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Effect of culture media and auxin on growth and betalain accumulation in the hairy root cultures of red chard (*Beta vulgaris* L. var. *cicla*)

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ABSTRACT

This study analyzes the response of biomass production and betalain biosynthesis in hairy root cultures of red Swiss chard (*Beta vulgaris* L. var. *cicla*). Among the media tested, half-strength Schenk and Hildebrandt (1/2 SH) medium yielded the highest dry weight (413.3±42 mg/flask DW), whereas full SH medium appeared to be more consistent (390.0±10 mg/flask DW). Similar trends were observed in B5 and Murashige and Skoog (MS) media, where halving nutrient concentrations had minimal impact on biomass yield but significantly enhanced betalain accumulation. In addition, in this study, we further assessed the influence of different concentrations and various types of auxins on root and metabolite production. The result showed that 0.1 mg/L naphthalene acetic acid (NAA) was the optimum concentration for biomass production (443.3±12 mg/flask DW), while 0.5 mg/L indole-3-acetic acid (IAA) was the best for the production of betacyanin (3.028 mg/g), betaxanthin (1.938 mg/g DW), and total betalain content (4.966 mg/g DW). The findings highlight that reduced nutrient concentrations, particularly in SH medium, and precise auxin optimization can enhance both biomass and betalin production. These results provide valuable insights into optimizing hairy root culture systems for commercial applications in enhancing the secondary metabolite production in *B. vulgaris*.

KEYWORDS: *Beta vulgaris* L. var. *cicla*, Red chard, Media, Hormones, Betalain

INTRODUCTION

In recent years, considerable phytochemicals in natural food have attracted attention as they have a variety of physiological activity and significant potential. Additionally, plant-derived drugs containing plant pigments became crucial to the diagnosis, treatment, and prevention of diseases. As quality of life improves, interest in and demand for good-looking foods, such as originally colored foods, are increasing (Gaona-Ruiz *et al.*, 2024). Carotenoids and flavonoids, representative plant-derived colorants, are widely used as food additives because of their color features and antioxidant capacity. Among the major plant pigments, betalain's abundance is far less, so it does not receive that much attention. However, research on betalain continues to rise according to many consumers' preferences and the embrace of non-chemically treated pigments. Betalain was repeatedly described as having several biological activities, including free radical-scavenging, inhibiting of DNA damage (Esatbeyoglu *et al.*, 2014), anticancer, antiviral, (Georgiev *et al.*, 2010), and hepatic protective activity (da Silva *et al.*, 2019).

Betalain is a representative hydro-soluble pigment which is present in vacuoles (Jain & Gould, 2015). The indole-derived nitrogenated betalain has a variety of colors depending on the amount of red-violet betacyanin and yellow betaxanthin pigment content. Although betalain has a phenotypically

similar red hue to anthocyanin, they have such an exclusive relationship that the same species or other species in the same family did not detect the two pigments together. In beetroots (*Beta vulgaris* L.), consisting of betanin, a subset of betacyanin, ranging up to 95% of the main colorant and a great electron donor (Gliszczynska-Swiglo *et al.*, 2006). While betaxanthin exists at a lower content than betacyanin, it has comparable antioxidant capacity and is relatively stable in external factors (Skalicky *et al.*, 2020).

Phylogenetically, betalain is not widely dispersed, and red Swiss chard (*B. vulgaris* L.) is a member of the family Chenopodioideae, which is the only family that produces betalain (Sawicki *et al.*, 2016). Betalain was established and coined from red beetroot, which has the same scientific name as red Swiss chard. Red Swiss chard is mainly cultivated in the United States, Mediterranean countries, and northern India to make fresh salads, pasta, Korean soups, and seasoned Swiss chard (Trifunovic *et al.*, 2015). Swiss Chard has traditionally been used for advancing cognitive function to treat Alzheimer 's-related symptoms (Szymański *et al.*, 2023) and for hypoglycemia (Gao *et al.*, 2009). Thus, the aim is to identify the biomass and compounds obtained depending on the culture conditions.

Of the numerous benefits of unrestricted propagation, stabilizing biochemical properties, and keeping the same genetic composition, the most notable advantage of hairy roots in this study is the proliferation of secondary metabolites (Park *et al.*, 2016). To increase the betalain in red Swiss chard, diverse media, and an auxin environment were tested for culturing hairy roots. Having Ri-plasmid, soil-living *Agrobacterium rhizogenes* mediate transformation by inserting root-inducing oncogenes into the plant genome. Due to the benefits of these amazing hairy roots, this technology has been used a lot industrially, such as in the field of studying pharmaceuticals, food additives, and cosmetics (Lee *et al.*, 2016). It is especially meritorious to get the natural compounds produced in a small amount.

The various plants grow well in various artificially made mediums. Selecting appropriate facilitating factors like media and phytohormones is very influential in the rooting process. A basic carbohydrate supply is present in plant growth media in order to sustain a carbon source that comes from photosynthesis. Further, inorganic elements and trace elements are provided by tissue culture media (George *et al.*, 2008). Hairy roots can multiply and uptake nutrients with the aid of liquid B5 (Gamborg *et al.*, 1968), MS (Murashige & Skoog, 1962), and SH (Schenk & Hildebrandt, 1972) media.

Auxin is a fundamental growth regulator that facilitates both cell division and cell expansion. The most renowned, naturally occurring auxins in many plants are indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Naphthalic Acetic Acid (NAA) is also generally used in plant tissue culture and root formation. IAA and NAA exert a more similar role in root formation than IBA; these three hormones stimulate root induction in even lateral and adventitious roots (Schlicht *et al.*, 2013). They can develop roots from different parts of the plants cut for vegetative propagation. (Shekhawat & Manokari, 2016) Auxins are, therefore, essential for discovering the index of root growth so long as they have adequate levels. This study aimed to optimize suitable culture medium and appropriate auxin concentrations to enhance the betalain content in *B. vulgaris* L. var. *cicla*.

MATERIAL AND METHOD

Plant Material

Red chard (*Beta vulgaris* L. var. *cicla*) red seeds were obtained from a seed company. Before sowing, the seeds were surface sterilized by immersing them in 70% (v/v) ethanol solution for 30 s and then immersed in 2% sodium hypochlorite for 10 min. The clean seeds were rinsed with sterile water five times. The seeds were placed on Petri dishes containing Murashige and Skoog (MS) medium (30 g/L sucrose, and 0.8% (w/v) agar, pH 5.7, and no plant growth regulators were added for germination.

Establishment of Hairy Roots

The wounded tissue was prepared with young leaves from seedlings of 10-day-old red chard. The young leaves were cut into small pieces using a scalpel in the fresh liquid suspension of A. *rhizogenes* (strain R1000) and then incubated for 20 min. After inoculation, seedlings were dried on sterile paper and incubated on MS medium lacking antibiotics for two days in darkness at 25 °C. Following 2 days of co-cultivation, infected leaves were washed several times with sterile water, blotted dry on sterile paper, and then placed on half-strength MS (1/2 MS) solid medium, supplemented with 250 mg/L cefotaxime. The cultures were maintained under dark conditions for 2 to 3 weeks. Hairy roots that developed at the wounded sites were excised from the leaves and further cultured under the same conditions. A single fast-growing clone was selected for subsequent experimental use and subjected to further culture.

Treatment of Culture Medium and Auxins

Hairy root cultures were maintained in liquid media comprising half-strength and full-strength B5, MS, and SH formulations. To ensure uniformity, hairy roots were weighed to 3 g (fresh weight) and transferred into 30 mL of each culture medium in sterile flasks. Following a cultivation period of 4 weeks, treated samples from each medium were collected, rapidly frozen in liquid nitrogen, and subsequently prepared for betalain analysis. Samples were ground to a fine powder with a pestle and freeze-dried at -80 °C for 72 h.

To understand the effect of different types and concentrations of auxin on hairy root cultures, half-strength MS medium was used as basal medium, and additionally different auxins (IAA, IBA, and NAA) concentrations 0.1, 0.5, and 1 mg/L were supplemented. Three grams of fresh weight hairy roots were placed in each liquid medium supplemented with an auxin. After 4 weeks, the culture was harvested and frozen in liquid nitrogen, subsequently freeze-dried at -80 °C for 72 h, followed

by finely grounding with a pestle to carry out betalain analysis. Each experiment is done in triplicate.

Betalain Content

Spectrophotometric analysis was employed to quantify the betalain content in the samples according to the methods described by Cano *et al.* (2017). From each analysis, 1ml of extract in corresponding 100 mg of lyophilized red Swiss chard hairy root sample powder contained and dissolved in 2 mL of distilled water. The extract was sonicated for 10 min and centrifuged at 12 000 rpm for 15 min at 4 °C, and the supernatant was carefully removed, and the 0.2 mL of supernatant was diluted 10 times by adding 1.8 mL of distilled water. A UV-Vis spectrophotometer was used to determine the betacyanin and betaxanthin contents at the absorbance of 535 nm and 483 nm, respectively. The formula was used for calculations by the following.

$$BC\,or\,BX\left(\frac{mg}{g}\right) = A.DF.MW.V\ /\varepsilon.L.S$$

All data were measured as the means of three replicates. Three subsamples were randomly generated from each treated sample of red chard, and each replicate sample was separately subjected to extraction.

Statistical Analysis

All data were measured as the means of three replicates. Three subsamples were randomly generated from each treated sample of red chard, and each replicate sample was separately subjected to extraction. Data were analyzed by using SAS Software (release 9.2; SAS Institute Inc., Cary, NC, USA), and the means significance was determined by using Duncan's multiple range test ($p<0.05$).

RESULTS

Among the media tested, the highest mean biomass yield was observed in 1/2 SH medium, with a value of 413.3±42 mg/flask dry weight (DW) (Figure 1). This result indicates that the reduced concentration of SH medium significantly enhanced root growth, although it also exhibited the highest variability, as indicated by the standard deviation (SD) of 42 mg/flask DW. In comparison, the full-strength SH medium produced a lower mean biomass of 390.0±10 mg/flask DW but demonstrated the least variability among all tested media, as reflected by its minimal SD of 10 mg/flask DW. These findings suggest that while the 1/2 SH medium supports superior growth, the full-strength SH medium provides more consistent results.

For both the half and full-strength B5 media, the biomass production was similar, with mean values for growth of 381.5±36 mg/flask and 386.7±21 mg/flask DW for 1/2 B5 and full-strength B5 media. The full-strength B5 medium shows a slightly lower SD than the half-strength B5, suggesting that it supports a better and more stable biomass production (but only marginally). Likewise, the biomass obtained on MS media was 379.9±20 mg/flask DW for 1/2 MS and 383.3±35 mg/flask DW for full-strength MS, whereas the SD of full-strength is slightly higher than that of the ½ strength MS.

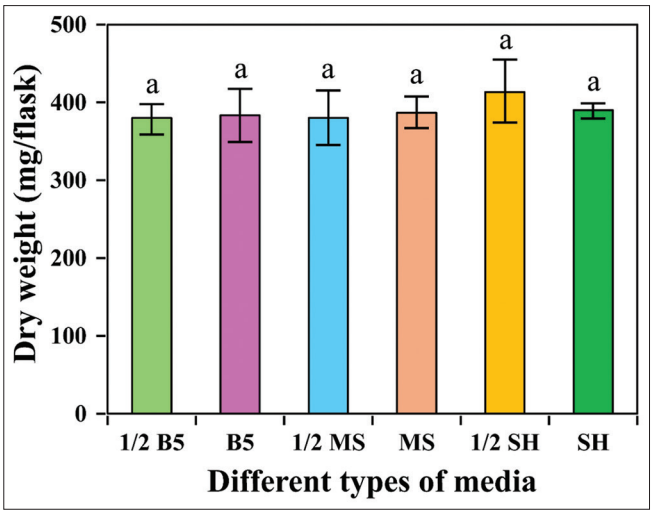

Figure 1: The effects of different media on the dry weight of red Swiss Chard hairy roots. Values are means from three independent replicate results±standard deviation (SD). The letters on the bar represent the results of the analysis of variance ($p<0.05$, ANOVA, DMRT; Duncan's multiple range test)

Among the tested conditions, the highest biomass yield was observed with NAA at 0.1 mg/L (443.3±12 mg/flask DW) (Figure 2). Dark treatment showed a higher dry weight whereas it showed lower SD, which means that although replicates were similar, this treatment was by far the best dry weight producer. Similarly, the concentration of 0.5 mg/L of IBA also produced high biomass yields as 436.6±47 mg/flask DW. Nonetheless, the variability associated between these treatments was that the IBA at 0.1 mg/L showed greater SD than that of NAA at 0.1 mg/L. In contrast, treatments with IAA displayed relatively lower biomass yields. IBA treatments showed a concentration-dependent trend in biomass production. Both concentrations of IAA, 0.1 mg/L and 1 mg/L, yielded a mean biomass of 413.3±20 and 413.3±12 mg/flask DW, respectively, while the 0.5 mg/L concentration of IAA produced a slightly lower biomass (403.3±20 mg/flask DW). These results suggest that while IAA supports moderate biomass production, its performance is less effective compared to NAA and IBA. As the concentration of IBA increased, the biomass concentration also increased to 406.6±41, 426.6±25 and 436.6±47 mg/flask DW at 0.1, 0.5, and 1 mg/L IBA concentration, respectively. Lastly, 1 mg/L of NAA as opposed to its lower concentrations, produced a low biomass yield of only 393.3±5 mg/flask DW. While the SD of 5 indicates high consistency, the reduced biomass suggests a potential inhibitory effect at higher NAA concentrations.

Among treatments, the highest betacyanin content (2.35 mg/g DW) was shown in the 1/2 SH medium, whereas the betacyanin contents in treatments with SH (2.26 mg/g DW) and 1/2 MS (2.25 mg/g DW) were not significantly different from this

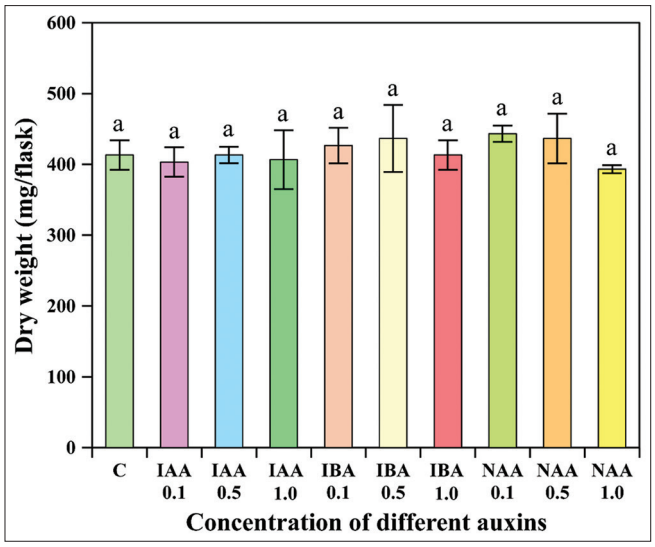

Figure 2: The effects of different concentrations of auxins on the dry weight of red Swiss Chard hairy roots. Values are means from three independent replicate results±standard deviation (SD). The letters on the bar represent the results of the analysis of variance ($p<0.05$, ANOVA, DMRT; Duncan's multiple range test). C=Control

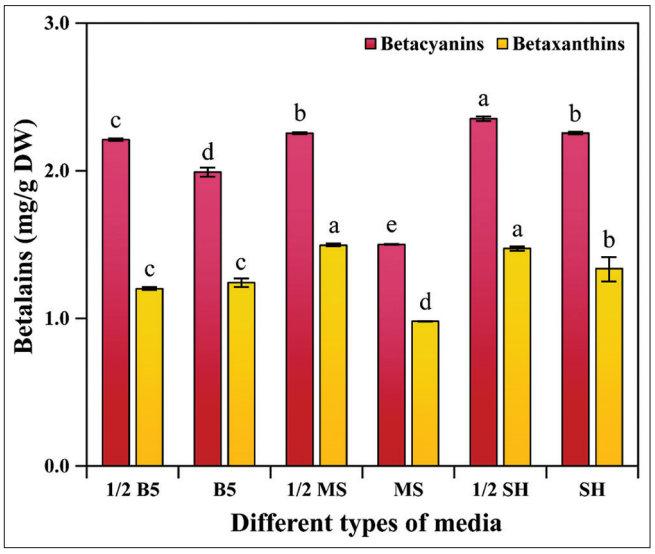

Figure 3: The graph represents the effects of the media on the betacyanin and betaxanthin contents in the red Swiss chard sample. Values are means from three independent replicate results±standard deviation (SD). The letters on the bar represent the results of the analysis of variance ($p<0.05$, ANOVA, DMRT; Duncan's multiple range test)

treatment (Figure 3). However, the MS medium produced the lowest betacyanin content (1.50 mg/g DW), which was significantly lower than in all other treatments. Betacyanin was made at intermediate levels in 1/2 B5 (2.21 mg/g DW) and B5 (1.99 mg/g DW). In a similar manner, the highest betaxanthin content occurred in 1/2 MS medium (1.5 mg/g DW) followed by the 1/2 SH (1.47 mg/g DW), and moderate levels were found in SH (1.34 mg/g DW) and B5 (1.24 mg/g DW). The amount of betaxanthin was low in the MS medium (0.98 mg/g DW), which suggests that synthesis is reduced under these conditions. The same trend was observed for total betalain content, in which the total amount (3.85 mg/g DW) was the highest in 1/2 SH, whereas 1/2 MS (3.75 mg/g DW) and SH (3.59 mg/g DW did not show any significant difference (data not shown) and MS medium (2.481 mg/g DW) showed the lowest total betalain content ($p<0.05$). Total betalain was not high in 1/2 B5 (3.41 mg/g DW), and B5 (3.23 mg/g DW) media. These results indicate that lower nutrient concentrations, like that of 1/2 SH and 1/2 MS, improve the production of betalains compared to those of full-strength media, but the MS medium works consistently as the worst in terms of betalain levels, indicating non-optimal conditions for its biosynthesis.

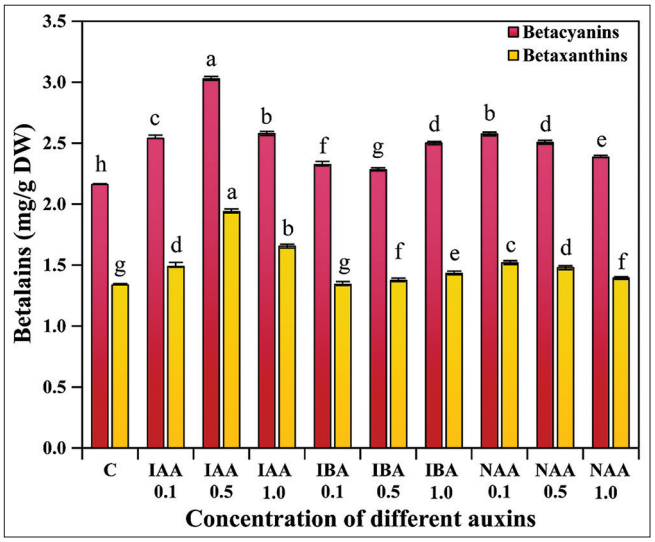

Figure 4: The graph represents the effects of auxin on the betacyanin and betaxanthin contents in the red Swiss chard sample. Values are means from three independent replicate results±standard deviation (SD). The letters on the bar represent the results of the analysis of variance ($p<0.05$, ANOVA, DMRT; Duncan's multiple range test). C=Control

Under different auxin treatments, the analysis of betalain content in hairy roots revealed that IAA (especially at 0.5 mg/L) was the most effective with respect to promoting betalain synthesis (Figure 4). Maximum betacyanin reached IAA 0.5 (3.03 mg/g DW), significantly higher than IAA 1 (2.58 mg/g DW) and IAA 0.1 (2.54 mg/g DW), which indicates that the IAA was the most effective among other auxins. On the other hand, IBA treatments showed the lowest content of betacyanin (2.28 mg/g DW (IBA0.5) to 2.50 mg/g DW (IBA 1)), while moderate betacyanin contents were obtained from NAA treatments, which reached 2.38 mg/g DW (NAA 0.1) to 2.51 mg/g DW (NAA 0.5). In the same way, maximum betaxanthin production was obtained with IAA 0.5 mg/g (1.94 mg/g DW) followed by IAA 1(1.66 mg/g DW) and IAA 0.1 (1.49 mg/g DW). Moreover, at NAA 0.1 (1.52 mg/g DW) and NAA 0.5 (1.48 mg/g DW), whereas in IBA 0.1-1.0 treatment, the betaxanthin content ranges from 1.34-1.43 mg/g DW. From the overall results, the control condition showed the lowest betacyanin (2.16 mg/g DW), betaxanthin (1.34 mg/g DW), and total betalain (3.50 mg/g DW) contents, which showed that auxin treatment has a positive effect on the

betalain accumulation. The total betalain showed a similar trend in which maximum accumulation was observed with IAA 0.5 (4.97 mg/g DW) followed by IAA 1 (4.23 mg/g DW) and NAA 0.1 (4.09 mg/g DW). Among the various auxin-treated hair root cultures, slight variation was observed in the various auxin treatments. In detail, NAA treatments displayed intermediate levels (4.09-3.79 mg/g DW), while IBA treatments showed slightly lower total betalain content (3.67-3.93 mg/g DW). Overall, the findings highlight that IAA, especially at 0.5 mg/L, was the most effective auxin for enhancing betalain accumulation in red Swiss chard hairy roots.

DISCUSSION

The current study revealed that the type and concentration of media significantly influence biomass production in red Swiss chard hairy root cultures. Among the tested media, 1/2 SH medium supported the highest biomass yield (413.3±42 mg/flask DW), while full-strength SH medium produced slightly lower biomass (390.0±10 mg/flask DW) but demonstrated greater consistency. These results align with studies in *Scutellaria baicalensis* hairy root cultures, where half-strength media enhanced secondary metabolite production while maintaining robust growth rates (Kim *et al.*, 2012). Similarly, half- and full-strength B5 and MS media yielded comparable biomass values, with no significant difference between the two nutrient concentrations. This is consistent with findings in *Nasturtium officinale*, where SH medium supported the highest growth, followed by B5 and MS media (Park *et al.*, 2019). The uniformity of biomass yields across media types suggests that nutrient availability in B5 and MS media was sufficient to sustain growth but not optimized for maximal production.

Auxin treatments had a marked effect on biomass production. NAA at 0.1 mg/L resulted in the highest biomass (443.3±12 mg/flask DW), corroborating previous studies where NAA enhanced growth and secondary metabolite accumulation in *Rubia akane* and *Astragalus membranaceus* hairy root cultures (Park & Lee, 2009; Park *et al.*, 2018). However, increasing the NAA concentration to 1 mg/L led to reduced biomass (393.3±5 mg/flask DW), likely due to auxin-induced feedback inhibition, a phenomenon observed in *Eruca sativa* (Park *et al.*, 2021).

IBA demonstrated a concentration-dependent effect, with 1 mg/L yielding the highest biomass (436.6±47 mg/flask DW). However, this treatment also exhibited the greatest variability, suggesting that higher IBA concentrations may induce inconsistent cellular responses, similar to findings in *S. baicalensis* (Kim *et al.*, 2012). IAA treatments produced moderate biomass yields, with the highest at 0.1 mg/L (413.3±20 mg/flask). While IAA was less effective than NAA or IBA, its consistent performance across concentrations aligns with reports in *Brassica oleracea* var. *acephala* (Lee *et al.*, 2016).

Betalain content was significantly influenced by the choice of medium. The highest betacyanin (2.35 mg/g DW) and total betalain content (3.825 mg/g DW) were observed in 1/2 SH medium, suggesting that reduced nutrient concentrations enhance secondary metabolite biosynthesis. This aligns with findings in *A. membranaceus*, where half-strength SH medium supported higher astragaloside production compared to full-strength media (Park *et al.*, 2018). Conversely, MS medium yielded the lowest betalain content, reflecting suboptimal conditions for betalain biosynthesis, as similarly noted in glucosinolate studies on *N. officinale* (Park *et al.*, 2019).

IAA at 0.5 mg/L was the most effective auxin for betalain biosynthesis, yielding the highest betacyanin (3.03 mg/g DW) and total betalain (4.97 mg/g DW). This supports earlier findings that IAA enhances secondary metabolite production by upregulating biosynthetic pathways, as seen in *S. baicalensis* (Kim *et al.*, 2012) and *E. sativa* (Park *et al.*, 2021). NAA and IBA treatments showed intermediate efficacy in promoting betalain accumulation. Moderate concentrations of NAA (0.1 mg/L) and IBA (1 mg/L) yielded notable increases in betacyanin content, similar to patterns observed for anthraquinone production in *R. akane* (Park & Lee, 2009). These results suggest that auxins play a critical role in enhancing secondary metabolite biosynthesis but require precise optimization to avoid inhibitory effects. This study highlights the significant impact of media composition and auxin supplementation on biomass yield and betalain biosynthesis in red Swiss chard hairy roots. The superior performance of 1/2 SH medium and the efficacy of IAA at 0.5 mg/L underscore the potential for tailored cultural conditions to maximize production. These findings provide a foundation for optimizing hairy root culture systems for both biomass and secondary metabolite production.

CONCLUSIONS

This study highlights the impact of media composition and auxin supplementation on biomass production and betalain biosynthesis in red Swiss chard hairy root cultures. The 1/2 SH medium was most effective for biomass and betalain production, while NAA at 0.1 mg/L yielded the highest biomass, and IAA at 0.5 mg/L enhanced betalain content. These findings support the use of optimized media and auxin treatments to improve the production of valuable metabolites, offering scalable biotechnological applications for various industries. Further research should explore the molecular basis of these effects.

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