Improving yield of a recombinant biologic in a Brassica hairy root manufacturing process

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March 11, 2022

Abstract

Hairy root systems have proven to be a viable alternative for recombinant protein production. For recalcitrant proteins, maximizing the productivity of hairy root cultures is essential. The aim of this study was to optimize a *Brassica rapa rapa* hairy root process for secretion of α-L-iduronidase (IDUA), a biologic of medical value. The process was first optimized with hairy roots expressing eGFP. For the biomass optimization, the highest biomass yields were achieved in modified Gamborg B5 culture medium. For the secretion induction, the optimized secretion media was obtained with additives (1.5g/l PVP + 1mg/l 2,4-D + 20.5g/l KNO $_3$) resulting in 3.4 fold eGFP secretion when compared to the non-induced control. These optimized conditions were applied to the IDUA-expressing hairy root clone, confirming that the highest yields of secreted IDUA occurred when using the already defined additive combination. The functionality of the IDUA protein, secreted and intracellular, was confirmed with an enzymatic activity assay. A >150-fold increase of the IDUA activity was observed using an optimized secretion medium, compared with a non-induced medium. We have proven that our *B. rapa rapa* hairy root system can be harnessed to secrete recalcitrant proteins, illustrating the high potential of hairy roots in plant molecular farming.

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Running title: A recombinant biologic in a Brassica hairy root

ACKNOWLEDGEMENTS

This research was funded from the European Union's Horizon 2020 Research and Innovation Program (Pharma-Factory project under grant agreement No 774078). The authors highly appreciate Pharma-Factory project coordination of Prof. Julian Ma and Dr. Matthew John Paul (St George´s hospital medical school, London UK). The authors thank Jaana Rikkinen, Kaisa Rinta-Harri and Tuuli Teikari for skillful technical assistance.

Author contributions: NGV, STH, CL, JDG conception and design, experimenting, data analysis, interpretation of data, drafting the manuscript; JPEE providing the eGFP clone, revising the manuscript; MG interpretation of data, revising the manuscript; FC and AR conception and design, interpretation of data, drafting and revising the manuscript. All authors have given final approval of the version to be published.

ABSTRACT

Hairy root systems have proven to be a viable alternative for recombinant protein production. For recalcitrant proteins, maximizing the productivity of hairy root cultures is essential. The aim of this study was to optimize a *Brassica rapa rapa* hairy root process for secretion of α-L-iduronidase (IDUA), a biologic of medical value. The process was first optimized with hairy roots expressing eGFP. For the biomass optimization, the highest biomass yields were achieved in modified Gamborg B5 culture medium. For the secretion induction, the optimized secretion media was obtained with additives (1.5g/l PVP + 1mg/l 2,4-D + 20.5g/l KNO_3) resulting in 3.4 fold eGFP secretion when compared to the non-induced control. These optimized conditions were applied to the IDUA-expressing hairy root clone, confirming that the highest yields of secreted IDUA occurred when using the already defined additive combination. The functionality of the IDUA protein, secreted and intracellular, was confirmed with an enzymatic activity assay. A >150-fold increase of the IDUA activity was observed using an optimized secretion medium, compared with a non-induced medium. We have proven that our *B. rapa rapa* hairy root system can be harnessed to secrete recalcitrant proteins, illustrating the high potential of hairy roots in plant molecular farming.

KEYWORDS

alpha-L-iduronidase, eGFP, hairy root, plant molecular farming, recombinant protein, secretion

INTRODUCTION

Over the last three decades, transgenic plants have been established as an alternative system to produce recombinant proteins. At the moment of writing, there are 27 studies for plant-derived biopharmaceuticals at different stages of clinical trials in the world (U.S. National Library of Medicine, 2022). An example of an already-in-the-market and FDA-approved plant-manufactured biopharmaceutical is the recombinant glucocerebrosidase, a therapeutic for Gaucher disease from Protalix BioTherapeutics(Fox, 2012). Currently, the most interesting example of plant biopharmaceuticals is the well-advanced (Phase 3 Clinical Trials) recombinant coronavirus-like particle COVID-19 vaccine from Medicago(Medicago, 2021).

Plant systems for recombinant protein production offer unique advantages such as cost-effective mass production, absence of inherent human or animal pathogens and high possibility of glycoengineering. Nevertheless, the associated environmental factors and social acceptance factors limit the implementation of transgenic plant cultivation particularly in open fields (A. M. Shelton et al., 2002; Lucht, 2015). Hairy root cultures integrate both the intrinsic advantages of plant-based protein synthesis together with a production in confinement. Hairy root cultures have been widely studied and used for production of high value plant secondary metabolites and recombinant proteins (Häkkinen et al., 2014; Vasilev et al., 2014; Ele Ekouna et al., 2017a; Cardon et al., 2019). Hairy roots also offer genotypic and phenotypic stability and, more importantly, possibility to secrete the expressed proteins (Gutierrez-Valdes et al., 2020; Halder et al., 2019). Optimizing the protein secretion in a given hairy root culture system, can be advantageous particularly for purification of the target proteins (Madeira et al., 2016).

After hairy root culture has been created with molecular farming and synthetic biology tools, the two main approaches for optimization of a hairy root culture system are the biomass production capacity and the target protein secretion. Different kinds and combinations of reagents can be used during the liquid culture. For instance, to ensure better nitrogen availability for the tissue [e.g. using KNO_3) (Rini Vijayan and Raghu, 2020)], to allow wall permeabilization for secretion of target proteins that would otherwise remain bound to the biomass [e.g. using DMSO (Wongsamuth and Doran, 1997)], to protect the integrity of secreted proteins [e.g. using BSA, PVP, PEG) (Alvarez, 2014)], or, to minimize the cell lysis by regulating the osmotic pressure in the medium [e.g. using mannitol (Halder et al., 2019)]. Statistical modeling can be helpful to design proper

experiments that need to include several culture reagents at the same time (Häkkinen et al., 2014; Häkkinen et al., 2018). Ultimately, a well-rounded optimization should offer synergistic effects of the used compounds in the culture to ensure high yields of good-quality target protein.

The therapeutic Alpha-L-iduronidase (IDUA), laronidase from Genzyme, is a recombinant form of the human IDUA that is produced by recombinant DNA technology using mammalian Chinese Hamster Ovary (CHO) cell culture (He et al., 2012). The plant-based analogue of IDUA produced in transgenic *Brassica rapa* hairy roots has demonstrated to have reproducible and highly homogeneous glycosylation profiles, as well as similar affinity and specific activity when compared to the one produced by CHO cells (Cardon et al., 2019). IDUA is clinically important as an enzyme replacement pharmaceutical for the treatment of mucopolysaccharidosis type I (MPS I), a progressive lysosomal storage disorder. IDUA (EC 3.2.1.176) is a secreted (71kDa) lysosomal enzyme that presents a signal peptide (^{1}M-^{23}A, released in its final secretion form), and six potential N-glycosylation sites as well as hydroxylation. Optimizing a hairy root system that already consistently produces functional plant-based IDUA such as the one described by Cardon *et al.* (2019), represented an opportunity to evaluate if it can be further harnessed to generate higher recombinant protein yields and/or ease the downstream processing.

The aim of this study was to optimize a hairy root process for secretion of α-L-iduronidase (IDUA). The process was first optimized with hairy roots expressing eGFP (Green Fluorescent Protein). As a production host we used *Brassica rapa rapa* hairy roots, which are currently used in commercial production purposes and which have shown to possess high recombinant protein production capacity (Huet et al., 2014). Our optimization approach intended to identify a range of culture medium additives that, when used alone or in combination, would increase the productivity of the process for "hard-to-produce" recombinant proteins. In addition, we wanted to evaluate if the eGFP secretion optimized conditions would also result in high secretion of the actual target protein, IDUA, a biologic of medical value.

MATERIALS AND METHODS

Establishment of Brassica rapa rapa hairy root lines and their maintenance

The eGFP *Brassica rapa rapa* hairy root lines were developed by BIOPI (Plant Biology and Innovation, Amiens, France) resulting from transformation with a construct portraying a double 35S promoter-SP-His tag-eGFP-CaMV polyA as described by Huet et al (2014). Similarly, the human alpha-L-iduronidase (IDUA) *B. rapa rapa* hairy root line was developed with the same vector set up but as a gene of interest encoding IDUA, as presented by Cardon et al. (2019).

Fresh hairy roots were subcultured every three weeks onto solid modified Gamborg B5 medium containing 10-fold higher concentrations of copper sulfate and cobalt chloride, and no casein when compared to the original Gamborg B5 (Oksman-Caldentey et al., 1991) and grown in the dark at +24°C. Prior to inoculation of liquid media, the hairy roots were subcultured on solid plates for seven days as a standardized time.

Optimization of induction of eGFP secretion in B. rapa rapa hairy root lines

B. rapa rapa hairy roots were cultured for 14 days in modified Gamborg B5 medium. Inoculum of 50 mg fresh weight (FW) was taken into 100 ml shake flask with 20 ml of culture medium. Cultivation was performed at + 24 °C, 90 rpm (shake radius of 3.2 cm), in the dark. After growth period, the recombinant protein secretion was induced by changing the culture medium and continuing the cultivation for another 14 days. Induction medium consisted of standard amounts of polyvinyl pyrrolidine (PVP) (1.5 mg/l) and 2,4-D (1.0 mg/l) with varying concentrations of 1- naphthaleneacetic acid acid (NAA) [1-20mg/l], methyl jasmonate (MeJA) [9-250µM], and potassium nitrate (KNO_3) [2-35g/l]. Sampling was performed by filtrating the hairy roots under suctions using Miracloth filter, and both hairy roots and culture medium were collected separately, frozen via liquid N_2 and stored at -20 °C. Both, fresh and dry weight (FW and DW) were recorded. For the DW measurement, samples were freeze-dried over three days in CHRIST ALPHA1-4 LD plus chamber.

Statistical experimental design and statistical analyses

A full factorial design at three levels (Modde v12.0 software, Umetrics, Sweden) was used to investigate the effects of studied factors on the yield and secretion of the recombinant protein. Studied factors were KNO_3, NAA and MeJA.

Statistical analyses were conducted with IBM SPSS Statistics 25 software. Normality of the data was assessed with Shapiro-Wilk's test. Unless otherwise described, one-way ANOVA was used together with Tukey HSD as post-hoc test when homogeneity of variances allowed (Levene test $p>0.05$). With data comprising unequal variances, Dunnett T3 test was used. Confidence level $p<0.01$ was used throughout the data.

Protein analysis

Fluorometric analysis of the green fluorescent protein

The green fluorescent protein (eGFP) concentration in culture medium and in TSP extracts of *B. rapa rapa* hairy roots was determined by fluorometry. Undiluted spent medium samples coming from different treatments were pipetted in pre-cooled black microtiter plates (Microfluor 2; Thermo Fisher Scientific, Waltham, MA) as triplicates. The fluorescence of the samples was determined at 485/527 nm using a VICTOR2 plate reader (Perkin Elmer, Waltham, MA) at 12 nm bandwidth and 100 ms measurement time. Sample readings were compared to a standard curve constructed with a purified GFP standard with known concentrations. For analyzing remaining intracellular eGFP after induction, TSP was extracted and analyzed as with the abovementioned fluorometric analysis.

Western Blot analysis of alpha-L-iduronidase expressing B. rapa rapa hairy roots

Western Blot analysis of alpha-L-iduronidase (IDUA) samples (crude culture media) was performed as previously described (Cardon et al., 2019) with the exception that after the mouse IDUA-binding primary antibody (ABIN603316 from Antibodies-online), the subsequent secondary antibody used was the IRDye 680RD Goat anti-mouse (926-68070 from Li-cor). Scanning of the blots after incubation and washing was performed with the Odyssey CLx Imaging System. Relative densitometries were analyzed using Image Studio Lite Ver 5.2.

Determination of alpha-L- iduronidase activity

Spent medium from the alpha-L- iduronidase (IDUA) expressing hairy root line after induction of secretion were analyzed as previously described (Cardon et al., 2019).

Microscopy and photographs of B. rapa rapa hairy root lines

B. rapa rapa hairy root fragments were excised from the liquid culture, mounted on microscopy slides in water or in toluidine blue, and analyzed with an inverted microscope (IX73 OLYMPUS). Macroscopic pictures were captured using a Nikon D5200 camera.

RESULTS AND DISCUSSION

Growth behavior of Brassica rapa rapa hairy root line

A Murashige and Skoog-based growth medium for *Brassica rapa rapa*hairy roots had been previously established by Samabriva (*Brassica* Growth Medium (BGM)). In this study, we assayed whether modified Gamborg B5 -based medium (B5mod) could also result in good biomass accumulation with *B. rapa rapa* hairy roots, since earlier it has been successfully used for cultivation of *Solanaceae* hairy roots (Häkkinen et al., 2005; Häkkinen et al., 2014). In addition, the growth in basic Gamborg B5 (B5) medium was studied in parallel (**Fig. 1A&B**). When the accumulation of fresh biomass weight (FW) was evaluated, it was observed that the biomass accumulation in studied media differed statistically (**Fig. 1A**) and the highest biomass was achieved with B5mod media. However, based on dry weight (DW), there were no differences in biomass accumulation in the studied media (**Fig. 1B**). Differences in FW and DW results reflected the differences in sucrose concentrations, as BGM medium contained higher sucrose concentration (50 g/l) when compared to the other two (30 g/l). The *B. rapa rapa* hairy roots grown in BGM medium with high sucrose level did not absorb as much intracellular water as hairy roots grown in B5 and B5mod (**Fig. 1A**). Due to the equal

growth with lower sucrose level, B5mod was selected as a cultivation medium for biomass production in the rest of the study.

In order to find out the optimal secretion induction phase, the growth behavior of *B. rapa rapa* hairy root line in B5mod was detrmined (**Fig. 1C**). An increase in biomass accumulation was observed until day 14, when stationary stage was reached. Thus, 14 days was selected as the induction point for the test protein, eGFP secretion. As a base line, without induction, amount up to 40 mg/l eGFP was detected in the culture medium already after 21 days when the B5-mod medium was applied (**Fig. 1D**).

Optimization of induction of eGFP secretion in Brassica rapa rapa hairy roots

Factors to be assessed for secretion induction in *B. rapa rapa*hairy roots were selected according to earlier studies, which have shown to improve recombinant protein secretion in hairy root platforms, including KNO_3, NAA and PVP as a stabilizer (Drake et al., 2003; Drake et al., 2009; Häkkinen et al., 2014; Wongsamuth and Doran, 1997). In addition, we wanted to see whether methyl jasmonate (MeJA), a known elicitor for a broad range of plant metabolites, could play a role in recombinant protein secretion. Some studies have reported the use of MeJA to increase the expression rate of recombinant proteins (Fraissinet-Tachet et al., 1998; Karimzadegan et al., 2019). The idea behind using this organic compound as reagent for recombinant protein optimization is that when plants experience herbivore attacks and wounding, plant defense mechanisms get triggered through jasmonate signaling pathways, resulting in re-allocation of plant resources to defense mechanisms instead of developmental processes. Hence, energy and resources like amino acids can be shifted to synthesize other proteins, and the heterologous proteins can be one of them.

Preliminary secretion induction assays in *B. rapa rapa* hairy roots had shown that addition of 2,4-D in the culture media was able to increase the extracellular levels of recombinant proteins such as lipase or eGFP (Ele Ekouna et al., 2017b). Thus, the starting 2,4-D level was initially set to 1 mg/l. In addition, PVP level was also set at 1.5 g/l according to Häkkinen et al., 2014. The studied factors included to the design of experiment (DoE) consisted of KNO_3, NAA and MeJA. The optimization iteration was performed in three experimental rounds (**Table 1**).

The outcome of secretion induction optimization as model descriptors and equations are shown in **Table 2** . The 1stsecretion induction optimization round indicated that KNO_3 and MeJA had significant effect on the eGFP secretion whereas NAA had no influence. The highest eGFP secretion, 22 mg/g DW *B. rapa rapa* hairy root biomass, was obtained in the studied range with low MeJA and high KNO_3 levels (**Fig. 2A**).

The 2nd secretion optimization experiment was performed so that the optimal secretion point was placed in the center of the experimental range (**Table 1**). Again, the highest eGFP secretion was gained with high KNO_3 induction. Noteworthy is that the role of MeJA was observed to be minimal. At highest the eGFP secretion was reaching 16 mg /g DW *B. rapa rapa*hairy root biomass (**Fig. 2B**). Indeed, the insignificant role of MeJA was confirmed in the 3rd secretion induction experiment were only KNO_3 had a significant effect on eGFP secretion. It was shown that the optimal concentration based on the model would be 20.4 g/l KNO_3 which would yield 16.2 mg/g DW eGFP (˜ 260 mg/l) (**Fig. 2C**). In the study of Häkkinen *et al.* , 2014, the addition of 14 g/l KNO_3 and 19 mg/l NAA to the B5mod culture medium increased the amount of recombinant M12 antibody recovered 30 fold.

as described above, for *B. rapa rapa* hairy root expressing eGFP, NAA and MeJA did not give additional benefit in secretion induction. Earlier, Drake et al. (2009) showed that the addition of growth regulators (NAA, indolebutyric acid (IBA), 6-benzylaminopurine (BAP) and kinetin (KIN)) to hydroponic plants all cased an increase in the root biomass, induced the secretion of recombinant proteins and also increased their stability in the hydroponic culture medium. NAA had the greatest effect on rhizosecretion with improving the antibody yields into the liquid media ca. 50-fold when compared to non-supplemented hydroponic media. In the current study, auxin was added in the form of 2,4-D and it is likely that additional auxin supplementation with NAA did not extend the auxin-induced effect in hairy roots.

In order to verify the identified optimal secretion induction conditions for *B. rapa rapa* hairy roots expressing

eGFP, an empirical set up shown in **Fig. 3A** was designed. The biomass accumulation was statistically significantly lower when secretion induction, PVP+2,4-D+KNO_3, was applied when compared to all other treatment and non-induced samples (**Fig. 3B**). Nevertheless, the PVP+2,4-D+KNO_3 secretion induction resulted in highest eGFP secretion of 342 mg/l being 3.4-fold higher than in the non-induced sample that reached level of 101 mg/l. The highest secretion value corresponded to 42 mg/g DW hairy roots.

Secretion induction of B. rapa rapa hairy root clone expressing IDUA

To understand whether *B. rapa rapa* hairy root clone carrying IDUA expression vector would show similar secretion behavior as eGFP clone, the setup shown in **Table 1** was applied. The construct configuration of both clones was the same, for a secretory protein, the only difference was the recombinant protein. In this sense, with the same empirical configuration of inducers, we were evaluating the protein-dependency in each system by analyzing how each protein was secreted.

In terms of DW biomass, there was a statistically significant reduction of biomass with the samples '2,4-D' and 'PVP+2,4-D', compared to 'no induction' **(Fig. 4A).** However, in general terms, the dry weights were in the same range as those for the eGFP clone. This is in line with the general set-up of the secretion induction experiment, in which the biomass for all treatments was propagated using the same B5mod medium. At that point, all bottles were replicates using the same conditions, and then changing the medium for the different treatments of inducers reflects how these affect the secretion of the respective protein.

The secretion of IDUA protein was analyzed in recovered media from the different treatments after the period of induction. The immunodetection protocol for IDUA detection was performed as described in the 2.5.3 section. The 'PVP+2,4-D+KNO_3' (treatment F) produced the highest IDUA secretion, as reflected in the highest value for relative densitometry **(Fig. 4B).** Additionally, treatments with' KNO_3' (treatment C) and with 'PVP+2,4-D' (treatment E) still induced the secretion of detectable amounts of IDUA protein. In treatments E and F, there are lower bands that depict protein degradation. However, we cannot say they present more protein degradation, possibly it is at the same level but there is just more overall IDUA protein present in treatment F.

The results of IDUA secretion obtained by immunodetection were confirmed using an enzymatic assay to measure the activity of secreted IDUA in the culture media from the different treatments **(Fig. 4C)** . Treatments C, E and F gave the best results in terms of IDUA activity, especially treatment F with PVP, 2,4D and KNO_3 which is higher and statistically different than treatments C and D. Therefore, treatment F allows to obtain the highest amount of active IDUA protein.

The treatments of induction affected the morphology of the hairy roots (**Supporting Figure 5)** . The treatment with 'PVP+2,4-D+KNO_3´, for instance, eliminated the tendency of the hairy roots to grow upwards in the flask when compared to the roots with the 'no induction´ treatment. PVP and KNO_3, when used independently, did not affect the structure of the tissue when compared to the 'no induction´ treatment. However, in all treatments in which 2,4-D was used, the root tips got swollen and they developed hump-like structures like the ones previously reported by Ele Ekouna (2017) in *B. rapa rapa* 2,4-D-treated hairy roots; and by Rage et al. (2020) in *Nicotiana benthamiana* 2,4-D treated roots. For our experiment, the hump-like structures were spotted after 4-6 days in treatments supplemented with 2,4-D. The morphology changes were the same in the case of the eGFP hairy root clone (data not shown).

2,4-D, affects the morphology of the roots by cell wall remodeling. In presence of a high auxin concentration, the cell wall is loosened, and the turgor pressure against the loosened wall leads to elongation. The general auxin-induced elongation mechanism has been explained before and, briefly, it states that the auxins activate the H+ export, lowering the cell wall pH. This provokes disruption of hydrogen bonding between cellulose microfibrils which in turn loosens the cell wall and consequently elongates cells. After the cell wall elongation, there is a consequent influx of water into the vacuole (Taiz, 1994). For the case of hairy roots, the treatment with 2,4-D only affects the cell wall expansion in cortex and epidermis cells as suggested in previous studies (Ele Ekouna et al., 2017b; Rage et al., 2020). Also, this auxin pressure may have stimulated the formation of lateral roots primordia from pericycle cells. The swelling of roots and of the generated hump-like structures

was a consequence of cell wall remodeling and elongation that in turn provoked uptake of water by those specific newly formed structures.

Nitrogen is a crucial macroelement for protein synthesis (Scheible et al., 2004). As already demonstrated by previous studies (Häkkinen et al., 2014; Holland et al., 2010), culture media supplemented with nitrate improves the intra- and extracellular levels of recombinant proteins in tobacco hairy roots, mainly by improving the protein synthesis and stabilizing the secreted proteins.

PVP, a water-soluble polymer, has colloidal and stabilizing properties in different cell cultures while being inert physiologically and metabolically (Magnuson et al., 1996). Some studies have reported that intracellular recombinant proteins were not significantly affected by the addition of PVP, nonetheless, due to the protein stabilizing effect of PVP, the stability of those proteins was significantly improved (Martínez et al., 2005; Pham et al., 2012). On the other hand, in older reports, PVP (up to 3g/l) is reported to have an effect on organ culture and growth of plant cells (LaCount et al., 1997; Magnuson et al., 1996; Sharp and Doran, 2001).

In an experiment with *Withania somnifera* hairy root culture producing a recombinant globular adiponectin (gAd) as a secretory protein, Dehdashti et al. (2020) showed that their MS media supplemented only with PVP (2g/l) stabilized by 5-fold the extracellular protein being secreted when compared to their control with no PVP added. They also reported that a combination of PVP (1 g/l) and KNO_3(2 g/l) resulted in the highest extracellular and intracellular gAd production (1877 µg/l and 21.3 µg/g FW, respectively) significantly higher than their control containing no PVP or KNO_3. This illustrates that PVP alone can stabilize the protein when it has been secreted to the apoplast, however, it is only with an extra nitrogen source (i.e. KNO_3) that the overall protein production is boosted and in turn stabilized by the PVP already in the media. The analysis of the secreted IDUA using an enzymatic assay to measure the activity of the protein has proved that the produced recombinant protein was indeed active. This protein in such a production system has been already characterized in terms of glycosylation, homogeneity and reproducibility (Cardon et al. 2019).

The improvement of the recombinant protein production by hairy roots of*Brassica rapa rapa* by culture medium optimization gave promising perspectives regarding the industrial production of lysosomal enzymes by a hairy root platform. Indeed, it was already proven that the culture of hairy roots in large scale bioreactors for the production of recombinant proteins, such as IDUA, is possible(Cardon et al., 2019; Gutierrez-Valdes et al., 2020). Hairy root culture in large scale bioreactors for the production of therapeutic compounds is suitable for GMP industrial purpose thanks to a controlled and sterile environment. Moreover biomass growth and molecule production can be monitored on-line thanks to identified growth-markers (Samabriva´s internal data) to ensure reproducible batches. The optimized culture medium developed in this study could be applied in large scale cultures in order to improve the production of recombinant protein by hairy roots.

CONCLUSIONS

Hairy root cultures represent a promising, scalable manufacturing system for high-value biopharmaceuticals. In order to maximize the productivity a careful optimization of the whole production process is required, including an optimization both of the culture medium used for hairy root growth and of the culture medium used during the recombinant protein production phase of the hairy root system. We studied suitability of*Brassica rapa rapa* hairy roots for secretion of a biopharmaceutical, namely α-L-iduronidase (IDUA). Our data first showed that with careful process optimization high level of secretion of a model protein such as eGFP can be obtained. We also demonstrated that such optimization can also allow to increase the ability of hairy roots to produce and secrete complex proteins. Thus *Brassica rapa rapa*hairy roots hold high potential as a production platform for complex human glycoproteins such as IDUA and indeed also for other biologics.

CONFLICT OF INTEREST

FC, CL, and MG are employed by the company Samabriva SA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLES

Table 1. The studied factors and design of experiment (DoE) of*Brassica rapa rapa* hairy root eGFP secretion induction.

Factor	**1st secretion induction**	**2nd secretion induction**
KNO	5-15 g/l	2-26 g/l
NAA	1-20 mg/l	-
MeJA	50-250 µM	9-91 µM
DoE	Full Factorial design in two levels	Central Composite Orthogonal (CCO) d
Number of experimental (and central) points	11 (3)	11 (3)

Table 2. Descriptions and equations of the models created in*Brassica rapa rapa* hairy root eGFP secretion induction experiments.

Model descriptor	**1st secretion induction**	**2nd secretion induction**
R^2	0.930	0.936
Q^2	0.852	0.898
Validity	0.950	0.991
Reproducibility	0.826	0.789
Equation	7501.4 + 1228.3[KNO_3] + 1.93 [MeJA] - 4.47 [KNO_3] x [MeJA]	7587.7 + 399.4[KNO_3] - 29.0 [MeJA

FIGURE LEGENDS

Figure 1. Biomass accumulation, growth behavior and eGFP secretion of *Brassica rapa rapa* hairy roots carrying eGFP encoding gene. **A)** Biomass accumulation in fresh weight (FW, g/l) with B5, B5mod and BGM after growth period of 14 days. **B)**Biomass accumulation in dry weight (DW, g/l) with B5, B5mod and BGM after growth period of 14 days. **C)** Growth behavior on B5mod medium (Circles: biomass expressed in FW; squares: biomass in DW).**D)** Extracellular eGFP yield (mg/l) during the cultivation (only one replicate assayed). Error bars represent the standard deviation of four (A, B) or three (C) biological replicates. Letters (a,b,c) indicate the statistically significant differences ($p<0.01$).

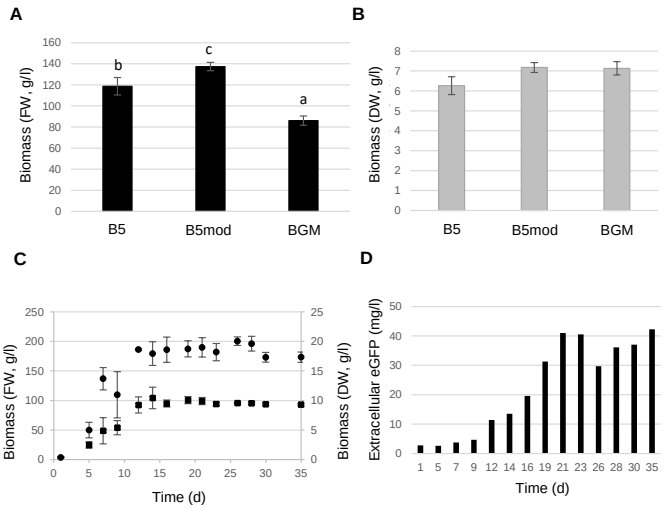
Figure 2. *Brassica rapa rapa* hairy root eGFP secretion induction experiments. **A)** A contour-plot based on the model constructed from the empirical secretion data of the 1st secretion induction experiment (Tables 1 & 2) showing secreted eGFP related to MeJA and KNO_3.**B)** A contour-plot based on the model constructed from the empirical secretion data of the 2nd secretion induction experiment (Tables 1 & 2) showing secreted eGFP related to MeJA and KNO_3. One outlier (N6) was selected.**C)** A contour-plot based on the model constructed from the empirical secretion data of the 3rd secretion induction experiment (**Tables 1 & 2**) showing secreted eGFP related to KNO_3.

Figure 3. Biomass accumulation and eGFP secretion in*Brassica rapa rapa* hairy root clone in the secretion induction verification **A)** Experimental set-up, **B)** Biomass accumulation (DW, g/l) **C)** Amount of secreted eGFP to culture medium after induction period (mg/l) [one-way ANOVA, post-hoc Tukey HSD, $p < 0.01$], **D)** Secreted eGFP after secretion induction expressed in mg/g DW hairy root biomass. Error bars represent the

standard deviation of five biological replicates. Letters (a,b,c,d) indicate statistically significant differences ($p<0.01$).

Figure 4. Biomass accumulation and α-L-iduronidase (IDUA) secretion in *Brassica rapa rapa* hairy root clone with optimized parameters. **A)** Biomass accumulation (DW, g/l). Dry weight biomass after secretion induction (Dunnett T3). Asterisks indicate significant differences in levels $p<0.05$ (*). Error bars represent the standard deviation of four biological replicates**B)** Immunodetection of IDUA protein in equivalent amounts of recovered media from the different treatments. A positive control of a commercial standard protein (+) (ABIN1464142, Antibodies-online) and a negative control (-) of clean non-induction media. The red arrow shows the 70.8 kDa band corresponding to the mature non-glycosylated standard protein. In the contiguous table, the relative densitometries of the different treatments are displayed. [A=PVP; B=2,4-D; C=KNO_3;D=PVP+2,4-D; E=PVP+2,4-D; F=PVP+2,4-D+KNO_3; G=No induction.] **C)** IDUA activity assay on the culture medium of the different treatments applied. Error bars indicates standard deviations. * indicates significant difference based on Student test ($p<0.05$).

Supporting Figure 5. Visual changes in the IDUA-expressing*Brassica rapa rapa* hairy root clone after the induction of secretion. **A)** Color and geotrophic changes in representative bottles of each treatment. **B)** Close-up photographs of representative pieces of hairy roots after the different treatments. Yellow arrows point out swollen tips and hump-like structures in the treatments with 2,4-D. The photographs are augmented four times the actual size. **C)** Bright field microscopic photography of the hump-like structures resulting from any treatment in which 2,4-D was used. **D)** Bright field microscopic photographies from 'No induction' and 'PVP+2,4-D+KNO_3'. **1** and**2** show the non-induced tissue (water and TBO, respectively), there is callus/starch (unstained) in some of the tips which might denote possibility of the tissue to keep growing. Blue in the very tips denotes lignified cell walls and the root apical meristem. **3**and **4** show the tissue induced with 'PVP+2, 4-D+KNO_3' (water and TBO, respectively). 2,4-D induced hump-like structures can be seen as a proliferation of possibly lateral roots primordia from pericyclic cells. Also, the root-tip vascular cylinders seem to abruptly deviate due to the formation of the formed humps. Additionally, root tips are swollen and there is absence of root apical meristems (i.e. not blue parts as those in the non-induced root tips).



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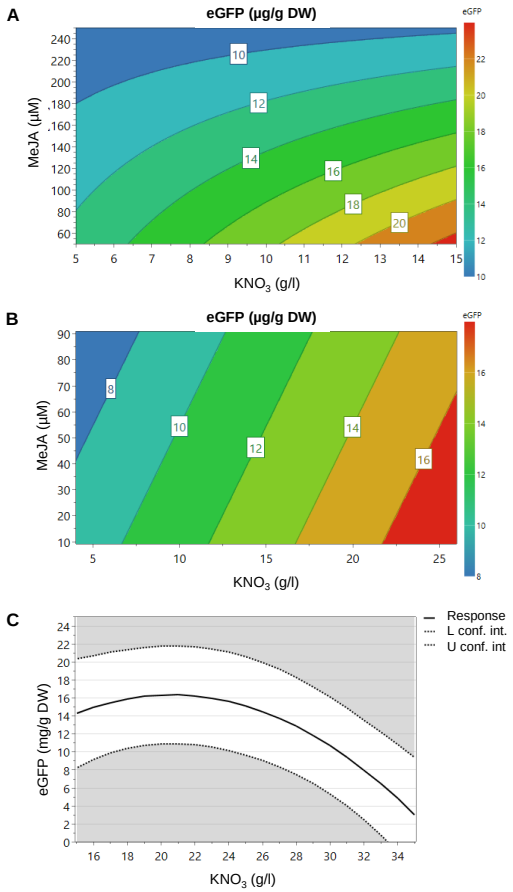

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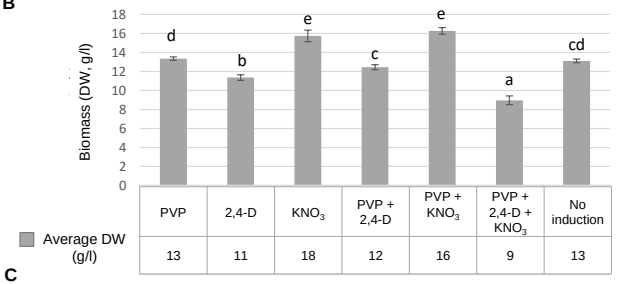
A

Tested parameters in the verification experiment

Label		PVP (g/l)	2,4-D (mg/l)	KNO_3 (g/l)
A	PVP	1.5		
B	2,4-D		1.0	
C	KNO_3			20.5
D	PVP+2,4-D	1.5	1.0	
E	PVP+KNO_3	1.5		20.5
F	PVP+2,4-D+KNO_3	1.5	1.0	20.5
G	No induction	-	-	-

B



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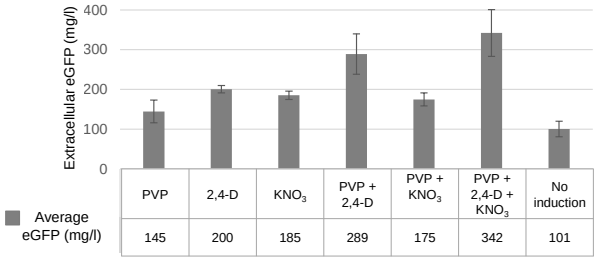
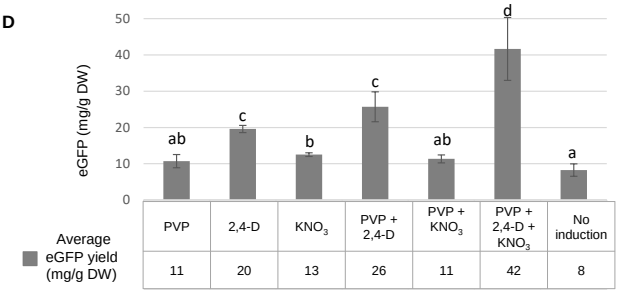


D



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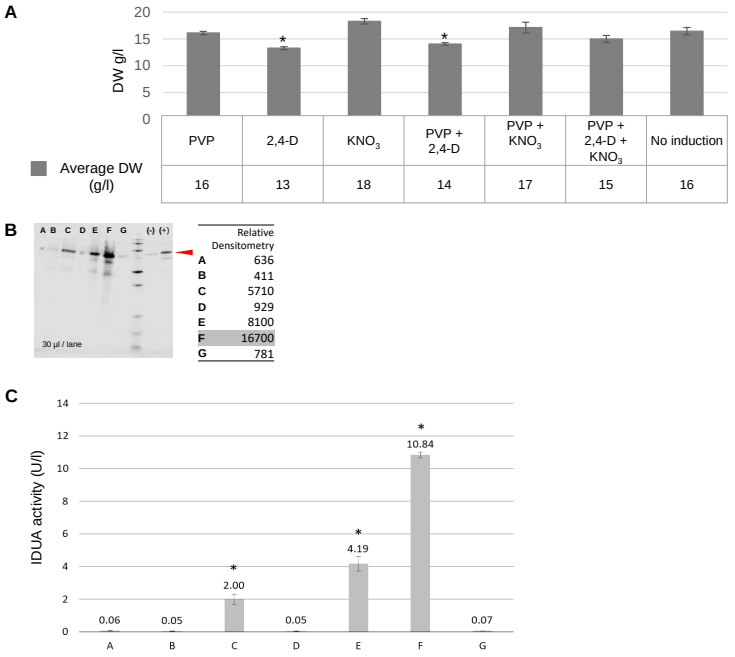

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