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Carrot hairy roots (*Daucus carota* L.) characterisation and optimisation for high β-carotene extraction

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ABSTRACT Hairy roots are widely known as a biological system for the production of highly diverse biomolecules. β-carotene – a precursor for vitamin A – is known to be an anti-oxidant and anti-gastric cancer and protection agent against cardiovascular disease, heart disease and stroke. β-carotene has been chemically synthesised and consumed by humans. However, the chemical process often produces a by-product that may be harmful to human health. Therefore, this study established a protocol to induce hairy roots (HRs) from a Vietnamese carrot variety and produce natural β-carotene. The *Rhizobium rhizogenes* ATCC15834 harbouring Ri plasmid and a Vietnamese carrot variety were used as materials for genetic transformation and HR induction studies. The result showed that approximately 50 HR lines were obtained. Culture medium supplemented with 30 mg/L of sucrose that gave the highest biomass of HR was shown in carrot HR line 30, which had a doubling time of 6.5 days. The highest content of β-carotene extraction, at 128 mg/100g hairy roots, was achieved with a ratio volume (v/v) of 2-propanol and plant samples of 20:1, followed by two hours' incubation with 2-propanol at 60 °C. Our study reveals a highly efficient protocol for Vietnamese carrot hairy root establishment and multiplication. A very efficient protocol for β-carotene extraction from the hairy root was established to produce natural β-carotene that achieves the same β-carotene quantity as that produced by normal roots. This study provides new insight into the production of high-content and natural β-carotene for therapeutic application.

KEYWORDS β-carotene extraction; *Daucus carota* L.; hairy root; *Rhizobium rhizogenes*; sucrose

1. Introduction

Rhizobium rhizogenes is a Gram-negative pathogenic soil bacterium able to induce 'hairy root' disease at the infection site on dicotyledonous plants (Tepfer 2017). For the past decades, hairy roots (HR) have emerged as a promising expression system to produce valuable secondary metabolites and fully functional recombinant proteins (Guerineau et al. 2020). HR can also help elucidate biosynthesis pathways and physiological processes, assist molecular breeding and enhance phytoremediation (Georgiev et al. 2012). Their fast growth, genetic stability for a long time, low doubling time, ease of maintenance, and ability to synthesise an extensive range of chemicals offer additional advantages over undifferentiated plant cells (Parr 2017). Moreover, HR is highly branched and can be cultured indefinitely on simple hormone-free media containing only minerals, vitamins, and sugar (Parr 2017). Furthermore, they are also less sensitive to mechanical damage than other tissues, and biomass can be easily separated from the culture medium. These advantages make hairy roots a proper expression system for producing secondary metabolites (Gutierrez-Valdes et al. 2020; Baek et al. 2022).

A large number of plant species have been successfully transformed by *R. rhizogenes* to induce HR. Various plant tissues that can be used to induce hairy roots are tissue within the seeds, leaves, stems, hypocotyls, petioles, root tips, cotyledons, and protoplasts. However, different plant species require different plant materials for successful HR induction. *Arabidopsis thaliana*, *Panax vienamensis*, and *Salvia corrugata* Vahl. used hypocotyls, shoot explants and leaves explants, respectively, as materials for inducing HR (Ha et al. 2016; Mai et al. 2016; Kentsop et al. 2021)

Carrot (*Daucus carota* L.) is a popular vegetable food worldwide and is among the world's most economically essential vegetables. Thanks to its metabolites, such as a rich source of fat-soluble hydrocarbon or β-carotene - a precursor for vitamin A, it is also known as a medicinal plant (Rachamallu 2016). Carrots, which accumulate high levels of β-carotene in the root, are one of the best sources of β-carotene (Ellison et al. 2017). Recently, more attention has been paid to the role of beta-carotene as an

anti-oxidants (Kasperczyk et al. 2014), anti-gastric cancer (Chen et al. 2021) and other health benefits such as the protection against cardiovascular disease, heart disease and stroke (Huang et al. 2018).

Traditionally, secondary metabolites, including β-carotene, can be directly obtained by raw plant material extraction or chemical synthesis. Several studies showed the feasibility of the production of carotenoids from low-cost substrates, such as sugarcane bagasse and corn starch hydrolysate (Hernández-Almanza et al. 2014). However, these processes often have poor or inconsistent yields (Pedreño and Almagro 2020). Although carrot is a rich source of β-carotene and could be used to extract β-carotene, the production of carrot also depends on the season, pathogens and geographical conditions. The commercial β-carotene is mostly synthetic β-carotene, not natural and sometimes may contain the by-product from the synthesis process. More than 90% of the total β-carotene produced worldwide chemical synthesis (Yang and Guo 2014). The β-carotene extracted from the HR of carrot will have a natural origin, thus avoiding chemical contamination, and the production does not depend on the environmental conditions, leading to feasible improved production. Therefore, HR can be considered a promising system to produce β-carotene. The primary factor that affects the yields of metabolites produced by HR is nutrient components (Mehrotra et al. 2015). Even though HR can grow on a simple medium containing only minerals, vitamins, and sugar; the source of carbon, nitrogen, sugar concentration and other macro elements can be optimised to maximise metabolite production. Therefore, this study established an efficient protocol for inducing hairy roots of a Vietnamese carrot variety. The effects of different parameters: sucrose and silver nitrate concentration, for hairy root growth were investigated. Finally, a β-carotene extraction protocol was optimised to gain high production of β-carotene.

2. Materials and Methods

2.1. Preparation of carrot discs

Freshly harvested storage roots of *Daucus carota* L. were purchased from the Vinmart supermarket in Hanoi (Vietnam). Carrot discs were prepared as described by Danesh et al. (2006) with some modifications. Carrot storage roots were washed thoroughly with tap water for 5 min to remove all soil, and then they were dipped in ethanol 70% for one min and flamed three times. After that, carrots were surfaced sterilised with 2% sodium hypochlorite for 20 min. Finally, they were rinsed five times with sterile distilled water. Carrots were then peeled to remove the cortex, cut into 0.5 cm thick discs, and placed with the basal sides faced upwards on plates containing half-strength Murashige and Skoog (0.5× MS) basal medium supplemented with 3% (w/v) sucrose and Phytagel at 3.5 g/L.

2.2. Plant genetic transformation and hairy root growth

Daucus carota L. and *Rhizobium rhizogenes* strain ATCC15834 harbouring Ri plasmid, which contains root inducing genes (*rol*), genes for auxin synthesis, genes for opine synthesis located in T-DNA region, virulent genes (*vir genes*), and genes for opine catabolism, were used for genetic transformation (Mai et al. 2016). The protocol for inducing hairy carrot roots was adapted from Danesh et al. (2006) with some modifications (Danesh et al. 2006). Hairy root cultures of *D. carota* were established by infecting carrot discs with *R. rhizogenes* ATCC15834. One mL of bacteria suspension with OD_{600} 0.5 were inoculated on the basal faces of carrot discs, and the plates were placed in the dark at 26 °C for 3 d in 0.5× MS medium supplemented with 3% (w/v) sucrose and Phytagel at 3.5 g/L. After 3 d, carrot pieces were transferred to a new 0.5× MS medium supplemented with 300 mg/L cefotaxime and incubated in the dark at 26 °C for one month. Roots showing a hairy phenotype were placed on a new MS medium containing 3% (w/v) sucrose, 300 mg cefotaxime, and 3.5 g/L Phytagels at pH 5.8 (Mai et al. 2016). Roots were grown in 90-mm diameter petri dishes for maintenance. The sub-culture was performed every month. Cefotaxime was added in a culture medium for 3-4 times of sub-culture to eliminate all remained bacteria. Hairy roots were transferred in 250 mL Erlenmeyer flasks containing 100 mL of MS cultural medium to scale the hairy root production. The cultivation took place in the dark at 26 °C on a shaker orbiting at 110 rpm. The sub-culture was also performed every month.

2.3. Polymerase chain reaction (PCR)

Four putative HR lines: 51, 26, 30, 34 were chosen to perform the PCR reaction. The *Rhizobium rhizogenes* bacteria and mili-q water was used as the positive and negative control, respectively. The PCR reaction was performed in the total volume of 20 µL including 2 µL Dreamtaq buffer (10×) (Dreamtaq buffer; Thermo Fisher Scientific, Waltham, MA, USA), 200 nM deoxyribonucleotide triphosphates (dNTPs; Thermo Fisher Scientific, Waltham, MA, USA), 250 nM forward primer, 250 nM reverse primer, 100 ng DNA template, 0.75 units of DreamTaq DNA polymerase (Dreamtaq DNA polymerase; Thermo Fisher Scientific, Waltham, MA, USA) and H_2O up to 20 µL. The PCR was performed to amplify *rolC* and *virD2* gene sequences. The primers for detecting *rolC* gene were 5′-ATGGCTGAAGACGACCTGTG-3′ and 5′-TTAGCCGATTGCAAACTTGCAC-3’ (Yang and Choi 2000). The primers for detecting *virD2* were 5’-ATGCCCGATCGAGCTCAAG-3’ and 5’-GACCCAAACATCTCGGCTG-3’ (Ruslan et al. 2012).

After an initial denaturation and polymerase activation step of 12 min at 95 °C, 35 cycles were performed with each cycle consisting of denaturation at 95 °C for 30 sec, primer annealing at 56 °C for 30 sec, and elongation at 72 °C for 45 sec. An additional elongation was per-

formed at 72 °C for 2 min after the last cycle. PCR reactions were implemented in an Eppendorf versatile Mastercycler® nexus.

2.4. Effects of sucrose concentration and silver nitrate ($AgNO_3$) on biomass accumulation of carrot HR

Sucrose and $AgNO_3$ concentrations ranging from 20 to 60 mg/L and 1 to 5 mg/L respectively were used to investigate their effects on carrot HR line 30 growth, which showed the nicest phenotype. Carrot hairy root line 30 at 0.5 mg of weight was used as starting material. Different concentrations of sucrose or $AgNO_3$ were added to the liquid MS culture medium. The medium without $AgNO_3$ was used as a control in the $AgNO_3$ experiment. Cultures were kept in the dark at 26 °C on a shaker orbiting at 110 rpm. Root biomass was collected and weighed at 30 d of culture in the experiment with sucrose and $AgNO_3$ concentration, respectively. At least ten samples were used in each experiment. The experiments were replicated three times.

2.5. β-carotene quantification

Carrot HR line 30 and 34 were collected and used as plant materials for β-carotene extraction. The extraction protocol was carried out according to the method of Fikselová et al. (2008) with some modifications as follows (Fikselová et al. 2008). Harvested HR was freeze at -20 °C before extraction. Then, 3 g of fresh HR was ground into a fine powder using a pestle and mortar and added to a 2-propanol solution before incubating in the water bath at 60 °C. The volume of 2-propanol was investigated with the range from 5 mL to 40 mL to get the optimal volume needed for extraction. Then, the solution was shaken every 10 min. After one h or two h, a 5 mL sample from the upper layer was taken out and mixed with 4 mL petroleum ether. A volume of 1 mL water was added to separate the petroleum-ether-carotenoid phases and have the final volume of 10 mL. The β-carotene was taken out from the upper layer of the mixture. The absorbance was determined at the wavelength of 450 nm by a UV-1800 UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan).

$$\beta - carotene = \frac{A \times d \times v}{E \times w} \quad (1)$$

Where A: Absorbance, d: dilution, E: coefficient of absorbance (2592 for petroleum ether), w: weight of the sample (g), v: volume (mL).

2.6. Statistical test

All experiments were repeated three times. The differences between treatments were analysed using Student's t-test in R software v3.6.

3. Results and Discussion

3.1. Carrot hairy root induction

Our study induced hairy roots from a Vietnamese carrot variety for β-carotene production and extraction to obtain the native β-carotene for human treatment. In detail, the sterilised carrot was cut into a 0.5 cm thick disc (Figure 1a) and placed in petri dishes containing MS medium. The suspension of bacteria *R. rhizogenes* harbouring Ri plasmid was added to the surface of carrot pieces. After 15 days, the sign of hairy roots started to appear (Figure 1b). After one month, long hairy roots were obtained (Figure 1c). A very high rate, which was approximately 80% of carrot pieces having hairy roots. Approximately 50 HR lines were obtained. These long hairy roots were cut and grown in solid for maintaining (Figure 1d) and liquid MS medium for biomass production (Figure 1e, f) without any additional plant growth regulator. Hairy roots were thicker, had more branches, and had a higher growth rate compared to normal roots. Hairy roots grew vigorously and sometimes formed yellowish calli under dark conditions.

3.2. Molecular confirmation of hairy roots

The putative HR, decontaminated with bacteria using cefotaxime at 300 mg/L, were used to confirm whether they were the real HR. PCR confirmed the successfully transformed roots by determining the presence of a T-DNA sequence in their genome. A pair of primers were designed and synthesised to analyse the presence and absence of *rolC* and *virD2* genes. 0.8 kb *rolC* was obtained in four putative carrot HR by PCR using the genome DNA of carrot hairy roots as genetic material, confirming the success of the transformation. The absence of *virD2* gene in all four investigated lines showed that all four HR were free of bacteria (Figure 2).

3.3. Effects of sucrose concentration and silver nitrate ($AgNO_3$) on biomass accumulation of carrot HR

Sucrose is the most significant carbon source for HR cultures in many plant species (Thiruvengadam et al. 2014). This study used a range of sucrose concentrations from 20 to 60 mg/L to investigate their effects on HR growth. HR line 30, which had the most stable growth, was chosen as a material for studying factors affecting the growth of HR. The results showed that from 10 mg/L to 30 mg/L sucrose in liquid MS media, the increased sugar concentration increased the fresh biomass of HR. Sucrose at 30 mg/L gave the highest HR biomass production (Figure 3). However, the lower HR biomass was obtained when sucrose in the culture medium was increased from 40 mg/L to 60 mg/L. At these high sucrose concentrations, the HR also became fairly brown. The preference for sucrose concentration on the HR growth can be observed differently in different plant species. Sucrose at 30 mg/L gave the highest HR biomass and metabolite production in *Momordica charantia* (Thiruvengadam et al. 2014), *Cucumis anguria* L. (Yoon et al. 2015). HR of *Arabidopsis thaliana* preferred 50 mg/L of sucrose for biomass accumulation (Mai et al. 2016). Moreover, HR of *Talinum paniculatum* Gaertn preferred 60 mg/L for HR biomass production (Manuhara et al. 2015).

Silver ions in the form of nitrate, such as $AgNO_3$, significantly influence somatic embryogenesis and forma-

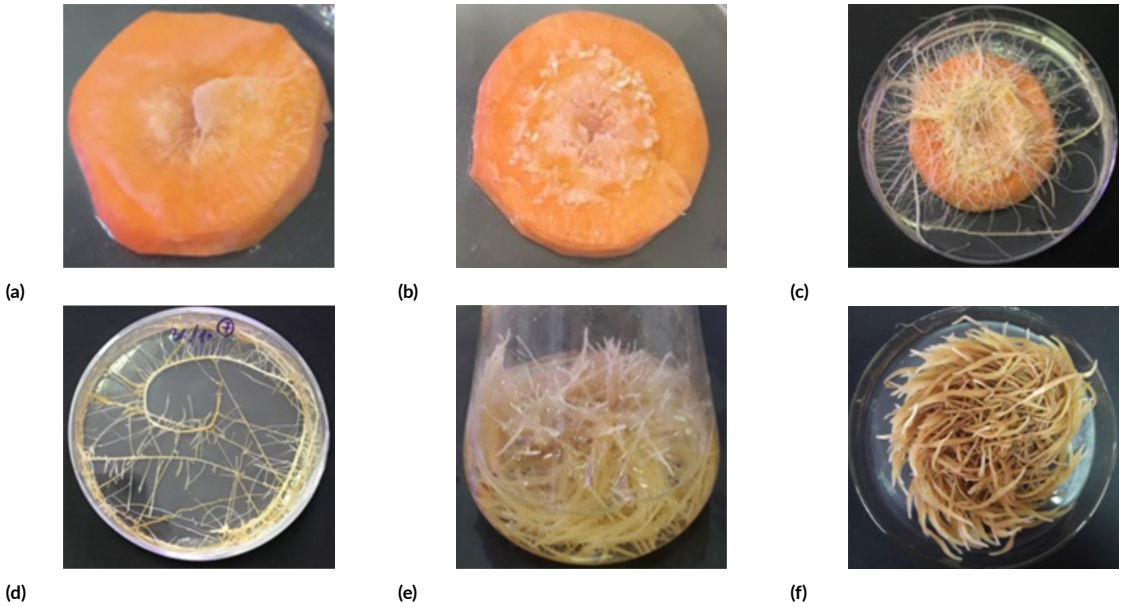

FIGURE 1 Carrot hairy root induction. Carrot piece (a), 15-day hairy root (b), one-month carrot with hairy root (c), hairy roots in solid MS medium (d) hairy root in liquid MS medium (e), and (f) hairy root was taken out from liquid culture medium.

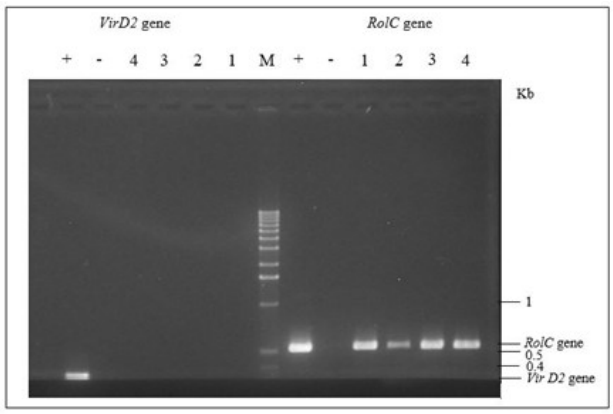

FIGURE 2 Amplification of *R. rhizogenes virD2* and *rolC* sequences. 1 to 4: DNA extracted from hairy root lines 51, 26, 30, 34, respectively; (+): from *R. rhizogenes* bacteria; (-): no DNA.

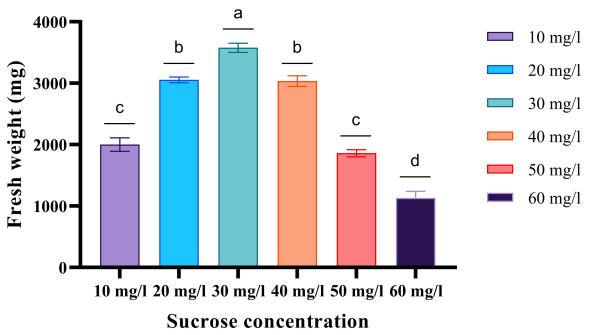

FIGURE 3 The dry weight of hairy root line 30 grown in liquid MS medium supplemented with different concentrations of sucrose after four weeks. The letters above the dataset indicate statistically difference. Relative values are represented as mean values ± standard deviation calculated from three replicates.

tions of root and shoot (Tahoori et al. 2018). Recently, $AgNO_3$ has been used widely in tissue culture since it inhibits ethylene action (Mahendran et al. 2018). In this study, the $AgNO_3$ with a range of concentrations from 1 mg/L to 5 mg/L were used to investigate the effect of $AgNO_3$ on carrot HR growth. Surprisingly, $AgNO_3$ inhibited the carrot HR growth (Figure 4). The degree of growth inhibition increased gradually with the increase in the concentration of $AgNO_3$. When $AgNO_3$ was used at low concentrations (1 and 2 mg/L), there was no significant difference in the growth inhibition effect between them ($p > 0.05$); however, even at low concentrations (1 and 2 mg/L), $AgNO_3$ strongly inhibited the hairy root growth compared to the medium without $AgNO_3$. At the concentration of 3, 4, and 5 mg/L, the growth of hairy root was reduced compared to the medium containing $AgNO_3$ at 1 or 2 mg/L, but there was no significant difference in the inhibition effect of medium containing 3, 4, or 5 mg/L on the carrot HR growth ($p > 0.05$). Several reports indicated that in some species, $AgNO_3$ could enhanced multiple shoot regeneration (Geetha et al. 2016), and increase the shoot quality (Cardoso 2019) while in other species, $AgNO_3$ inhibited shoot growth of *Brassica* seedlings (Vishwakarma et al. 2017). Therefore, these results indicated that the effect of $AgNO_3$ on shoot regeneration was species-specific. The effect of $AgNO_3$ on root formation and development was also widely investigated. The usage of $AgNO_3$ in the culture medium of liquorice (*Glycyrrhiza glabra* L.) induced shoot and root growth (Tahoori et al. 2018). The combination of 0.3 mg/L $AgNO_3$ and 0.1 mg/L BA gave the best rooting in *Hypericum perforatum* plants (Syahid and Wahyuni 2019).

The effect of $AgNO_3$ on hairy root growth was fairly

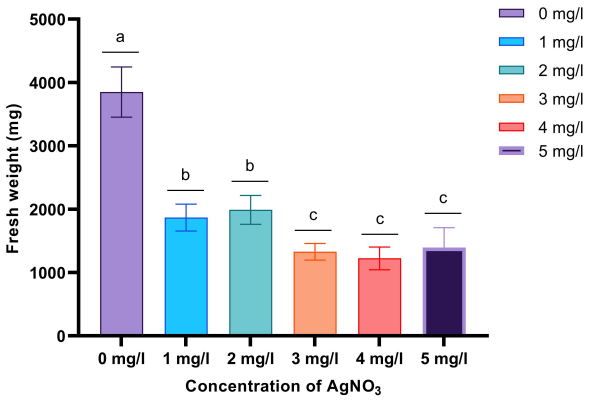

FIGURE 4 Effect of $AgNO_3$ on the growth of carrot hairy root line 30 after 45 d grown in liquid MS culture medium. Relative values are represented as mean values ± standard deviation calculated from three replicates. Letters above dataset indicate statistically difference.

studied. In our study, $AgNO_3$ inhibited carrot HR growth. Similar results were also obtained in hairy root cultures of *Cucumis anguria* where $AgNO_3$ at 1 and 2 mg/L decreased the HR biomass (Chung et al. 2018). Meanwhile, the application of 1.5 mg/L $AgNO_3$ in the HR culture medium of sesame increased to 109.6% biomass compared to untreated roots (Chun et al. 2007). These results also demonstrated that the effect of $AgNO_3$ on HR growth was genotype-dependent.

3.4. Growth kinetics

The hairy root line 30, which has fast growth, light yellow colour, and excellent sharpness, was chosen to study growth kinetic in MS medium supplemented with 30 mg/L sucrose (Figure 5). The results showed that hairy root line 30 had 10 first days, which showed the slow growth and could be considered the lag phase, and then it had rapid growth from day 10 to day 25, which could be considered the exponential phase. Hairy roots experienced a stationary phase from day 25 to day 40 due to the limitation of nutrients. The supplementation of nutrients could restart the growth of hairy roots.

The double-time of this HR line was about 6.5 days; it means that HR line 30 needs about 6.5 days to double its biomass. This result is different from other hairy roots coming from different plant species, some species have faster growth, and some have slower growth compared to the growth of Vietnamese carrot hairy roots. *Fagopyrum tataricum* hairy roots exhibited a rapid linear growth period between days 12-24, after 9 days of a slow growth phase (Zhao et al. 2014). Cucurbits plants had the first sixth day of the lag period, the next 21 days of the exponential phase, and 15–25 days of the stationary phase (Rekha and Thiruvengadam 2017). The doubling time of *Vitis vinifera* cv Pinot hairy roots was 10.9 days (Tisserant et al. 2016).

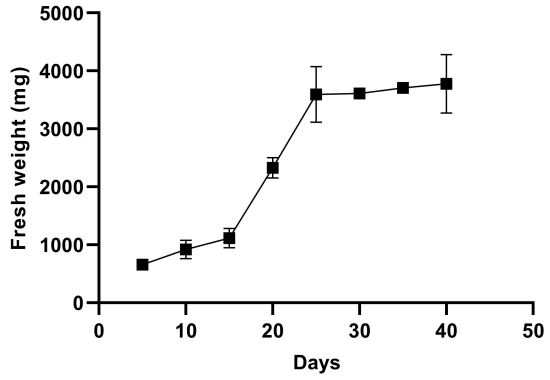

FIGURE 5 Growth kinetic of hairy root line 30 in MS medium supplemented with 30 mg/L sucrose over the period of 40 days. Relative values are represented as mean values ± standard deviation calculated from three replicates.

3.5. β–Carotene quantification

The β-carotene concentration in non-transformed roots and two transformed roots, HR lines 30 and 34, were quantified using the volume ratio (v/v) between 2-propanol and plant samples was 4:1 suggested by Fikselová et al. (2008). The β-carotene concentration in three samples, which consists of the non-transformed roots and two HR lines (lines 34 and 30) were quantified and shown in Figure 6. The results showed that the concentration of β-carotene after one h extraction in 2-propanol in non-transformed roots (2 mg/100 g root) was approximately 3 and 9 times higher than that in HR line 30 (0.67 mg/100 g hairy root) and line 34 (0.24 mg/100 g hairy root), respectively. The results also showed that HR line 30 produced β-carotene approximately three times higher than HR line 34 (Figure 6). The quantification was also performed after 2 h of extraction in 2-propanol. However, the results of HR lines 34 and 30 were not increased. These results give us two hypotheses. First, the extraction time was not enough for 2-propanol to extract total β-carotene. Second, the ratio volume between 2-propanol and plant samples was also not optimal for the extraction process. Therefore, we decided to optimise the time for extraction and the volume ratio between 2-propanol and plant samples.

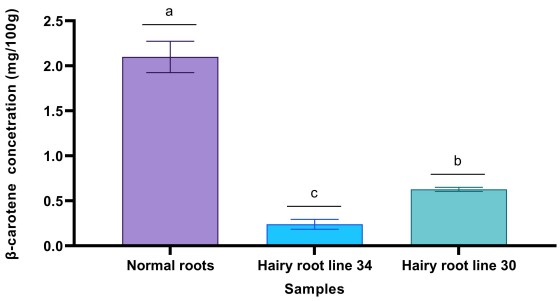

FIGURE 6 β-carotene concentration (mg/100 g) at the ratio of 2-propanol and plant samples was 4:1. Letters above dataset indicate statistically difference. Relative values are represented as mean values ± standard deviation calculated from three replicates.

Overall, our results showed that much higher β-carotene content was obtained in the conditions of two hours of incubation with 2-propanol and the increase of volume ratio (v/v) between 2-propanol and plant samples than those obtained in the conditions of 1 h of incubation with 2-propanol and the 4:1 volume ratio (v/v) of 2-propanol and plant samples (Figure 7). In a series of volume ratios (v/v) between 2-propanol and plant samples, after 1 h of incubation, the 15:1 volume ratio gave the highest β-carotene content, where it reached approximately 44 mg/100 g HR sample. After 2 h of incubation, the 20:1 volume ratio gave the highest value of β-carotene content, where it reached approximately 128 mg/100 g HR sample. This β-carotene content is similar to β-carotene content obtained from the normal roots, ranging from 60-120 mg/100 g (Kesharlal et al. 2001). Other volume ratios (v/v) of 2-propanol and plant samples extracted low β-carotene contents. Our modified extraction conditions for β-carotene obtained much higher β-carotene content than the results obtained by Fikselová et al. (2008), who obtained the maximum β-carotene extraction content at approximately 6.45 mg/100g HR after 5 h of incubation with 2-propanol and with a 4:1 volume ratio (v/v) of 2-propanol and plant samples (Fikselová et al. 2008). Our results also showed that when the volume ratio (v/v) of 2-propanol and plant samples increased up to 20:1, the extraction process needed only 2 h of incubation with 2-propanol to get maximum β-carotene extraction content which will save more time than 5 h of incubation as Fikselová et al. (2008) suggested (Fikselová et al. 2008). A higher incubation time than 2 h with 2-propanol ether did not give higher β-carotene extraction content. Our extraction protocol also gave much higher β-carotene content than the results obtained by Mohammed and Al-Mallah (2013) (approximately 1.9 mg/100g) using stem callus and taproots of regenerated carrot (Mohammed and Al-Mallah 2013) and by Fikselová et al. (2008) using roots of carrot (approximately 6.45 mg/100g HR) (Fikselová et al. 2008)

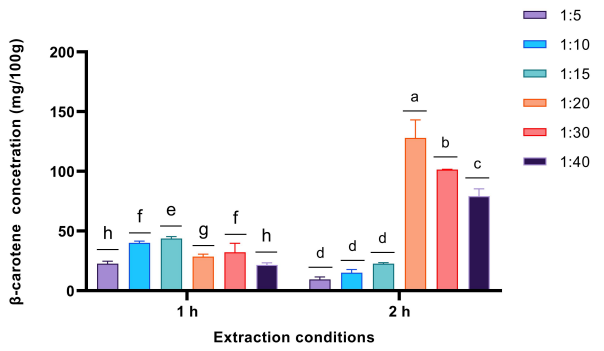

FIGURE 7 β-carotene concentration (mg/100 g) at different ratios of hairy root line 30 and 2-propanol after 1 and 2 h of incubation with petroleum ether at 60 °C. Letters above dataset indicate statistically difference. Relative values are represented as mean values ± standard deviation calculated from three replicates.

4. Conclusions

Our study showed that *Rhizobium rhizogenes* ATCC15834 was able to induce HR in a Vietnamese carrot variety. Sucrose at 30 mg/L was the most suitable concentration for HR growth, whereas $AgNO_3$ inhibited HR growth even at 1 mg/L. The volume ratio (v/v) of 20:1 between 2-propanol and root samples and 2 h of incubation with 2-propanol at 60 °C gave the highest β-carotene extraction content among tested conditions. Further studies about the safety and effectiveness of β-carotene produced by carrot HR, and the scaling-up conditions in big bioreactors for industry processes should be investigated to produce a large amount of safe β-carotene for human consumption.

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Authors' contributions

NTPM conceived ideas and designed the research plans. NTPM, VATL conducted all the experiments. NTPM analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript.

Competing interests

All authors declare that there are not any conflicts of interest.

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