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Advancing *Glycyrrhiza glabra* L. Cultivation and Hairy Root Transformation and Elicitation for Future Metabolite Overexpression

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Abstract: The production of valuable bioactive compounds in the medicinal plant *Glycyrrhiza glabra* L. (*G. glabra*) would benefit from biotechnological approaches for the cultivation and induction of metabolite-producing hairy roots. Germination trials were tested to overcome seed dormancy, achieving high germination rates with sulfuric acid treatment. Hairy root cultures of cotyledons using *Rhizobium rhizogenes* strain 1724 showed the highest transformation efficiency. A fast-growing line, line S, was subsequently exposed to light treatments (red, blue, and blue and red combined) to evaluate their effects on growth, phenolic content, and Ferric Reducing Antioxidant Power (FRAP). Hairy root cultures grown in blue light and in blue and red light combined had higher growth rates than those grown in red light only or in control conditions (dark). FRAP increased over time under all light treatments, including the control, and those cultures exposed to blue and red light combined had higher FRAP than the control. These findings provide valuable insights into conditions for optimal seed germination and hairy root transformation. Treatment of the line S with different qualities of light induced changes in antioxidant capacity and phenolic content, indicating promise for its use in upregulating secondary metabolite production in *G. glabra* for future biotechnological applications.

Keywords: *Glycyrrhiza glabra* L.; seed germination; hairy root; light induction; polyphenols; antioxidant activity

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Academic Editors: Silvana Nicola and Alessandra Carrubba

Received: 24 October 2024
Revised: 8 December 2024
Accepted: 18 December 2024
Published: 9 January 2025

Citation: Afsharzadeh, N.; Paltram, R.; Jungwirth, A.; Tabrizi, L.; Nazeri, V.; Kalantari, H.; Halbwirth, H.; Samiei, L.; Sheehan, H.; Shokrpour, M. Advancing *Glycyrrhiza glabra* L. Cultivation and Hairy Root Transformation and Elicitation for Future Metabolite Overexpression. *Horticulturae* **2025**, *11*, 62. https://doi.org/10.3390/horticulturae11010062

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1. Introduction

Glycyrrhiza glabra L. (licorice; *G. glabra*), a medicinal plant belonging to the Fabaceae family, is renowned for its diverse range of bioactive compounds, particularly flavonoids, which are responsible for its extensive therapeutic applications. The roots and rhizomes of licorice contain a rich array of secondary metabolites, including triterpene saponins, chalcones, and over 300 types of flavonoids [1–5]. These compounds, such as glycyrrhizin, glabridin, and isoliquiritigenin, are effective in treating various health conditions, including

acute liver disorders, chronic hepatitis B and C, hemophilia, and immune system disorders [6]. Glycyrrhizin, a key compound in licorice, is widely used in the food industry and in treating pulmonary, gastrointestinal, hepatic, and biliary disorders, as well as peptic ulcers [1].

These valuable metabolites are typically extracted from the roots and rhizomes of the licorice plant, using methods such as solvent extraction, supercritical fluid extraction, or microwave-assisted extraction [7]. However, sourcing these metabolites from the plant poses several challenges, including the overharvesting of wild licorice populations, which threatens biodiversity [8–11], and the complex and often low-yield extraction processes that can be costly and time-consuming [7]. Additionally, environmental factors such as soil quality, climate, and cultivation practices can significantly affect the concentration and quality of the bioactive compounds in licorice, leading to variability in the therapeutic efficacy of the extracted metabolites [12]. For instance, the glycyrrhizin content of *G. uralensis* (Chinese licorice) has been shown to be lower in cultivated versus wild plants [13,14]. *G. glabra* is cultivated and this is the primary source for licorice's bioactive compounds, but multiple processes such as overharvesting, the shortage of licorice in some areas such as Iran [15], the increasing demand for licorice [16], and the insecurity in land resources due to a changing climate [17] makes research into alternative methods for production of valuable metabolites from licorice a desirable goal.

To improve the production of valuable metabolites from *G. glabra*, tissue culture and biotechnological approaches, such as hairy root cultures, provide a promising supplement to cultivation. Hairy root cultures, induced by infection with different strains of *Rhizobium rhizogenes* (formerly known as *Agrobacterium rhizogenes*), offer a stable system for the continuous production of secondary metabolites under controlled conditions [18]. Recent studies have demonstrated the potential of hairy root induction for enhancing the production of valuable metabolites in *G. glabra*. For example, a study by Shirazi et al. [19] successfully induced hairy root cultures using the *R. rhizogenes* strain ATCC15834, which significantly increased the accumulation of glycyrrhizin, a key triterpene saponin known for its anti-inflammatory and antiviral properties. Multiple other studies have used hairy root induction along with abiotic and biotic elicitors to enhance the production of various metabolites in licorice [20–23], offering a way to boost the output from this approach. Sourcing bioactive compounds from licorice hairy root cultures is still in a developmental phase [24], but it offers promise for the future as a way to complement the production of compounds from cultivated plants.

Light quality and exposure duration are known to significantly influence the accumulation of secondary metabolites, both in endogenous plant tissues as well as in hairy root cultures [25]. Blue LED light has been shown to be particularly effective in promoting hairy root growth and increasing the accumulation of isoflavonoids and astragalosides in *Astragalus membranaceus* by upregulating biosynthetic pathway genes [26]. Yang et al. [27] reported that UV-B and specific LED light spectra, such as different ratios of combinations of red and blue, increased rosmarinic acid in *Salvia miltiorrhiza* hairy roots over an induction period of three weeks. In *Glycyrrhiza uralensis*, various light treatments such as blue, red, and UV light, have been shown to increase melatonin levels and glycyrrhizin levels in endogenous tissues [28]. In particular, red light and UV-B exposure significantly increased glycyrrhizin levels in *G. uralensis* roots, with concentrations 1.5 times higher than in controls. Three- to six-month-old plants produced glycyrrhizin levels comparable to 3–4 year-old field-grown plants, demonstrating the effectiveness of controlled environments in enhancing glycyrrhizin production [29]. To our knowledge, no study has yet tested light induction as a tool to enhance the growth and metabolite production of hairy root cultures in *G. glabra*.

A major challenge in cultivating *G. glabra* lies in the germination of its seeds, which have a hard seed coat that acts as a barrier to water and gas exchange. This requires specific pretreatment methods to break dormancy and enhance germination rates [30]. Optimizing these pretreatment conditions is essential to overcoming germination barriers and obtaining high-quality plants for further cultivation [31]. Common treatments for enhancing germination by breaking seed dormancy are sulfuric acid immersion and mechanical scarification, and these are well-established in *Glycyrrhiza* species [32,33]. Hot water treatments at different ranges of temperatures have proven effective for other species [34,35] but have not been extensively tested at high temperatures for *G. glabra*.

In this study, we aimed to optimize approaches to improve the biotechnological production of important metabolites, such as glycyrrhizin and isoliquiritigenin, in *G. glabra*. We tested approaches for the cultivation of *G. glabra*, specifically the breaking of seed dormancy, to ensure timely and efficient seed germination. We tested the effect of different pretreatments, sterilization treatments and growth media on the germination success of *G. glabra*. We further aimed to optimize the induction of hairy roots in *G. glabra*. Four strains of *R. rhizogenes* were tested to identify the most effective strain for inducing hairy root formation, and we tested different tissue explants and methods of *R. rhizogenes* infection in these tissues. Finally, we aimed to test different qualities of light on hairy root cultures in order to induce higher growth and possible enhanced metabolite production. Eight distinct hairy root lines were established, and one of the lines with the highest growth rate, line S, was selected for growth under different light treatments: red light, blue light, and a combination of blue and red light. To assess the overall impact of these treatments, the growth of line S, as well as the increase in Ferric Reducing Antioxidant Power (FRAP) and total phenolic content (TPC), were tracked over this induction period.

2. Materials and Methods

2.1. Plant Material, Seed Germination, and Growth Conditions

Seeds of *G. glabra* were obtained from the Institute of Medicinal Plants (IMP; Karaj, Iran) and originate from the Kermanshah province of Iran. To optimize seedling germination, seeds underwent four pretreatments: immersion in 75% sulfuric acid for 30 min (SA75), 96% sulfuric acid for 45 min (SA96), hot water at 96 °C for 2 min (HW2), and hot water at 96 °C for 4 min (HW4). Following pretreatment, seeds were kept in sterile water for 24 h. Thereafter, seeds were surface sterilized with 70% (*v*/*v*) ethanol for 30 s, rinsed with sterile distilled water, and treated with solutions of 1% (NaOCl_1) or 2.5% (*w*/*v*) solution (NaOCl_2_5) of sodium hypochlorite (NaOCl; containing 5% available chlorine) with 2% (*v*/*v*) Tween-20 for 15 min. Disinfection was followed by three 3 min rinses with sterile distilled water. The pretreated sterilized seeds were cultured on either Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, Holland) or MS medium supplemented with B5 vitamins (MS + B5; Duchefa Biochemie, Haarlem, Holland), both adjusted to pH 5.7–5.8, solidified with agar and enriched with 3% sucrose for explant preparation. The seeds were then incubated at 25 ± 2 °C under a 16 h photoperiod with a light intensity of 35 µmol $m^{-2}s^{-1}$, and the evaluation of seed germination was carried out at 2 weeks and 4 weeks post-treatment. Seedlings were then maintained under these same growth conditions until required for hairy root transformation. A total of 473 seeds were included in the experiment, divided between all possible treatment combinations (pretreatment, disinfection, medium, and sampling time; see Table S1 for details of sample sizes for treatment combinations).

2.2. Induction and Proliferation of Hairy Root Cultures

Four strains of *R. rhizogenes*, R1000, A4, ATCC15834, and 1724, were obtained from the IMP. These were selected based on their successful induction of hairy roots in various plant species [7,23,24]. The strains were cultured on LB plates containing 50 mg/L rifampicin. A single colony from each strain was inoculated into liquid LB, followed by incubation at 28 °C (140 rpm) for 48 h. At OD_{600} 0.6–1.0, the cultures were harvested by centrifugation at 4000 rpm and resuspended in liquid MS to an OD of 0.6. Cotyledon and hypocotyl explants from in vitro-grown plants of varying ages (2, 3, 4, and 8 weeks) were used for inoculation with *R. rhizogenes*. To stimulate bacterial signaling and improve absorption, the bacterial suspension was supplemented with crushed *G. glabra* tissue (leaves and shoots) for 30 min before inoculation. The plant samples were then wounded using a sterile scalpel and immersed in the bacterial suspension for varying durations, namely ten minutes, one hour, and twenty-four hours, with gentle shaking, either by hand or on a rotary shaker at 70 rpm. Alternatively, bacterial solution (0.1 mL) was injected on the abaxial side of the cotyledons and at various points along the hypocotyls using a small syringe without a needle. For each condition, explants were split into five Petri-dishes, representing five technical replicates. As a control, explants wounded with a sterile scalpel and dipped in sterile distilled water were incubated under the same conditions. After inoculation, the infected explants were blotted dry on sterile filter paper and placed on co-cultivation medium (3% sucrose, 8% agar) for 2–3 days in a dark room at 28 °C. Following co-cultivation, the explants were washed with sterilized water containing 500 mg L^{-1} cefotaxime to eliminate bacterial contamination. They were then transferred to either MS or ½ MS medium supplemented with 3% sucrose and 500 mg L^{-1} cefotaxime. Once putative hairy roots emerged (approximately 3–4 cm in length; Figure S1), they were excised and subcultured, with the antibiotic concentration gradually reduced over 4 to 5 cycles of subculturing, and then maintained in hormone-free ½ MS liquid medium containing 2% sucrose. These cultures were kept at 25 ± 2 °C on a rotary shaker (110 rpm) with a 16 h photoperiod and were sub-cultured every two weeks into fresh medium.

2.3. mRNA, cDNA Synthesis, and PCR Verification of Transformed Roots

mRNA was extracted from hairy roots using the μMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. RNA integrity and purity were verified by gel electrophoresis and spectrophotometric analysis at 260/280 nm. cDNA was synthesized from the isolated mRNA using the LunaScript RT SuperMix (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's protocol. Standard PCR (GoTaq Green Master Mix; Promega, Madison, WI, USA) was performed on diluted cDNA using specific primers for rolB, rolC, and VirD (Table S2) to confirm the expression of these genes in the plant tissue. As a positive control, plasmids from all strains were purified by the Agrobacterium Plasmid Miniprep DNA Purification Kit (EURx, Gdańsk, Poland), according to the manufacturer's instructions, and were amplified with the same primers (Table S2).

2.4. Growth Curve of Hairy Roots

Growth kinetics of hairy roots were examined using 100 mg of fresh inoculums of four-week-old hairy roots, with one replicate for each time point. Hairy roots of each line were inoculated into 100 mL Erlenmeyer flasks containing 20 mL of half-strength MS liquid medium supplemented with 3% sucrose. The flasks were incubated in the dark at 28 °C with shaking at 120 rpm. Three culture flasks were sampled every 10 days until day 40, and harvested roots were blotted dry on sterilized Whatman® filter paper and weighed to record their fresh weight.

2.5. Light Induction Experiment

Light treatments were carried out in a custom-designed plant growth chamber equipped with various LED light sources. The chamber included shelves fitted with LED lamps emitting red light (660 nm), blue light (460 nm), a combination of blue and red light (1:1 ratio), and an additional shelf without illumination was used as a control. To prevent any unintended light exposure from adjacent shelves or the external environment, all shelves were covered with blackout fabric. Hairy roots were exposed to an LED light intensity of 110 µmol m^{-2} s^{-1} for 16 h per day. The chamber was maintained in a shaking incubator at a stable temperature of 27 °C with 110 rpm shaking to ensure consistent growth conditions. The experimental set-up was as follows: approximately 100 mg of hairy root inocula from line S was subcultured for 10 days in the dark and was then exposed to four different light treatments for 45 days. Sampling was performed at days 27, 34, 41, 48, and 55, with day 10 representing the start of the light induction. These timepoints were chosen to allow tissue to recover after cutting the initial hairy root into replicates, and to allow at least two weeks of light treatment before sampling. Three biological replicates were included for each sampling point for each light treatment, and these replicates were removed from the experiment at the point of sampling in order to record fresh weight and perform further analyses (FRAP and TPC).

2.6. Ferric Reducing Antioxidant Power (FRAP) Assay

A proportion of the hairy root samples were weighed and then ground fresh in liquid nitrogen using a mortar and pestle. These were extracted with 70% (*v*/*v*) ethanol at a ratio of 1 mL extraction volume per 50 mg of fresh weight. The mixture was vortexed for 3 min subjected to ultrasonic-assisted extraction for 1 h, and then centrifuged at 13,000 rpm for 5 min, and the resulting supernatant was collected for the assay. The FRAP assay was carried out according to a modified version of the protocol established by [36], with 10 µL of the root extract and 240 µL of a freshly prepared FRAP working reagent (10 mmol/L 2,4,6-tripyridyl-s-triazine in 40 mmol/L HCl, 20 mmol/L ferric chloride, and 300 mmol/L acetate buffer, pH 3.6). Following an incubation in the dark for 30 min, the absorbance measured at 593 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Ortenburg, Germany). The reducing power of the extracts was subsequently quantified by comparison to a standard curve prepared with ascorbic acid, and results were expressed as ascorbic acid equivalents (AAE) in milligrams of ascorbic acid equivalents per 100 mg of fresh weight of hairy roots. Three samples could not be measured in the FRAP assay: only one biological replicate was available for the control at day 55 and two were available for blue light at day 41.

2.7. Total Phenolic Content (TPC) Assay

A proportion of the hairy root samples from the light induction experiment were freeze-dried and finely ground into a uniform powder using a mortar and pestle. Fifty mg of powder was mixed for 3 min with 1 mL 70% (*v*/*v*) ethanol and the extraction method followed the same procedure as described for the FRAP assay. TPC of the samples was measured using the original procedure described by [24], with modifications as outlined in [25]. After ensuring complete color development, absorbance was measured at 765 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Ortenburg, Germany). The phenolic content was quantified against a standard curve of gallic acid, and the results were expressed as gallic acid equivalents (GAE), calculated in milligrams of gallic acid equivalents per 100 mg of dry weight of hairy roots.

2.8. Statistical Analyses

All statistical analyses were carried out in R (version 4.4.1 [37]), *p*-values and other model outputs were derived using 'summary' and 'drop1' commands where appropriate, and model fit was checked using 'testResiduals' in DHARMa (version 0.4.6; [38]). To analyze the effects on germination success, we first fitted a binomial generalized linear model (bGLM). In this model, we aimed to investigate whether our different treatments (i.e., pretreatment, disinfection scheme, planting media) either interactively affected each other and/or whether either of them affected germination timing. We thus initially fitted a full model and step-wise removed non-significant terms until we arrived at a final (reduced) model that included only significant interaction terms and any main effects not included in the remaining interactions. The full model used germination success (binomial variable: seed germinated or not; n = 473 seeds) as the response variable. As explanatory variables, we included all pair-wise interactions between sampling time (two-level factor: 2 weeks or 4 weeks since planting) and our treatments (pretreatment—four-level factor: HW2, HW4, SA75, SA98; disinfection scheme—two-level factor: NaOCl_1 or NaOCl_2_5; medium—two-level factor: MS or MS + B5), as well as all pair-wise interactions between the treatments for a total of 6 explanatory terms. In the final model, we retained time since planting as a main effect and the two-way interactions between pretreatment and planting media and between disinfection scheme and planting media. Subsequently, we used Tukey's post hoc test to assess the effects of all retained explanatory variables on germination success separately, adjusting for multiple testing.

To analyze our light induction experiment, we fitted three different linear models (LMs) that had the same general structure: we fitted a given variable of interest as response variable, and the two-way interaction between light treatment (four-level factor: blue, red, blue and red, or dark) and sampling time point (five-level factor: measurement occurred in week 1, 2, 3, 4, or 5) was included as explanatory variable. If this had a non-significant effect on the model's fit, we removed the interaction and refitted the model with the two main effects light treatment and time point as explanatory variables. As such, the model on phenol levels in our plants included TPC (as mg of gallic acid equivalents per 100 mg sample dry weight) as the response variable and the interaction was retained in it. The model of FRAP included FRAP as the response variable (as mg of ascorbic acid equivalents per 100 mg sample fresh weight) and the interaction was removed. The model of fresh weight changes included relative change in fresh weight since the start of the experiment (in proportional change as weight-at-sampling divided by weight-at-start such that a value of 1 indicates no change, values below 1 indicate a reduction in weight, and values above 1 indicate weight gain) as the response variable and the interaction was removed. We subsequently performed pair-wise comparisons of all light treatments for FRAP and fresh weight changes, using Tukey's post hoc test and adjusting for multiple testing. Finally, we used simple LMs to assess whether observed changes in phenol content over time under specific light treatments could be explained by corresponding changes in FRAP. We thus fitted one LM each for the respective light treatments (dark control; blue) with phenol content as the response variable and FRAP as the explanatory variable.

3. Results

3.1. Pretreatment, Sterilization, and Growth Media Effects on Seed Germination in G. glabra

This study aimed to identify the best pre-treatment methods and culture medium for achieving high germination rates for *G. glabra* seeds. Seed germination was evaluated at 2 weeks and 4 weeks (Table S1). The results of the bGLM revealed a significant effect of sampling time on germination rates (likelihood ratio test (LRT) = 15.1, $p < 0.001$), with higher rates of germination after 4 compared to 2 weeks (Figure 1a), as might be expected given the

longer time. We detected no interactive effects between sampling time and any of the applied treatments. Both sulfuric acid pretreatments had a large significant positive effect on seed germination, achieving 90–100% germination rates compared to 30–50% germination rates under hot water pretreatments across all disinfection and medium conditions (Figure 1b). Our post hoc tests confirmed this, finding that sulfuric acid pretreatments differed from hot water pretreatments (Tukey: most pair-wise comparisons adjusted $p < 0.001$; see Table S3 for details) but there were no differences between the sulfuric acid pretreatments ($p = 1$) or between the hot water pretreatments ($p = 0.06$). Among the applied treatments, the type of growth medium used affected germination rates interactively with the pretreatments (LRT = 20.2, $p < 0.001$; Figure 1b) and the disinfection method (LRT = 18.8, $p < 0.001$; Figure 1c): SA75 and SA98 resulted in high germination success on both media and HW2 resulted in low germination success on both media, while HW4 had improved germination success on MS compared to MS + B5 (Figure 1b). Similarly, NaOCl_2_5 resulted in high rates of germination on MS, but not MS + B5, while NaOCl_1's effect was not modified by the planting media (Figure 1c). However, while pretreatment with 96% sulfuric acid effectively promoted germination, we observed that abnormalities began to appear in the seedlings from this pretreatment. Specifically, these seedlings died shortly after germination or aborted growth shortly after revealing the cotyledon (Figure S2). In contrast, seeds pretreated with hot water or 75% sulfuric acid did not appear to have this problem.

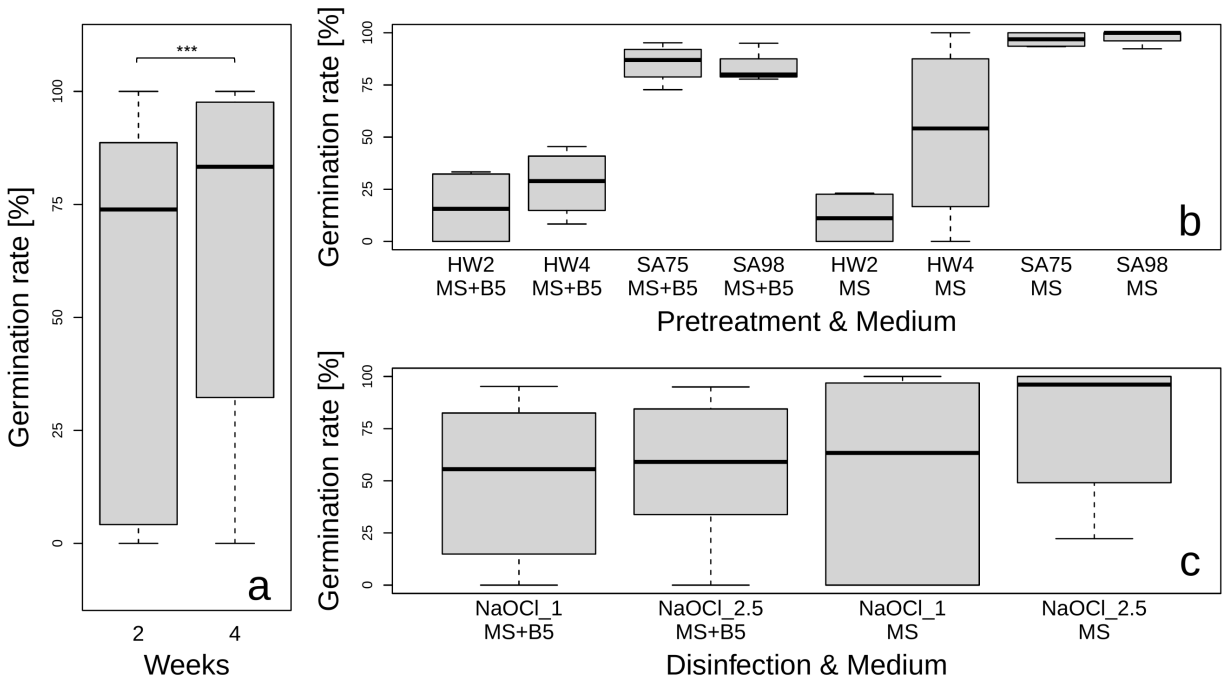

Figure 1. Boxplots of the effects of treatments and time elapsed after sowing on the germination success of 473 *Glycyrrhiza glabra* seeds. In each plot, the y-axis indicates the germination rate between 0% (no seeds germinated) and 100% (all seeds germinated). In (**a**), data are arranged according to the time since sowing (either 2 or 4 weeks), after which the seeds were scored as germinated or not. In (**b**), data are arranged according to the pretreatment the seeds received (HW2, HW4, SA75, or SA98) and the medium on which they were planted (MS or MS + B5). In (**c**), data are arranged according to the disinfection they received (NaOCl_1 or NaOCl_2_5) and the medium in which they were planted (MS or MS + B5). In (**a**), *** indicates $p < 0.001$ for the post hoc comparison; for all pair-wise post hoc comparisons in (**b**), see Table S3; in (**c**), no significant differences between categories were found. HW2, hot water at 96 °C for 2 min; HW4, hot water at 96 °C for 4 min; SA75, 75% in sulfuric acid for 30 min; SA96, 96% sulfuric acid for 45 min; MS, Murashige and Skoog; MS + B5, MS with B5 vitamins; NaOCl_1, 1% (*w*/*v*) NaOCl for 15 min; NaOCl_2_5, 2.5% (*w*/*v*) NaOCl for 15 min.

3.2. Optimal Conditions for Induction and Proliferation of Hairy Root Cultures in G. glabra

In order to determine optimal conditions for the induction of hairy roots in *G. glabra*, three key variables were tested: *R. rhizogenes* strain, method of infection, and type of explant (cotyledon/hypocotyl). The rates of hairy root transformation under these different conditions are presented in Table 1. Strains 1724 and ATCC15834 outperformed R1000 and A4 for almost all combinations of tissue type and infection method. In most cases, injections produced a higher percentage of hairy roots than wounding and soaking. Across the different bacterial strains and injection method, cotyledon explants were most responsive to transformation. The optimal combination across all these treatments was injection with strain 1724 in the cotyledons with 83.8% of explants successfully producing hairy roots.

Table 1. Hairy root transformation success in *G. glabra* for four strains of *Rhizobium rhizogenes*, using two explant types and various infection strategies. Averages and standard deviations of five technical replicates are presented.

Strain	Explant	Hairy Roots Produced by Injection (%)	Hairy Roots Produced by Wounding and Soaking (%)		
			10 min	1 h	24 h
1724	Cotyledon	83.8 (±21.4)	70.0 (±16.8)	30.1 (±8.0)	5.0 (±3.1)
	Hypocotyl	45.1 (±20.1)	50.2 (±15.3)	12.4 (±3.3)	1.7 (±3.7)
ATCC15834	Cotyledon	40.1 (±28.1)	30.2 (±14.8)	35.2 (±8.1)	20.1 (±7.8)
	Hypocotyl	15.7 (±11.5)	40.2 (±10.9)	31.3 (±10.9)	0.0 (±0.0)
R1000	Cotyledon	10.2 (±9.5)	3.3 (±4.7)	10.3 (±3.4)	0.0 (±0.0)
	Hypocotyl	12.1 (±11.5)	5.1 (±3.7)	4.7 (±2.7)	0.0 (±0.0)
A4	Cotyledon	26.2 (±14.0)	25.0 (±11.2)	4.9 (±2.8)	2.5 (±5.6)
	Hypocotyl	0.0 (±0.0)	10.3 (±7.4)	0.0 (±0.0)	10.3 (±3.3)

3.3. Growth Rate of Hairy Root Lines Showing Optimal Growth

Hairy roots produced from the successful infection of *G. glabra* tissue by the four *R. rhizogenes* strains were subcultured in order to gradually reduce the concentration of cefotaxime and enable the growth and proliferation of the transformed roots in MS medium. These lines were observed to determine those that appeared to accumulate the most biomass (Figure S1). Eight lines were selected for growth curve measurements to confirm these observations and to determine which lines could be suitable for producing high amounts of specialized metabolites (Table S4). To confirm the genetic transformation of these lines, cDNA made from these lines was used to amplify the rolB and rolC genes, demonstrating the successful integration of the T-DNA region into all eight lines and the subsequent expression from these integrated pRi genes (Figure S3). The growth rate experiment was initiated with 100 mg of fresh hairy root inocula from these eight lines. These were cultivated in liquid ½ MS medium over 40 days in the dark with biomass measurements recorded at 10, 20, 30, and 40 days. The growth curve established for the eight lines demonstrated varying degrees of biomass accumulation over the measurement period (Figure 2). Over half of the lines lost some weight or only maintained their starting weight during the first 20 days of the experiment. Biomass loss is due to necrosis and partial loss of hairy roots during growth, which were difficult to fully retrieve from the media for weighing (Figure S4). Others accumulated high amounts of biomass over the same period, but then lost biomass during the second 20 days of the experiment. Lines 1.4, T3, and S accumulated the most biomass over the entire duration of the experiment, reaching amounts that were 1.67-, 1.59-, and 1.42-fold higher than the starting weight, respectively.

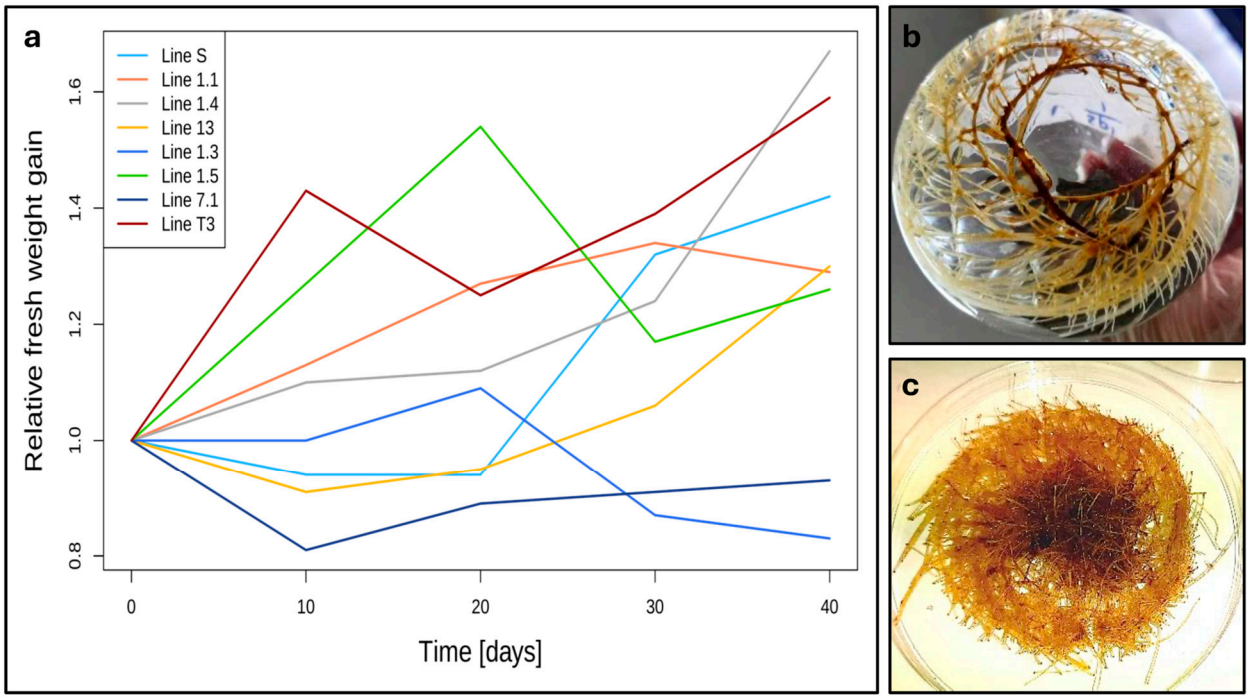

Figure 2. (**a**) Growth curves of eight hairy root lines of *G. glabra* over 40 days. For each time point, approximately one gram of initial fresh weight was used to start the culture and the fresh weight at each sampling point was standardized to the initial starting weight giving fold-change from starting weight. (**b**,**c**) Growth of line S from day 10 (**b**) to day 40 (**c**). In (**b**), the tissue is shown in a 100 mL flask with a diameter of 10 cm; in (**c**), the tissue is shown in a 9 cm diameter Petri dish.

3.4. The Effect of Light Quality on the Growth Rate, FRAP, and Phenolic Content of Hairy Root Line S

Line S, one of the lines with the highest growth rate (Figure 2a), was selected for exposure to different light treatments to determine if different qualities of light could induce differences in the rate of growth, the accumulation of phenols, and FRAP. The LM on fresh weight changes revealed a general increase in weight over time for all treatments (residual sum of squares, RSS = 20.24, $p < 0.001$). There were also differences in weight changes between the treatments (RSS = 19.1, $p < 0.001$): those samples exposed to blue light or to blue and red light combined had significantly higher weight gains than samples in the dark control (Tukey: adjusted $p = 0.032$ and $p = 0.011$, respectively; Figure 3a,b).

To determine whether differences in light quality affected the FRAP of line S, a Ferric Reducing Antioxidant Power (FRAP) assay was used on fresh tissue samples collected at the same timepoints as the growth measurements. The LM on FRAP found a general increase in FRAP over time across all treatments, including the control (RSS = 0.15, $p < 0.001$). There was also a general difference in FRAP between treatments (RSS = 0.1, $p = 0.042$): FRAP values of samples exposed to blue and red light combined tended to be higher than those in the dark control (Tukey: adjusted $p = 0.06$; Figure 3c,d).

We investigated whether differences in TPC could explain the increase in FRAP for the light treatments of hairy root line S, particularly for blue and red light combined, where FRAP was shown to be significantly higher than the control (Figure 3c,d). Our LM on phenol content revealed a significant interaction between light treatment and sampling point (RSS = 2.94, $p = 0.035$), finding that phenol levels in samples exposed to blue light or kept in the dark increased over time, with blue light showing on average an increase of 0.76 mg GAE per 100 mg dry weight from day 27 to day 48, and dark conditions showing an increase of 0.37 mg GAE per 100 mg dry weight over the same period (Figure 3e,f). However, phenol levels in samples exposed to red or blue and red light combined remained

mostly constant over the time course, as evidenced by the flat lines of our LM (Figure 3e,f). We looked for a positive relationship between FRAP and phenol content for each light treatment, and observed a positive correlation between FRAP and phenol content for blue light and the dark control, but these were not significant (blue: adjusted $R^2 = 0.07$, $p = 0.19$; dark: adjusted $R^2 = 0.12$, $p = 0.14$; Figure S5).

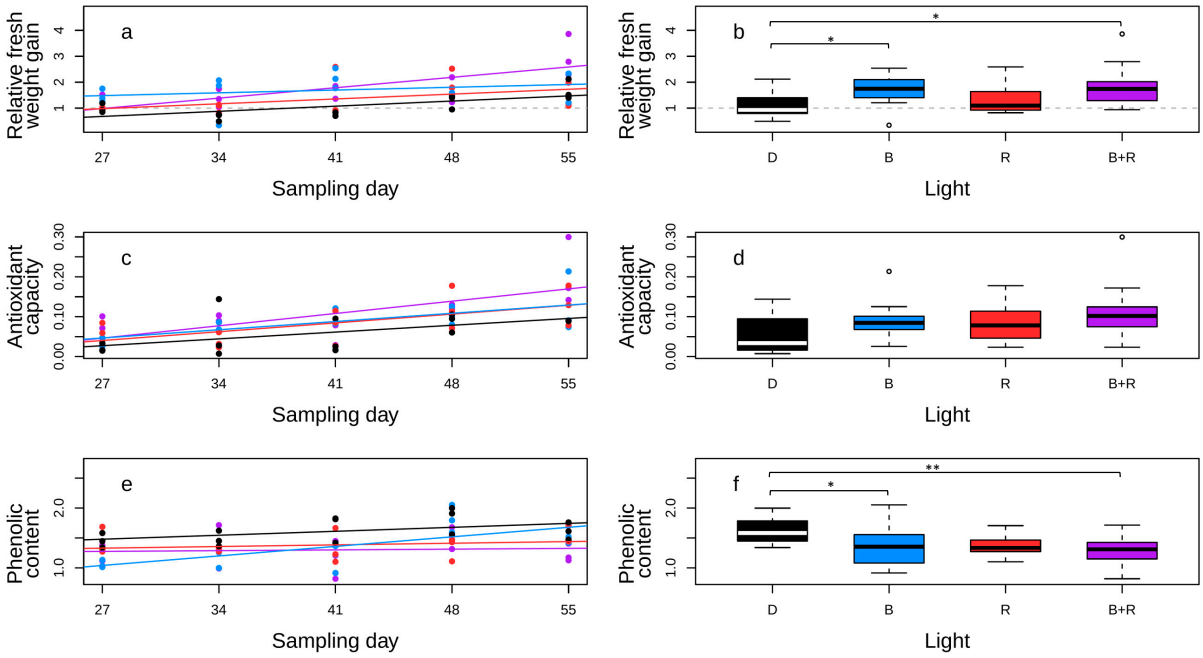

Figure 3. The effect of different qualities of light induction on growth rate, FRAP, and TPC of G. glabra hairy root line S over 45 days of light treatment. Scatterplots on the left (**a**,**c**,**e**) give values as measured on the respective day since start of the experiment. Boxplots on the right (**b**,**d**,**f**) give summaries across all values for a given light treatment. The top panel shows proportional fresh weight change (y-axis as fold-change from starting amount; 1 = no change); the middle panel gives FRAP (y-axis as mg ascorbic acid equivalents per 100 mg fresh weight); the bottom panel gives phenolic content (y-axis as mg gallic acid equivalents per 100 mg dry weight). Each point in the scatterplots represents a single sample with, in general, three samples measured per light treatment per time point for a total of 60, 57, and 60 estimates of fresh weight change, FRAP, and phenolic content, respectively. Lines give the treatment-specific linear regressions. Light induction was initiated on day 10. Black, D: dark (control); blue, B: blue light; red, R: red light; purple, B + R: blue and red light. For post-hoc comparisons, * indicates $p < 0.05$ and ** indicates $p < 0.01$.

4. Discussion

4.1. Effect of Pretreatments, Disinfection, and Media on Germination Rate of G. glabra

For the cultivation of *G. glabra* from seeds, it is important to break the seed dormancy caused by the seed coat. The typical strategies to achieve this in *G. glabra* are pretreatment with sulfuric acid or scarification of the seed coat [32,39]. Hot water has also been shown to release dormancy in many plant species [40,41]. We sought to determine if hot water would also be an effective dormancy breaker for *G. glabra* seeds, and we tested this along with the more typical treatment of sulfuric acid. We found, however, that seed germination rates were much lower when pretreated with hot water (2 min or 4 min) than when pretreated with sulfuric acid (75% for 30 min or 96% for 45 min; Figure 1, Table S1). This is similar to results from a previous study which showed that sulfuric acid 75% for 45 min has the highest germination rate in *G. glabra* seeds [30]. Studies in other species have measured high germination rates using longer exposure to hot water [42,43]. Hot water may also be a gentler method that ensures healthier seedlings post-germination. We observed that

many seedlings pretreated with 96% sulfuric acid had aborted growth, although this was less prevalent in those pretreated with the lower concentration of 75% sulfuric acid. This is similar to results observed in other studies, for example [34], where seed treatments of 98% sulfuric acid were found to damage seed viability in contrast to moderate concentrations of sulfuric acid (49%), which increased germination success without causing harm to the seedlings. We did not find a significant difference in germination rate between 96% and 75% sulfuric acid pretreatments; therefore, we would recommend choosing the lower concentration of sulfuric acid for seed germination considering the likely deleterious effects of sulfuric acid treatment on *G. glabra* seedling growth. Further studies in *G. glabra* should focus on identifying the lowest concentration of sulfuric acid for successful seed germination as well as the potential of longer durations of soaking in hot water.

We also wanted to determine if the disinfection method (1% sodium NaOCl or 2.5% NaOCl) and the media that the seeds were sowed on (MS or MS with B5 vitamins) would affect the success of germination. According to our model, neither pretreatment, disinfection method, nor media type alone showed an effect on germination rate. Rather, we found that medium type interacted with both pretreatment and disinfection scheme: first, soaking in hot water for four minutes improved germination success on MS compared to MS with B5 vitamins (Figure 1b), suggesting that hot water pretreatments may show higher germination rates when combined with sowing on MS medium as opposed to MS with B5 vitamins. Second, 1% NaOCl did not show an effect on germination rate for the different medium types while 2.5% NaOCl had higher rates of germination on MS than MS with B5 vitamins (Figure 1c). In these cases, it seems that MS media may be preferable to use as a substrate for germination, an effect that has been documented previously [44].

4.2. Effect of R. rhizogenes Strain on Hairy Root Transformation Rate of G. glabra

This study identified optimal conditions for inducing hairy root cultures in *G. glabra* by testing different combinations of *R. rhizogenes* strains, infection methods, and explant types. We managed to generate hairy root transformants from all four strains tested, but with varying degrees of success (Table 1). The strains with the highest rate of transformation were strains 1724 and ATCC15834, whereas strains A4 and R1000 produced much lower numbers of hairy roots. Strains ATCC15834 and A4 have been used successfully before for both *G. glabra* and *G. uralensis* [19–22,42,45–48], and R1000 has been used to generate hairy roots in *G. uralensis* [45]. We could not find any previous reports of successful transformation of *Glycyrrhiza* species with strain 1724, although it is well-established in other plant species [49–51], suggesting that it may be an underexplored resource for hairy root generation in *G. glabra*.

4.3. Effect of Explant Type and Infection Method on Hairy Root Transformation Rate

Besides *R. rhizogenes* strain, the type of tissue explant and the infection method also had an impact on the success of transformation. Across both types of infection method (injection and wounding with soaking), cotyledons mostly outperformed hypocotyls in terms of transformation success (Table 1). Several studies using *R. rhizogenes* have reported that cotyledons showed a higher transformation success rate than hypocotyls [52–54]. For the method of *R. rhizogenes* infection, injection and wounding with soaking for ten minutes produced the highest transformation rates, although injection when combined with cotyledon explants and the highest performing *R. rhizogenes* strain, 1724, produced a very high transformation rate of 83.8%, suggesting that this combination of strain, infection method, and explant type should be utilized for generating hairy roots in *G. glabra*, especially where transformation efficiency is a primary concern. The length of time the explants spent soaking in the bacterial culture after wounding also had a clear effect on transformation

success, with the shortest soaking duration tending to produce the highest frequency of hairy roots (Table 1). We observed a high degree of necrosis on the explants soaked in bacterial solution for 24 h, both during and after the soaking period, suggesting that this long exposure to the bacteria was deleterious for the survival of the plant tissue.

4.4. Testing Light Induction on the Growth of G. Glabra Hairy Root Line S

A main aim of our study was to establish optimal conditions for hairy root transformation in *G. glabra* with an eye to the biotechnological production of licorice's valuable specialized metabolites, such as glycyrrhizin and isoflavonoids. To this end, we chose to test the effect of different qualities of light on *G. glabra* hairy root cultures, an induction method that has been used in other plant species to upregulate the amounts of specialized metabolites [26,29,55,56]. From eight lines that were generated from our experiment testing optimal conditions for hairy root induction (Figure 2a), we selected one line, line S, which showed high growth, particularly in the second half of this experiment. We exposed line S to different qualities of light and initially measured growth rate. We found that growth rate increased, as might be expected, over the course of the experiment, with two light treatments, namely blue light and blue and red light combined, showing the highest amounts of fresh weight increase (Figure 3a,b). Both of these treatments included blue light at a wavelength of 460 nm. In other studies, blue light has been shown to promote the growth of both endogenous roots [57], as well as hairy roots [26,29,58]. For instance, Sani et al. [58] found that both blue light and red light induced higher biomass accumulation in *Eurycoma longifolia* hairy roots, and the effect of blue and red light combined produced a synergistic effect on biomass accumulation.

4.5. Enhancement of Antioxidant Capacity, as Measured by FRAP, in G. glabra Hairy Root Line S Exposed to Different Qualities of Light

To determine whether light induction influenced the accumulation of specialized metabolites in hairy root cultures, we tested the antioxidant capacity of the hairy root lines using a commonly used assay, FRAP [59–61]. A major contributor to antioxidant capacity in plants is the generation of specialized metabolites such as flavonoids, phenolic acids, and carotenoids, which possess antioxidant properties. They scavenge free radicals and neutralize ROS, thereby protecting cellular components like DNA, proteins, and lipids from oxidative damage [62]. Therefore, we used FRAP as a proxy for specialized metabolite accumulation, which is what we ultimately want to achieve through light induction in hairy root cultures. We found that FRAP on a per weight basis increased for all treatments over the duration of the experiment, including the control (dark; Figure 3c). This suggests an enhanced capacity for antioxidant ability over time, possibly due to accumulation of specialized metabolites in all conditions. However, between the light treatments, FRAP increased the most under the combination of blue and red light when compared to the control, indicating that this light condition may have accumulated specialized metabolites at a greater rate than the other light treatments (Figure 3d). This has also been observed in other studies, such that the highest antioxidant levels were revealed in treatments with blue and red light [63]. This contrasts with some current studies that showed that blue light causes higher antioxidant activity [53,54]. These findings collectively emphasize the role of specific light spectra, particularly red and blue, in boosting antioxidant production in plant systems, supporting the observed effects in *G. glabra* hairy roots under our experimental conditions.

4.6. Determining the Correlation Between FRAP and TPC

Phenolic compounds (also known as polyphenols), such as flavonoids and phenolic acids, are compounds with strong antioxidant activity. This activity is due to their ability to

donate an electron or a hydrogen atom to free radicals, causing a break in the chain reaction of oxidation. The accumulation of phenols in plant tissue can be influenced by factors such as light exposure [64]. Given that we had detected an overall increase in FRAP across all treatments (Figure 3c), and a particularly high rate under blue and red light (Figure 3d), and considering that licorice contains many bioactive phenolic compounds, such as the flavanone, liquiritigenin [5], we measured the TPC of our hairy root lines using the TPC assay [65,66] to determine if the observed increases in FRAP could be correlated with phenolic content. We found that TPC increased over time in hairy root cultures exposed to blue light or in the control (dark; Figure 3e). However, in samples exposed to red or red and blue light combined, TPC remained mostly constant (Figure 3e). Therefore, we did not find any overall correlation between FRAP and phenolic content across our different treatments and for the duration of the experiment. For red light and, in particular, for blue and red light combined, which showed a significantly higher rate of FRAP than the control (Figure 3d), the TPC thus cannot be said to explain any of the increase in FRAP (Figure 3f). For blue light and control treatments, the increased amount of FRAP might be partly attributed to TPC; however, we did not see a strong correlation between these variables for these two treatments.

Plants perceive light using photoreceptors, and different qualities of light trigger the activation of different types of photoreceptors. For instance, blue light activates cryptochromes and phototropins, while red light activates phytochromes [67,68]. These photoreceptors activate distinct signaling pathways that ultimately activate or repress transcriptional regulators controlling gene expression. Due to these different receptors, different wavelengths of light may trigger different responses, for instance, the accumulation of particular classes of specialized metabolites. For example, blue light is often associated with the upregulation flavonoids, a class of phenols [67–70]. Our results showing upregulation of phenol levels under blue light may therefore reflect the upregulation of the genes of the flavonoid biosynthetic pathway. Surprisingly, under control conditions, we saw an increase in TPC, showing that further light induction experiments must be carried out before we can draw firm conclusions about the effect of light quality on TPC in licorice. Overall, however, the weak correlation between total phenol content and FRAP suggests that light-induced changes in antioxidant capacity may involve other specialized metabolites besides phenols. Other metabolites that may contribute to antioxidant activity in *G. glabra* are triterpenoid saponins, such as glycyrrhizin, a major compound in *G. glabra* known for its strong antioxidant effects [71,72]. Additionally, polysaccharides found in *G. glabra* have also been reported to possess antioxidant properties [73,74]. This suggests that the light elicitation in our experiment may have induced the production in *G. glabra* hairy roots of other classes of specialized metabolites besides phenols, and future experiments will explore the contribution of these other metabolite classes in explaining the enhanced antioxidant capacity.

5. Conclusions

In this study, we found that breaking seed dormancy in licorice was best achieved using sulfuric acid as opposed to hot water, and we observed small but significant effects on the germination of licorice seeds on MS media compared to MS + B5 in some conditions. High rates of hairy root transformation were achieved with *R. rhizogenes* strain 1724, and cotyledons were more amenable to transformation, with the possibility of using injection of *R. rhizogenes* culture or wounding and short immersion periods in culture to successfully produce hairy roots. Using different qualities of light induction on a high-growing hairy root line, we found that the line accumulated fresh weight biomass under all conditions, but at higher rates under conditions containing blue light, suggesting that blue light

may specifically stimulate hairy root growth in licorice. We used FRAP as a measure of antioxidant capacity, and saw it increase in all treatments with the highest rate under red and blue light combined. Only in blue light and dark could the increase in FRAP be correlated with an increase in TPC, suggesting other antioxidant compounds that we did not measure in this study, like triterpenoid saponins, may be more important under red light. Our preliminary findings suggest that light induction holds promise for enhancing the concentration of key compounds in licorice, highlighting a distinct benefit of hairy root cultures that could be crucial for optimizing yield in this system.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11010062/s1. Figure S1. Typical examples of hairy root cultures from infection of *G. glabra* with *Rhizobium rhizogenes* strain 1724. Figure S2. Representative examples of *Glycyrrhiza glabra* L. (*G. glabra*) seedlings from different pretreatments on day 21 after germination. Figure S3. PCR verification of hairy root transformation in eight *G. glabra* lines. Figure S4. Morphology of hairy roots from two lines on day 20 of growth curve experiment. Figure S5. Correlation between phenolic content and Ferric Reducing Antioxidant Power (FRAP) in *G. glabra* hairy root line S exposed to different qualities of light. Table S1. *G. glabra* seed germination for different pretreatments, different concentrations of sodium hypochlorite and different media types. Table S2. List of oligonucleotide primers used in this study. Table S3. Results of Tukey's post hoc tests for all pair-wise combinations of pre-treatment and planting medium combinations. Table S4. Details of the eight hairy root lines selected for growth curve analysis.

Author Contributions: Conceptualization, N.A., L.S., L.T., V.N., H.K. and M.S.; methodology, N.A. and M.S.; investigation, N.A. and R.P.; resources, H.H., H.S., L.S. and M.S.; data curation, N.A., R.P. and A.J.; writing—original draft preparation, N.A. and A.J.; writing—review and editing, A.J., L.T., V.N., H.H., H.S. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge the University of Tehran and the Iran Vice Presidency of Science, Technology and Knowledge Based Economy [Project No. 1805040000] for their financial support. as well as the TU Wien Bibliothek for financial support through its Open Access Funding Program.

Data Availability Statement: The original data presented in the study is openly available in the repository TU Wien Research Data, at doi.org/10.48436/jczhc-srh29 (accessed on 17 December 2024).

Acknowledgments: The authors acknowledge Open Access Funding by TU Wien.

Conflicts of Interest: The authors declare no conflicts of interest.

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