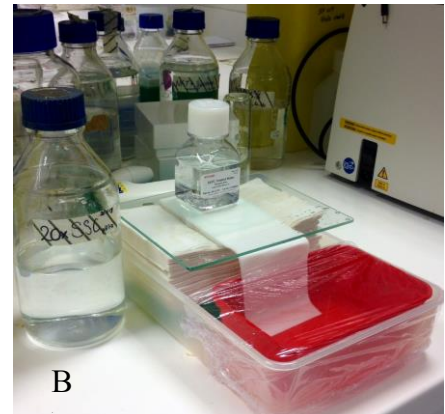
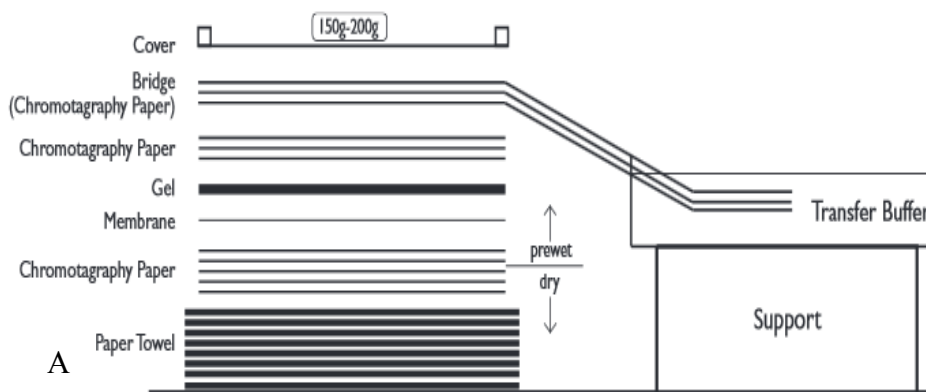


Figure 7: A: Downward transfer assembly (NorthernMax® Kit); B: Transfer setting assembled

3. Results

3.1 Impact of gelatin omission on rhizosecretion yields

Proteolytic degradation is a significant problem in recombinant protein production. In the hydroponic system, typically the hydroponic medium is harvested once a week during which time the secreted mAb is in the medium with a mixture of molecules including proteases (Luisa Madeira unpublished results). To enhance protein conservation, the medium composition must be optimised, and a few additives have been investigated in this regard. It has been shown that yields of the mouse monoclonal Guy's 13 secreted from tobacco hairy roots, improved by a factor of 4-8 with the addition of 9 g/L gelatin to the medium. More

additives have also been shown to improve antibody yields such as Polyvinylpyrrolidone (PVP) (Wongsamuth, 1996).

Consistent with this result, Guy's 13 rhizosecreted from transgenic tobacco plants grown hydroponically exhibited a similar 4 times enhancement in yield when grown in the presence of 8 g/L gelatin (Drake, 2003).

Gelatin is a protein obtained by the thermal denaturation of collagen and is mainly used in the food industry (jellies, dairy products stabiliser, juices, dried soups, *etc*). Industrial gelatin comes from animal by-products (i.e. demineralised bone, pigskin, cow hide, fish skin and in China, donkey hide) and its inclusion may present an ethical issue in a plant production system (Cole, 2000).

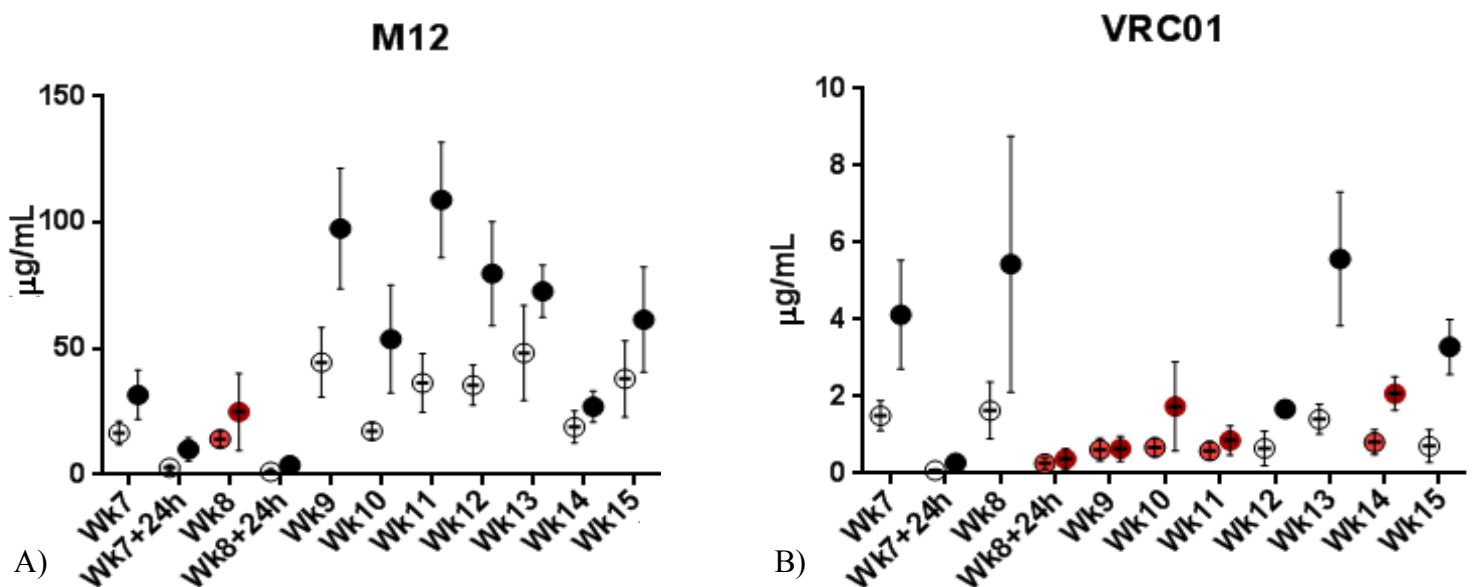
In this experiment we have investigated the effect on the yields of rhizosecreted antibody by the omission of gelatin to MSN hydroponic medium. The model monoclonal antibody M12, the anti-rabies χ E559 and anti-HIV VRC01 antibodies were included in this study.

3.1.1 Impact of gelatin omission on rhizosecreted yields

Regarding M12 secretion, better yields were always obtained in the gelatin-containing medium, ranging from 1.5x (week 14) to 3.5x (week 7+24h) higher than MSN medium alone. The highest average occurred in MSNg, week 11 (109 μ g/mL). The average yield for M12 in MSNg is 61 μ g/mL per week (Figure 8, A).

Despite lower yields and only statistically significance in five of the 11 weeks, it seems that gelatin improved VRC01 secretion by up to 4.5 times (week 15). The maximal average yield obtained (5.6 μ g/mL) was recorded in MSNg week 13. There seems to be no particular trend for the secretion of this antibody and gelatin appears to make the greatest impact in the early and late harvesting time points only (Figure 8, B).

A similar trend in yield is also noted for the rhizosecretion of χ E559, though better yields were obtained (9.3 μ g/mL on week 15 in MSNg). Gelatin improved the secreted antibody yields from 1.5 times in week 12 to 4.2 times in week 15 (Figure 8, C).



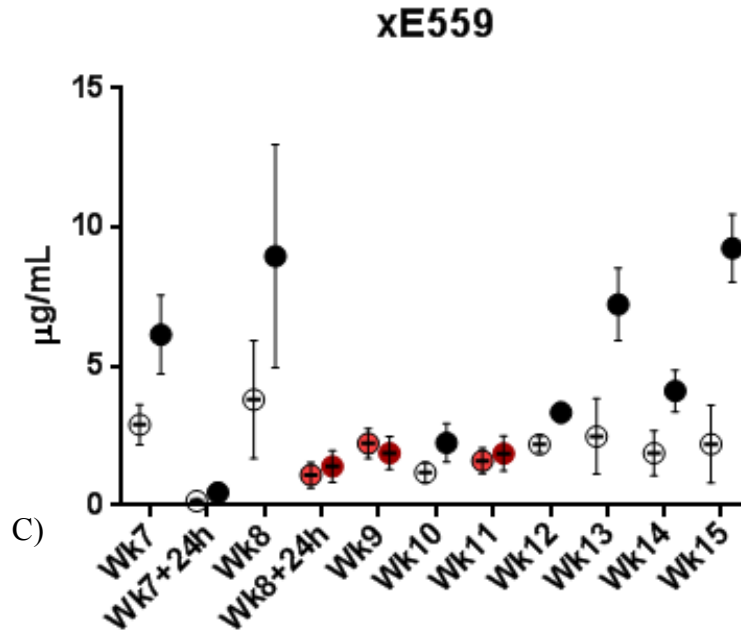


Figure 8: Average weekly secreted yields of A) M12, B) VRC01 and C) χ E559 as determined by ELISA; MSN (○); MSN+gelatin (●).
 Red = no statistical significance as determined by t-test.

Omission of gelatin causes a 50% antibody loss on average (53% M12; 55%VRC01; 43% χ E559); detail is provided in Figure 9. This represents a loss of 30 μ g/mL for M12, 1.9 μ g/mL for VRC01 and 2.3 μ g/mL for χ E559.

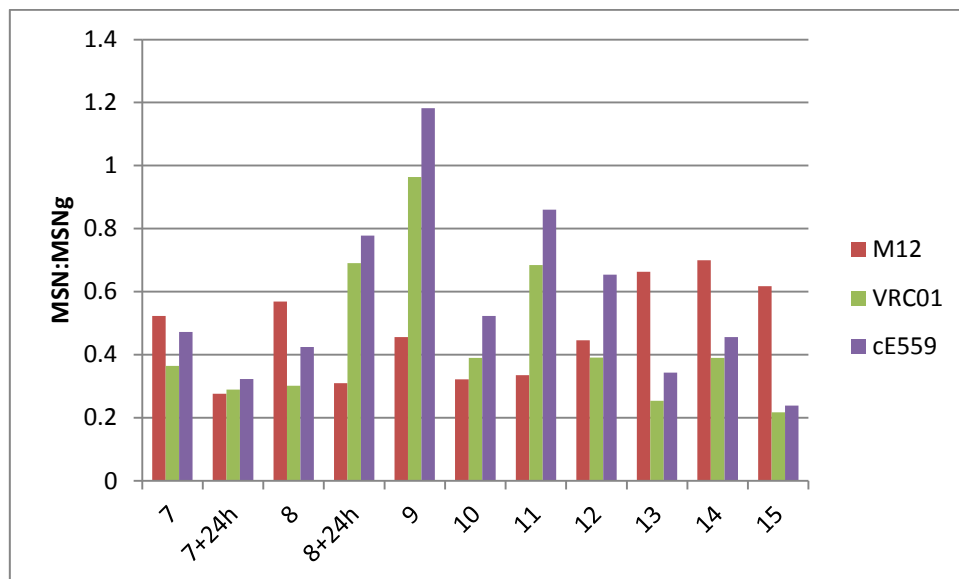


Figure 9: MSN:MSNg antibody yields ratios.

Conclusion:

Gelatin omission decreased the yields of M12 antibody by a factor of 2 on average, which confirms previous studies (Luisa Madeira, unpublished results). For VRC01 and E559, the results are quite similar but there are a number of weeks where gelatin addition had no effect on secretion. However, the addition of gelatin to the hydroponic medium never resulted in lower yields than MSN medium alone.

3.1.2 Impact of added gelatin on root growth

Addition of gelatin to the medium showed no significant difference in dry roots weight for any of the transgenic plants (Figure 10), indicating that secretion differences observed were not a result of a difference in root growth and therefore root mass. The average dry root weight was about 200 mg for all the different plants tested.

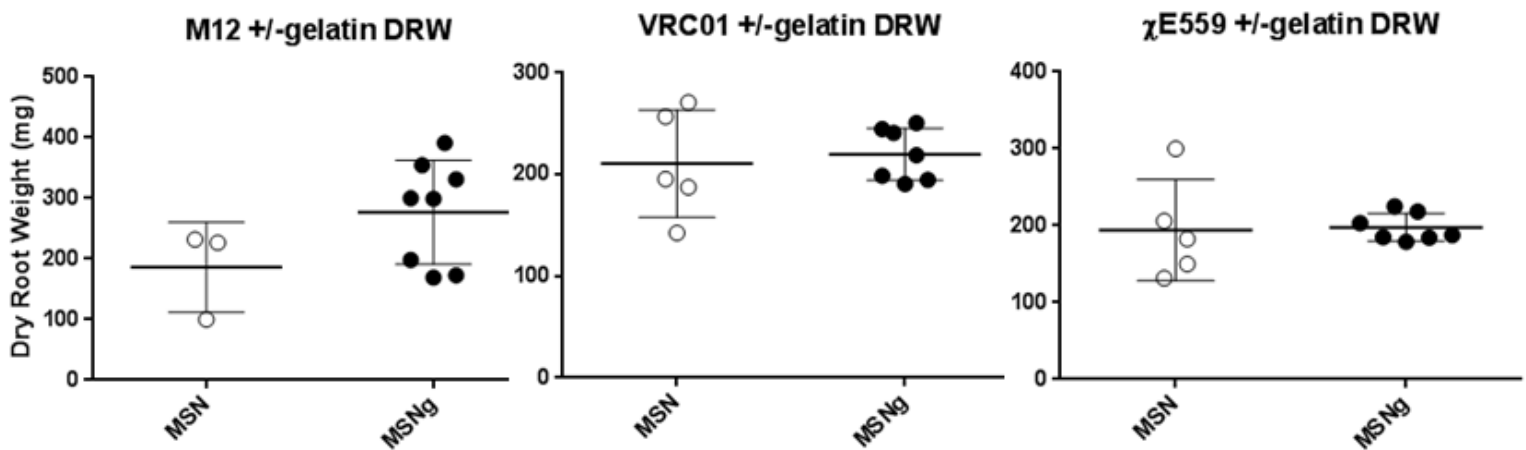


Figure 10: M12, VRC01 and χ E559 final dry root weight.

3.1.3 Discussion

Low yields for VRC01 plants made analyses difficult and resulted in more weeks showing no statistical significance in yield than for M12 and χ E559 (red points in Figure X, B)). Furthermore, several VRC01 plants did not secrete any detectable levels of antibody as measured by ELISA. This further compromised statistical analyses. This is unusual and is most likely the result of an unstable plant line. New VRC01 plant lines are currently being bred for stability of transgene expression.

The addition of gelatin seems to stabilise secreted antibodies and increase antibody accumulation in the medium. Its use in recombinant antibody high scale production may depend on the plant productivity; for high yielding plants the loss due to absence of gelatin might be economically acceptable (if not profitable due to savings on gelatin), but for low yielding plants gelatin (or another protein stabilizer) might be needed to produce enough material.

Interestingly, the mean M12 yield in this experiment was almost 3 times higher than found previously (Madeira, 2012), for identical culture conditions in Julian Ma's lab. Temperature

may have an effect on secretion and may fluctuate where the plants are kept, also manual harvesting sometimes causes injuries to plants and introduce stress, which could explain our results (Tim Szeto pers. comm.).

Dry root weight data showed that gelatin has no effect on root growth for any of the transgenic plant lines. From these results it would appear that antibody accumulation in the medium does not correlate with root mass. Indeed, the average root weight was similar for plants growing in MSN or MSNg, even though the antibody accumulation was significantly increased in the presence of gelatin.

Gelatin's mechanism of action is unknown. It has been proposed that gelatin acts as a substitute substrate for peptidases naturally secreted into the culture medium, allowing the antibody to accumulate (Drake, 2003). However, there is also experimental evidence to suggest that gelatin acts instead to prevent antibody lost by adsorption to glass flasks (Madeira, 2012).

The next stage in the optimisation of antibody stabilisation in MSN medium would be to search for an alternative molecule to gelatin. Being an animal product, it involves additional costs and risk of pathogens and reduces some of the advantages of plant systems. Polyvinylpyrrolidone (PVP), a non-animal food additive, has been shown to improve yields of recombinant antibodies in suspended plant cell cultures (LaCount, 1997; Sharp and Doran, 2001) and in hairy roots (Wongsamuth, 1996). However, in a recent study, PVP addition in hydroponics did not significantly improve secreted antibody yields (Madeira, 2012). Human serum albumin (HSA) is another potential stabilizer that could be investigated. Although also an animal product, recombinant HSA is currently being manufactured in non-mammalian systems, including transgenic rice (Ye, 2011). This work could be taken further in trying a wider range of concentration or testing an additive with similar properties.

3.2 Impact of nitrate supplementation on rhizosecretion

Tobacco Bright Yellow-2 (BY-2) suspension cells expressing an anti-HIV monoclonal antibody, 2G12, exhibited improved extracellular antibody yields up to 20-fold following the enrichment of MS medium with nitrate, MSN (Holland, 2012). The same enriched medium used for rhizosecretion from hydroponically grown tobacco plants was found to increase M12 antibody yields as much as 46 fold compared to MS medium alone (Madeira, 2012).

One of the aims of the Ma lab is to transfer the hydroponic rhizosecretion platform from an *in vitro* (sterile glass jars) to an *ex vitro* (non-sterile nutrient film technique - NFT) system. Despite achieving high antibody secretion yields in MSN medium in sterile glass jars, thus far, transfer to the NFT system has not yielded similar success. Previously, growth of transgenic plants in the NFT system has resulted in no detectable accumulation of secreted M12 antibody, as detected by ELISA (Tim Szeto pers. comm.).

The aim of this experiment was to determine *in vitro* if such effects of nitrate enrichment on antibody secretion would similarly apply for transgenic plants grown in NFT medium.

3.2.1 Impact of added nitrates on antibody secretion

M12 plants were grown in three different media: MSN, NFT medium supplemented with sucrose and nitrates (NFT+Suc+N) and NFT medium supplemented only with sucrose (NFT+Suc) (see Section 2.2.2. for details of media composition). Medium samples were collected weekly from week 4 for ELISA analysis. Antibody secretion is shown in Figure 11.

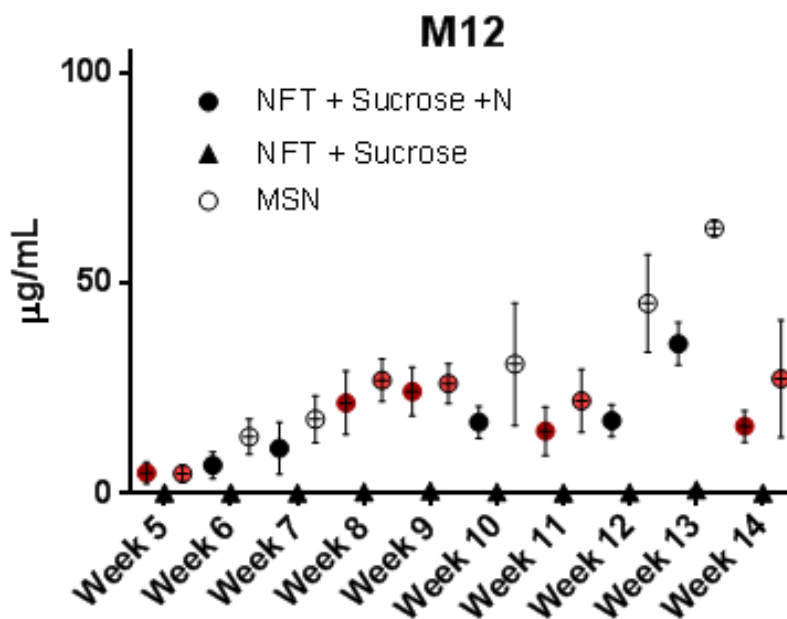


Figure 11: M12 yields for ●: NFT + Sucrose + N; ○: MSN; ▲: NFT + Sucrose.
Red pair: no statistically significant difference.

For all weeks, M12 yields obtained in NFT+Suc were barely detectable and consistently the lowest of the three growth media. Interestingly, the absence of nitrate resulted in a smaller and yellowish plant (see Figure 12, d), which underpins nitrate's essential role as a plant macronutrient. This is similar to previous findings in which plants grown in NFT medium alone showed undetectable levels of secreted antibody and poor growth (Tim Szeto unpublished data). The addition of sucrose was to provide a carbon source for plant growth and metabolism – but surprisingly doesn't seem to have helped.

Nitrate enrichment improved NFT+Suc medium yields by 25- (Week 5) to 109-fold (Week 14). This is the first ever demonstration of rhizosecretion in NFT medium. Yields obtained in NFT+Suc+N are less than for MSN but both globally follow a similar trend over time. For 5 weeks, they are not statistically different which gives nitrate enrichment of NFT+Suc the same effect on antibody yield as nitrate enrichment of MS medium. For 5 other weeks though, the yields obtained in MSN are significantly higher with a maximum of 2.6-fold in week 12

Overall, the enrichment in nitrates of NFT+Suc seems to have similar effect on antibody yield as the enrichment of MS medium. Despite the fact that generally better yields are obtained for MSN, NFT+Suc+N provides promising yields and merits further investigation for its use in antibody production using the NFT systems.

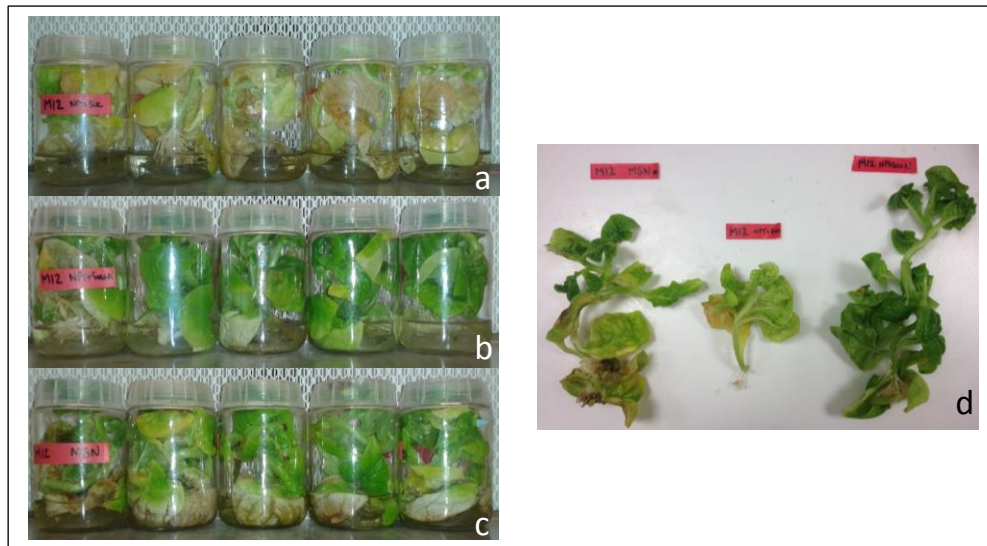


Figure 12: M12 plants – 8 weeks: a: NFT+Suc; b: NFT+Suc+N; c: MSN; d: plants after root harvest.

3.2.2 Impact of added nitrates on root growth

To investigate the effect of a higher concentration in nitrates on root growth, the roots were harvested at the end of the experiment and weighed after 48h of drying (Figure 13).

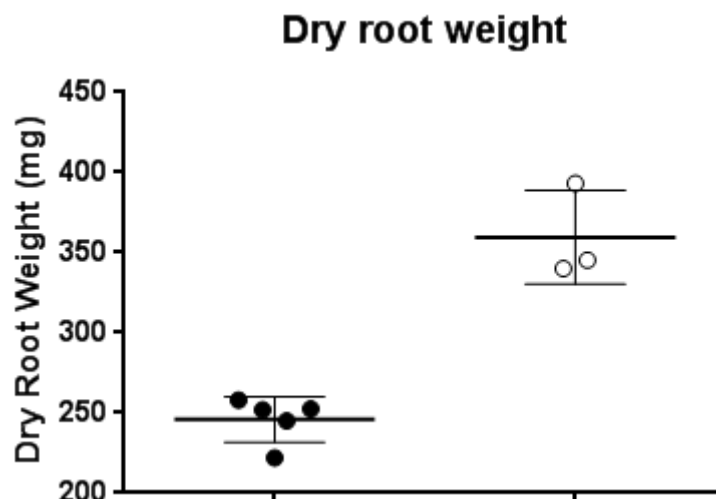


Figure 13: Dry root weight from ●: NFT + Sucrose + N; ○; NFT + Sucrose.

Added nitrates seem to have a negative effect on root growth as dry root weight of plants grown in NFT+Suc+N is on average 32% lower than those grown in NFT+Suc. According to

this observation, the high M12 antibody yields obtained with the enrichment in nitrates are not due to an increased amount of root mass in plants.

3.2.3 Discussion

Data variability towards the end of the experiment may be due to medium contamination in MSN plants. At week 14 the MSN sample size became too small for statistical significance and we stopped the experiment. This problem was also encountered in my first project and can be a recurrent issue in hydroponics.

NFT+Suc+N gave satisfying enough results to be taken for further investigation. A future aim is to set up a functional NFT system to scale up the production of mAbs. Currently, medium composition remains to be optimised. Enrichment of nitrates could be a key for better antibody yields. However, the presence of sucrose in NFT medium may increase bacterial and fungal contamination risk as NFT is an open culture system. Following my findings, an experiment looking at the yields obtained with NFT+N without sucrose has been initiated.

Several reasons have been proposed as to why/how additional nitrate increases M12 rhizosecretion. Elevated nitrate could: act as a stabiliser for antibodies; increase the secretion of high molecular weight molecules; increase antibody stability in hydroponic medium; or be responsible for an unknown mechanism driven by hyperosmolar conditions (Madeira, 2012). Indeed, a previous PhD student in the lab, Luisa Madeira has demonstrated that the “nitrate” effect of enhancing rhizosecretion is not specific to nitrates but can also be achieved by the addition of different salts (e.g., KNO₃, NaCl). Therefore, hyperosmolar conditions appear to be the driving force. However, the mechanism remains elusive. Experiments are currently underway to address whether the increase in rhizosecretion is a transcriptional, translational or post translational event.

3.3 Evaluation of transcript levels of transgenes in MS and MSN media

3.3.1 Introduction

The mechanism by which added nitrate improves recombinant antibody yields is currently unknown. M12 plants in MSN were found to secrete antibody up to 40 times more compared to MS (Madeira, 2012). To investigate M12 expression at the transcriptional level, different RNA probes are needed; against M12 heavy and light chain mRNA, and against an internal control.

Elongation factor 1 consists of a complex composed of four different subunits: alpha, beta, gamma and delta. EF1- α is a highly conserved and abundantly expressed cytosolic protein with an approximate molecular weight of 50 kDa (Riis, 1990; Proud, 1992). EF1- α is a ubiquitous eukaryotic protein coded by a maintenance gene. EF1- α mRNA sequence is 1671 bp long (<http://www.ncbi.nlm.nih.gov/>) and was selected to serve as an internal loading control in this experiment.

The aim of this experiment was to generate a DIG-dUTP labelled probe against EF1- α mRNA, in order to reveal experimental variability and be able to assess the effect of added nitrates on M12 transcription.

3.3.2 EF1- α DIG-dUTP probing results

To test the efficiency of the probes, a Northern blot (see Section 2.2.4) of total wild-type *N. tabacum* mRNA extraction was conducted. Distinct banding on gel electrophoresis indicated that rRNA was not degraded (Figure 14).

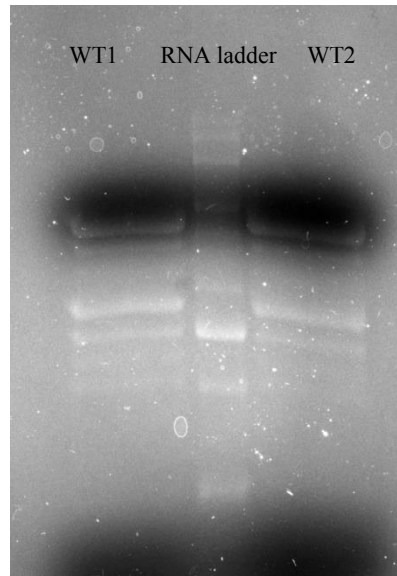


Figure 14: Gel electrophoresis of total *N. tabacum* mRNA extract (WT1/2: Wild Type).

Multiple bands (Figure 15) show that probe binding lacked specificity. However, higher intensity of the luminescence is observed just under the 2000bp marker. As EF1- α is 1671 bp it is likely to have been correctly detected by the DIG probes.

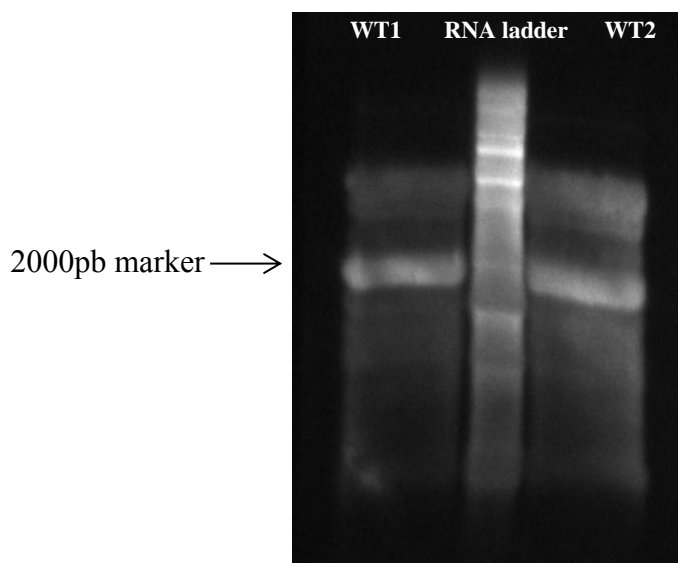


Figure 15: Chemiluminescent detection of EF1- α targeted probes on tobacco total mRNA.

3.3.3 Discussion

More stringent washes of the blot may be required to reduce the non-specificity of the EF1- α DIG-dUTP probes. A similar experiment should be conducted to verify these results, with particular attention applied to the washes (e.g. switching to high stringency washing).

Further investigation of the effect of added nitrates on M12 antibody secretion at a transcriptional level will be taken up. EF1- α DIG-dUTP and M12 probes will be used on total mRNA from M12 transgenic plants grown into either MS or MSN. The issue of this experiment is likely to be determinant in the understanding of nitrates effect on plants as it has never been undertaken before in plants; unfortunately I could not complete this study within my internship's time.

4. General conclusion and future works

Plant molecular pharming yields of biomolecules currently still lag significantly behind their mammalian cell counterparts. Despite this, it has long been recognized that plant based biotechnology offers unique benefits which include: substantial infrastructure cost reductions; ease of scalability; reduced risk of human-tropic contamination (e.g., viruses and prion); transferability of low-tech agricultural methods to developing countries; and, in the case for hydroponic rhizosecretion, reduced downstream processing costs (ref).

In this project, we have manipulated *in vitro* hydroponic medium composition with the view of scaling up rhizosecretion of mAbs to the *ex vitro* nutrient film technique (NFT) system. Although the mechanism is unclear, it has been shown by us and other groups that the addition of inert macromolecules such as gelatin, PVP, and BSA is able to increase recoverable yields of proteins secreted into hydroponic medium (Luisa, 2012; Wongasmouth, 1997). However, such macromolecules are not compatible with the circulatory system of NFT. Our results from this current study show that gelatin omission from MS hydroponic medium suffered an approximate 50% reduction in secreted antibody detected, irrespective of the mAb being secreted (M12, VRC01 or χ E559). This finding will be a crucial determining factor in assessing the choice of product to hydroponically rhizosecrete. Indeed, if the use of gelatin (or other stabilisers) might not be avoidable for low yield secretors (such as VRC01 and χ E559), only high yielding secretors (here M12) may be more profitable without associated additive and purification costs and considered for NFT scale up. Low secretors maybe restricted to the *in vitro* rhizosecretion platform and would benefit from investigation of other stabilizers such as polyvinylpyrrolidone and recombinant human serum albumin to maximize yields.

Detection of secreted M12 antibody into NFT medium in the NFT system has so far been underwhelming. Other plant systems have reported significant boosts in secretion with the addition of nitrates to the growth medium (Holland, 2010). Following up on this, we have for the first time successfully detected M12 secretion in NFT medium in the *in vitro* system as a result of nitrate enrichment. This is very encouraging for scaling up rhizosecretion to the NFT

system as a recombinant protein production platform. Further optimization of the NFT medium is required and an experiment is already underway in the lab to assess whether secretion levels will be maintained in the absence of sucrose (i.e., NFT medium+nitrate only) which would encourage fungi and bacteria growth in the non-sterile environment of the NFT system. Indeed, such plant manufacturing platforms could be of particular interest for the development of small scale, low-technology recombinant therapeutics production systems, highly needed to fight neglected diseases (e.g. rabies, tuberculosis) occurring in developing countries.

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Abstract – ENGLISH

Plants are being developed as an inexpensive alternative platform for the production of recombinant pharmaceuticals, including monoclonal antibodies (mAbs). Transgenic plants were previously demonstrated to secrete the recombinant proteins from their roots in a process called rhizosecretion. Thus hydroponic cultivation of transgenic plants that allows harvesting of recombinant proteins from the hydroponic medium has been investigated for some years, but has not yet provided sufficient yields of target proteins for commercial viability. Scaling up the current *in vitro* production system could consist of the elaboration of an appropriate media for Nutrient Film Technique (NFT) *ex vitro* system. Medium supplemented with gelatin has previously shown 20 times-improvement of rhizosecreted antibody yields but is incompatible with NFT. In this project we have assessed loss in yields in the absence of gelatin of three monoclonal antibodies: M12 (anti-vitronectin), VRC01 (anti-HIV) and χ E559 (anti-rabies). Consistently, a 50% yield decrease occurred without gelatin, for each antibody. The NFT system has so far been recalcitrant to rhizosecretion with no detectable yields. Nitrate enriched hydroponic medium has previously been found to increase antibody secretion, by a mechanism currently thought to be osmolarity-related. A similar enrichment was applied to NFT medium *in vitro* in this project, and for the first time M12 antibody secretion could be detected and quantified. These results are encouraging for the scaling up of rhizosecretion to the NFT system as a recombinant protein production platform. Presently, plant yields remain dramatically lower than mammalian-cell production and plant molecular farming has not been embraced by pharmaceutical companies. However, such plant manufacturing systems could be of particular interest for the development of small scale, low-technology recombinant therapeutics production systems, highly needed to fight neglected diseases (e.g. rabies, tuberculosis) occurring in developing countries.

Résumé – FRANÇAIS

Les nombreuses propriétés des plantes sont actuellement étudiées dans le but de développer une nouvelle plateforme de production de produits pharmaceutiques à bas prix (Plant Molecular Pharming – PMP). Des protéines recombinantes fonctionnelles ont été secrétées avec succès dans un milieu hydroponique par des racines de tabac transgénique. Cependant, les rendements obtenus par rhizosecretion n'ont jamais été assez élevés pour permettre l'exploitation industrielle du système. L'élaboration d'un milieu adapté à la technique de culture sur film nutritif (NFT) pourrait permettre le Scale-up de l'actuel système *in vitro*. Dans de précédents travaux, l'addition de gélatine dans le milieu de culture a permis d'augmenter 20 fois le rendement en anticorps secrétés, seulement, cet additif est incompatible avec le système NFT. Dans ce projet, l'impact de l'absence de gélatine sur le rendement de trois anticorps monoclonaux : M12 (anti-vitronectine), VRC01 (anti-VIH) and χ E559 (anti-virus de la rage). Une perte de rendement de 50% a pu être observée, indépendamment de l'anticorps étudié. D'autre part, une augmentation de la sécrétion d'anticorps recombinant a précédemment été obtenue grâce à l'ajout de Nitrates dans le milieu de culture hydroponique. Ici, un enrichissement similaire fut appliqué au milieu de culture utilisé en culture NFT. La première sécrétion quantifiable d'anticorps M12 à ce jour en milieu de culture NFT a pu être détectée, encourageant l'investigation de ce système pour la production de protéines recombinantes. Actuellement, les rendements en protéines pharmaceutiques obtenus en PMP restent considérablement inférieurs à ceux des plateformes de production en cellules animales, système bien établi dans les industries pharmaceutiques. Cependant, cette technologie accessible et low-cost pourrait s'avérer d'un intérêt majeur dans le développement à petite échelle de nouvelles plateformes production de produits pharmaceutiques. Cela permettrait aux pays en voie de développement de manufacturer leurs propres produits thérapeutiques pour le traitement de maladies actuellement négligées comme la rage ou la tuberculose, qui y sont pourtant toujours d'actualité.