An investigation of elicitation and precursor feeding to increased imperatorin synthesis in hairy root culture of *Urena lobata* L.

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Abstract

Imperatorin is a furocoumarin found in the natural roots of Urena lobata L., which has valuable medicinal properties. In this study, precursors (L-phenylalanine, L-tyrosine and umbelliferone), biotic elicitors (chitosan, yeast extract) and abiotic elicitors (salicylic acid, copper (II) sulfate) were applied to the hairy roots of U. lobata to enhance the imperatorin synthesis in hope of effectively creating a proactive source of materials for pharmaceutical production. The results revealed that the precursor was ineffective at increasing imperatorin accumulation. Meanwhile, all elicitors significantly revealed an outstanding result, which enhanced the imperatorin accumulation when compared to the non-feeding control.

The biotic elicitors group significantly enhanced imperatorin accumulation in hairy roots compared with the abiotic elicitors group. After 4 days of elicitation, the increase in imperatorin accumulation was prominent, with 498.29 µg in the 10 mg L^{-1} chitosan treatment, 36.08-fold higher than the non-feeding control. The findings of this study showed that chitosan could be an appropriate strategy to enhance imperatorin production in hairy root culture of U. lobata.

Keywords: *Urena lobata* L., hairy root, imperatorin, precursors, elicitors.

Introduction

Urena lobata L. is a tropical plant in the family Malvaceae, grows wild in tropical, subtropical. In Vietnam, it is distributed all over the wasteland, from the Northwestern hillsides to the Southern regions. This herb has been used for treating dry coughs, aphthae, sore throat, malaria, gonorrhea, leucorrhea, hematemesis, carbuncle, trauma, bleeding, diarrhea, dysentery, gingivitis and emmenagogue for a long time and has been proven effective by people's beliefs[11]. Various animal studies have also demonstrated the properties of the narural root, which include inhibiting lipid peroxidation, scavenging hydroxyl and superoxide radicals *in vitro*, being immunomodulatory, affecting blood glucose and hepatic function[18] and having anticancer properties. Imperatorin is a furocoumarin found by Ghosh (2004) in abundance in the roots of *U. lobata* with about 0.0023%[10]. It exhibits highly effective pharmacological properties in the treatment of neurological diseases, asthma, cancer, bacterial infections, myalgia and in hepatoprotection, anti–convulsion, inhibition of HIV virus replication[23], anti–oxidant acitivites[34], reduces obesity, hypertension, dyslipidemia and insulin resistance[5].

Recent research conducted on rats indicates that imperatorin can significantly reduce obesity, hypertension, dyslipidemia and insulin resistance. Imperatorin is a secondary metabolite plant and its biotransformation involves significant complexity. This biotransformation can proceed through the shikimic pathway to produce amino acids containing a phenol ring (i.e. L-phenylalanine, L-tyrosine) and subsequently utilize these precursors in the phenylpropanoid pathway for the biosynthesis of imperatorin (Fig. 1)[24,30].

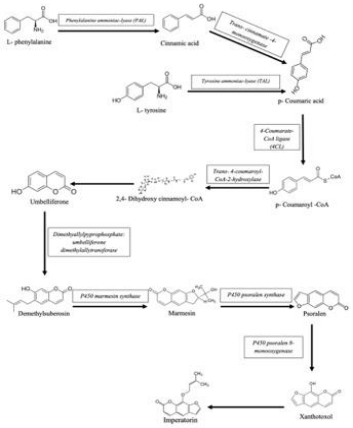

Figure 1: Biosynthesis pathway of imperatorin in plants originated from L-phenylalanine and L-tyrosine[24,29]

However, imperatorin is relatively rare, found in only a handful of plant species[8]. For example, the levels of imperatorin vary among different plant sources: it constitutes approximately 0.0002% in the roots of *Imperata cylindrica*, 0.0021% in the roots of *Radix glehniae* and 0.2775% in the roots of *Angelica dahurica*. Alternatively, it is notably abundant in the fruit of *Aegle marmelos*, where it can reach levels as high as 0.544%. However, research on plant culture for producing imperatorin is relatively limited and its poor acquisition follows an expensive extraction process, resulting in imperatorin being very expensive.

Hairy root (HR), a natural product of genetically transformed plant tissue cultures, was created by

transformation of *Agrobacterium rhizogenes* to plant genome. HR grows rapidly without hormones addition and retains the sustainable produce metabolites similar to the parent plant[3]. Recently, elicitors and precursors are crucial techniques for enhancing secondary metabolites production. Feeding precursors has emerged as a useful adopted approach, as it capitalizes on the notion that any intermediate compound in a secondary metabolite biosynthetic pathway has the potential to boost the final product yield[3]. The defense system of plant has been leveraged to develop a fresh tactic for augmenting metabolite production in plants, which involves utilizing elicitors which activated specific transcriptional factors and upregulated genes, thereby triggering biosynthetic pathways and boosting secondary metabolite production[28].

We have successfully created *U. lobata* HR containing imperatorin. Therefore, enhancing the imperatorin production in HR is the objective of this study. In this study, the effects of types and concentrations of precursors and elicitors were evaluated based on HR growth and imperatorin accumulation.

Material and Methods

Hairy roots culture: *U. lobata* HR clone source was studied by Phuong et al[20] in the Laboratory of Plant Biotechnology, Faculty of Biology and Biotechnology, University of Science - Vietnam National University, Ho Chi Minh City. *U. lobata* HR was cultured in liquid woody plant medium[19] (WPM) containing sucrose 4% (w/v) at pH 5.7 ± 0.1. A flask containing 30 mL of liquid medium was inoculated with 0.2 g fresh weight of HRs and shaken at 80 r.p.m. at 25 °C in fully dark condition.

Analysis of *U. lobata* HR growth and imperatorin accumulation

Growth analysis: Growth of *U. lobata* HR was assessed amidst varying biomass accumulation. Cultivated in liquid WPM under culture conditions as described, both fresh and dry biomass were measured weekly over an 8-week period. Dry biomass was obtained by continuous drying of fresh biomass in a 50 °C oven for 7 days. The data was collected for further analysis, including fresh weight (FW), dry weight (DW) and dry growth index (DGI) according to the formula:

$$DGI = \frac{DWx - DW0}{DW0}$$

where DW_0 is the dry weight at the initial time and DW_x is the dry weight at the harvested time.

Imperatorin measurement: The method used for measuring imperatorin accumulation was the high-performance liquid chromatography (HPLC) method which was validated and performed according to the procedure of Nie et al[17]. Aligent Zorbax 300SB C18 column (250 mm × 4.6 mm, 5 µm) was maintained at 30 °C. The mobile phase was composed of a mixture of methanol and water using a gradient program of (5%–95%:95%–5%, v/v) in 0–90 min, (95%:5%, v/v) in 90–100 min. The flow rate was 1 mL min^{-1}, detection wavelength was 260 nm and sample volume was 5 µL. For preparation of the standard curves, imperatorin (≥ 99%, Sigma, USA) (36 µg mL^{-1}) in methanol was serially diluted with an equal volume of distilled water. Imperatorin productivity (IM) was calculated as µg 30 mL^{-1} at each acquisition time.

Effect of precursors feeding on hairy root of *U. lobate*: Phenylalanine (PHE) (0, 0.05, 0.2 and 0.5 mM), tyrosine (TYR) (0, 0.28, 0.55 and 0.83 mM) and umbelliferone (UMB) (0, 0.03, 0.06, 0.15, 0.31 mM) were purchased from Sigma-Aldrich, USA. All the precursors have been supplemented simultaneously when the HR was being transferred to the new 250 mL flask containing 30 mL liquid WPM. The HRs were harvested each week. All experiments were repeated three times with three flasks per replication.

Effect of elicitation feeding on hairy root of *U. lobate*: Chitosan (CTS) (from shrimp shells, ≥ 75% deacetylated level) (0, 2, 10, 20 mg L^{-1}), salicylic acid (SA) (0, 16, 32 mg L^{-1}), yeast extract (YE) (0, 50, 200, 500 mg L^{-1}) and copper (II) sulfate (CUS) (0, 16, 64, 128 µg L^{-1}) were purchased from Sigma-Aldrich, USA. All the elicitors have been supplemented when the HR achieved the end of the exponential growth phase (week 4). The HRs were harvested after 4 days of elicitation. All experiments were repeated 3 times with three flasks per replication.

Statistical analysis: The obtained data were statistically processed using Statistical Package for Social Sciences, version 25.0 (SPSS, Chicago). Differences among samples were evaluated statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Values were expressed as a mean of three replicates.

Results and Discussion

Effects of precursors on growth and imperatorin accumulation in *U. lobata* HR

Effect of L-phenylalanine: When feeding PHE, there was a change in growth ability and imperatorin accumulation in *U. lobata* HR. The results (FW, DW, DGI and IM) are presented in fig. 2. The addition of 0.05 mM PHE significantly enhanced HR growth over 8 weeks, with the highest performance observed in the 8th week (0.57 g DW), showing a 1.6-fold increase compared to the 4th week control. However, higher PHE concentration (0.2 mM and 0.5 mM) did not improve biomass accumulation.

Imperatorin accumulation was highest at 14.91 µg in the 4th week with 0.05 mM PHE but was 1.3 folds lower than in the non-feeding control. Higher PHE concentrations (0.2 and 0.5 mM) restricted HR growth and reduced imperatorin yield.

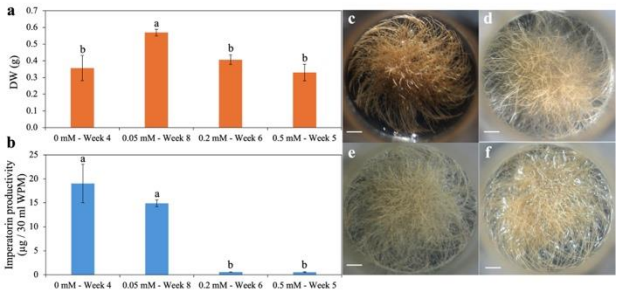

Figure 2: Effect of various L-phenylalanine concentrations on HR growth and imperatorin productivity. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 0.05 mM, (e) 0.2 mM and (f) 0.5 mM. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

In plants, metabolic pathways extend from primary to secondary metabolism. High activity in plant secondary metabolism results in slow-growing plants naturally allocating significant resources to defense compounds. Conversely, PHE originated from organic nitrogen source for plant growth to increase high activity in primary metabolism and this may slow down secondary metabolism. Therefore, the results of this study were similar when supplementing 0.05 mM PHE improved growth in HR but limited the ability to accumulate imperatorin. Moreover, Jiao et al[12] also showed that PHE improved the root growth in *Populus* × *canescens*. Excessive or insufficient nitrogen provision can result in stunted development or toxicity[16]. PHE is a final product of the shikimate pathway, leading to various phenolic compounds such as flavonoids, coumarins, hydroxycinnamic acid conjugates and lignans. PHE has been widely used to enhance secondary metabolite production in HR cultures, but high concentrations can induce cell stress and reduce production.

The study of Abyari et al[1] found that feeding 150 µM L-phenylalanine or more in cell suspension culture of *Spilanthes acmella* reduced scopoletin (coumarin) production. Manjula et al[13] also showed that higher concentrations above 3.0 mM hindered psoralen and bergapten (furocoumarin) accumulation in *Ruta graveolens* callus culture. In this study, it was found that the optimal concentration of 0.05 mM PHE is for enhancing *U. lobata* HR growth but not for increasing IM. Therefore, we conclude that supplementing the culture medium with PHE was not the most effective approach for enhancing imperatorin production.

L-tyrosine (TYR): There was a change in growth capacity as well as imperatorin accumulation when TYR was added to the culture medium of *U. lobata* HR. The results of FW, DW, DGI and IM are presented in fig. 3. An inverse linear correlation between TYR concentration and HR growth was evident. The non-feeding control peaked in 4th week: 34.67 of DGI, 3.47 g of FW and 0.36 g of DW. Increasing the TYR concentration to 0.28 mM resulted in a slight decrease in growth, with the best results observed in 5th week (31.00 of DGI, 3.09 g of FW, 0.32 g of DW). Certainly, as the TYR concentration is increased to 0.55 and 0.83 mM, growth inhibition intensifies. So, the addition of TYR also resulted in a change in imperatorin accumulation, but this change was not effective. Among the TYR treatments, 0.28 mM TYR in 5th week showed the highest effectiveness with an IM of 1.13 µg. However, the IM of 0.28 mM TYR was 16.8-fold lower than that of the control 4th week.

The decline in growth potential observed in the study may be attributed to the excessive concentration of TYR, which surpasses the threshold of organic nitrogen uptake in *U. lobata* HR. This resulted in severe cellular stress, causing a decrease in both biomass growth and imperatorin biosynthesis. Additionally, TYR acts as a precursor for secondary metabolites, which is susceptible to modifications, especially under the conditions of cellular redox imbalance, indicating oxidative stress, in which the conversion of the geometric isomer to the m-Tyrosine form is also the cause of cytotoxicity[33]. Tarasevičienė et al[31] have found that adding TYR at concentrations of 100 and 200 mg L^{-1} did not increase the phenolic acid content compared to the control group.

Furthermore, Singh et al[26,27] have shown that feeding TYR at higher concentrations (50, 100 and 150 mg L^{-1}) strongly inhibited biomass growth and plumbagin accumulation. Therefore, this study found that none of the investigated TYR concentrations was suitable for raising imperatorin accumulation.

Umbelliferone: When feeding UM, there was a change in growth ability and imperatorin accumulation in *U. lobata* HR. The results (FW, DW, DGI and IM) are presented in fig. 4. Supplementing with UMB at concentrations of 0.03, 0.06 and 0.15 mM significantly boosted HR growth from the 1st to the 8th week, with 0.03 mM UMB yielding the highest growth indices: DGI of 55.33 (7th week), FW of 5.06 g (8th week) and DW of 0.56 g (8th week), representing increases of 1.60, 1.41 and 1.56-fold respectively, compared to the 4th week, the non-feeding control. Contrarily, imperatorin accumulation was lowest under all UMB treatments, with the non-feeding control showing 260.19, 31.58 and 151.78-fold higher levels than UMB treatments 0.03, 0.06 and 0.15 mM respectively.

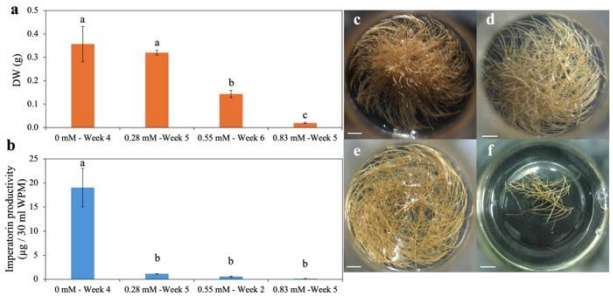

Figure 3: Effect of various L-tyrosine concentrations on HR growth and imperatorin productivity. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 0.28 mM, (e) 0.55 mM, (f) 0.83 mM. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

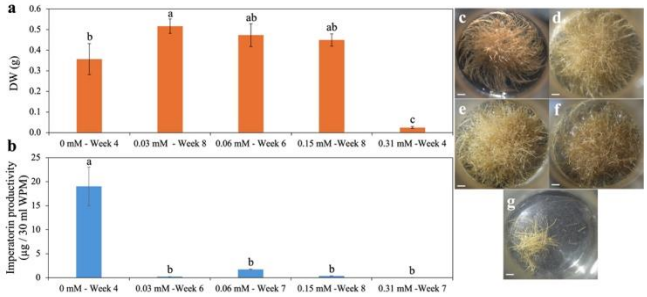

Figure 4: Effect of various umbelliferone concentrations on HR growth and imperatorin productivity. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 0.03 mM, (e) 0.06 mM, (f) 0.15 mM, (g) 0.31 mM. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

Moreover, feeding UMB at high concentration (0.31 mM) completely inhibited the growth and imperatorin accumulation. Although UMB is composed only of carbon, hydrogen and oxygen, it has a strong effect on the growth of HR as a plant growth regulator. Misra et al[15] published research on *Triticum durum* that UMB disrupts metabolites related to tryptophan metabolism and causes an imbalance in the shikimate pathway, leading to a significant increase in IAA and tryptamine. This experiment demonstrates the correlation between primary and secondary metabolism. The 0.03 mM UMB treatment was optimal for primary metabolism, enhancing biomass accumulation but less effective for secondary metabolism. This contrast is likely due to high primary metabolic activity overshadowing secondary metabolism. Secondary metabolism is high activity; it slows plant growth as resources are diverted to produce secondary metabolites[6].

Higher UMB concentrations (0.06 mM) reduce HR growth but increase IM compared with 0.03 mM. However, excessive UMB concentration limits growth and causes physiological disorders, resulting in decreased IM. In this study, it was found that the optimal concentration of 0.03 mM UMB is for enhancing *U. lobata* HR growth but not for increasing IM. Therefore, UMB was not found for enhancing imperatorin production.

A comparison between using precursors on growth and imperatorin productivity of the *U. lobata* hairy roots: The study investigates the impact of different precursors on the growth of *U. lobata* HR. Our results indicate that supplementation with 0.05 mM PHE and 0.03 mM UMB significantly enhances HR growth, as evidenced by a 1.58-folds and 1.44-fold increase in DW respectively, compared to the control at the 4th week (Fig. 5a). In contrast, 0.28 mM TYR did not yield any observable enhancement in growth. These findings suggest that PHE and UMB can effectively promote HR biomass accumulation whereas TYR lacks this effect under the tested conditions.

The influence of various precursors on imperatorin accumulation was also assessed. Despite significant fluctuations in imperatorin accumulation, these changes did not lead to an overall increase in imperatorin production (Fig. 5b). Specifically, treatment with 0.28 mM TYR neither promoted growth nor enhanced imperatorin accumulation. While 0.03 mM UMB substantially stimulated the growth of *U. lobata* HR, it markedly suppressed imperatorin accumulation. In contrast, 0.05 mM PHE exhibited no discernible effect on imperatorin accumulation. These findings highlight the complex and sometimes counterproductive effects of precursors on secondary metabolite production.

Effects of elicitors on growth and imperatorin accumulation in *U. lobata* HR

Salicylic acid: As shown in fig. 6, salicylic acid (SA) dosages had no effect on the reduction of *U. lobata* HR

growth when compared with the control. However, when elicited with SA at all dosages, the HR showed a significant increase in IM. After 4 days of elicitation, the imperatorin yield was compared between different dosages of SA. The results showed that 32 mg L^{-1} SA was the best (IM = 18.62 µg), which was 1.38-fold higher than the control (IM = 13.54 µg). However, these increases were not very prominent and it did not show a statistical difference compared to the 16 mg L^{-1} SA (IM = 18.29 µg) which was 1.35-fold higher than the control.

SA is famous as one of the multifunctional phytohormones, widely found in plants. Physiological and biochemical processes have revealed that SA enhances signaling molecules, increases enzyme activity and produces plant secondary metabolites in response to stress[20], that is, why eliciting SA is also utilized in plant cultures to acquire secondary plant metabolites. Miao et al[14] reported that feeding 8 mg L^{-1} of salicylic acid led to imperatorin being 3.8-folds higher than the control. At 16 mg L^{-1}, psoralen production was 1.7-folds higher than the control. At 28 and 30 mg L^{-1}, isoimperatorin and total coumarins shown a 2.33-folds increase over the control.

The effectiveness of SA was also reported by Singh et al[26]. Their results indicated that among the doses tested, 5 µM SA was the most effective in raising the accumulation of psoralen, daidzein and geniste in in *Cullen corylifolium* callus culture, resulting in a several-fold increase in the accumulation of these compounds compared to the control. In this study, it has been demonstrated that the addition of SA can enhance the production of imperatorin. While both 16 mg L^{-1} and 32 mg L^{-1} showed high imperatorin yield, the use of 16 mg L^{-1} SA is more cost-effective, making it the preferred option.

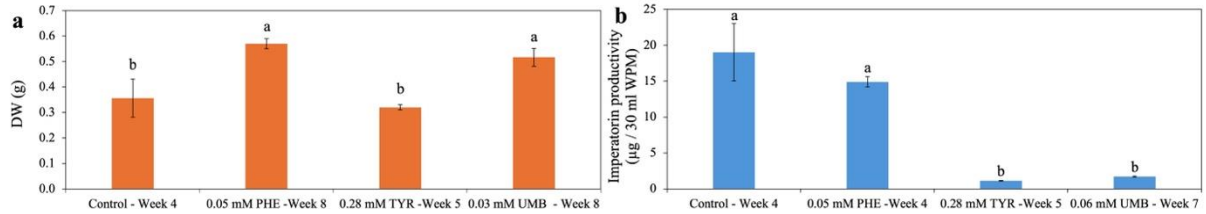

Fig. 5: A comparison between the greatest concentrations of each precursor to the growth and imperatorin productivity of *U. lobata* HR. (a) dry weight, (b) imperatorin productivity. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT).

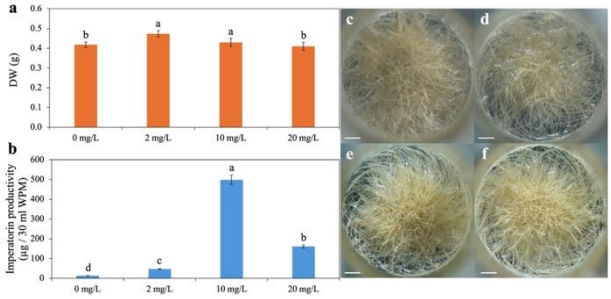

Fig. 6: Effect of various salicylic acid dosages on growth and imperatorin productivity of *U. lobata* HR after 4 days of elicitation. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 16 mg L^{-1}, (e) 32 mg L^{-1}. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

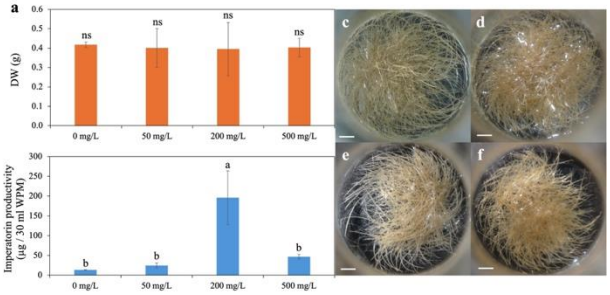

Fig. 7: Effect of various copper (II) sulfate dosages on growth and imperatorin productivity of *U. lobata* HR after 4 days of elicitation. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 16 µg L^{-1}, (e) 64 µg L^{-1}, (f) 128 µg L^{-1}. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

Copper (II) sulfate: As shown in fig. 7, after 4 days of adding copper (II) sulfate (CUS) to the culture medium, HR did not decrease in growth. IM gradually increased when increasing the additional CUS dosage with the results obtained in the 128 µg L^{-1} CUS treatment of 46.93 µg, 3.47-fold higher than the control (IM= 13.54 µg), 1.71-fold higher than the 16 µg L^{-1} CUS treatment (IM = 27.38 µg) and about 1.11-fold slightly higher than the 64 µg L^{-1} CUS treatment (IM = 42.23 µg).

Copper is an essential trace mineral for plant growth and development. Cu^{2+} ions are cofactors in many enzymes such as Cu/Zn superoxide dismutase, laccase, plastocyanin, ascorbate oxidase and polyphenol oxidase with roles in enzyme activation, nitrogen metabolism, respiration, photosynthesis, lignification, protein synthesis, auxin regulation and phenolic metabolism[4]. However, micromineral supplementation to enhance secondary metabolic activity must have limits. Because of its high redox properties, Cu^{2+} needs to be maintained at low levels, they can become extremely toxic causing symptoms such as yellowing, necrosis, stunting and inhibition of root growth.

Supplementing Cu^{2+} to increase the production of secondary metabolites in plants has been done by many researchers. Yaoya et al[35] reported that adding CUS to *Pharbitis nil* hairy root cultures increased umbelliferone and scopoletin over time. In addition, research on adding CS to *Angelica archangelica* suspension culture medium was conducted by Siatka et al[25] using CUS at a dose of 5-50 µM to obtain scopoletin with high yield. Increasing the CUS dose to 100 and 200 µM caused a gradual decrease in scopoletin. In this experiment, both 64 µg L^{-1} and 128 µg L^{-1} CUS treatments enhanced IM. Therefore, 64 µg L^{-1} CUS is a superior choice for improving IM in *U. lobata* HR culture.

Chitosan: As shown in fig. 8, the use of chitosan (CTS) had no significant impact on *U. lobata* HR growth when compared to the control. However, adding CTS at varying dosages resulted in a notable rise in imperatorin accumulation. Supplementing CTS at doses of 2 mg L^{-1} and 10 mg L^{-1} resulted in a gradual increase in imperatorin accumulation. The most remarkable outcome occurred at 10 mg L^{-1} of CS, where imperatorin significantly rose to 498.29 µg, marking a 36.80-fold increase compared to the control treatment of 13.54 µg. Nonetheless, further increasing the CTS dose to 20 mg L^{-1} led to a gradual decrease in imperatorin accumulation.

CTS is the deactylated form of chitin while chitin is derived from pathogens such as insects and fungi[29]. Upon exposure to CTS, plants produce chitosanase that breaks down chitosan into chitosan oligomers. The chitosan oligomers play a fundamental role in stimulating the enhancing of phenylalanine ammonia lyase activity, thereby inducing the biosynthesis of secondary metabolites such as phenolics, according to Thadathil and Velappan[32]. Likewise, a different study has showcased how various chitin and chitosan oligomers have resulted in elevating the activity of phenylalanine ammonia lyase and tyrosine ammonia lyase in soybean leaves, potentially prompting the production of secondary metabolites via the phenylpropanoid pathway. The effect of CTS on the ability to accumulate secondary metabolites was also reported by Ahmed[2], eliciting chitosan to *Lycium barbarum* suspension cultures at a concentration of 763 µM resulting in a 1.85-fold improvement over the control.

However, increasing the CTS concentration beyond 763 µM led to a reduction in coumarin accumulation. Furthermore, Singh et al[27] reported that the addition of 50 mg L^{-1} CTS to the callus culture medium of *Cullen corylifolium* resulted in 6 to 12-fold greater accumulation of psoralen, daidzein and genistein than the control group. The results of our study have shown the potential for applying CTS to increase imperatorin accumulation. The dosage of 10 mg L^{-1} CTS is the superior choice for improving imperatorin production in *U. lobata* HR cultures.

Yeast extract: As shown in fig. 9, YE did not reduce the growth of HR. In fact, YE slightly improved growth within just 4 days of supplementation (although not significantly). However, YE markedly affected secondary metabolism. As YE dosages increased to 500 mg L^{-1}, IM increased compared to the control but not in a linear way. The best result was with 200 mg L^{-1} YE which stimulated imperatorin production to 196.05 µg and 14.48-fold higher than the control (IM = 13.54 µg).

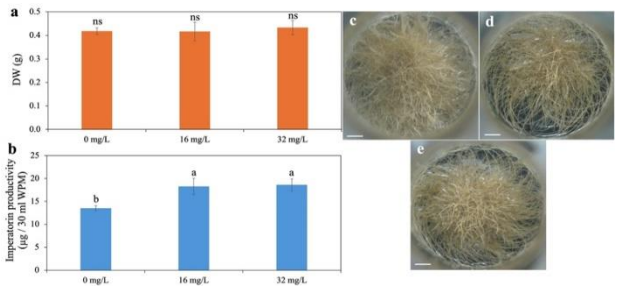

Fig. 8: Effect of various chitosan dosages on growth and imperatorin productivity of *U. lobata* HR after 4 days of elicitation. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 2 mg L^{-1}, (e) 10 mg L^{-1}, (f) 20 mg L^{-1}. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

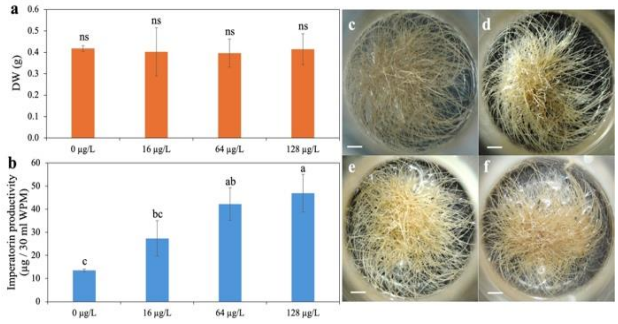

Fig. 9: Effect of various yeast extract dosages on growth and imperatorin productivity of *U. lobata* HR after 4 days of elicitation. (a) dry weight, (b) imperatorin productivity, (c) phenotype of HR in the control treatment, (d) 50 mg L^{-1}, (e) 200 mg L^{-1}, (f) 500 mg L^{-1}. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT). Bars = 1 cm.

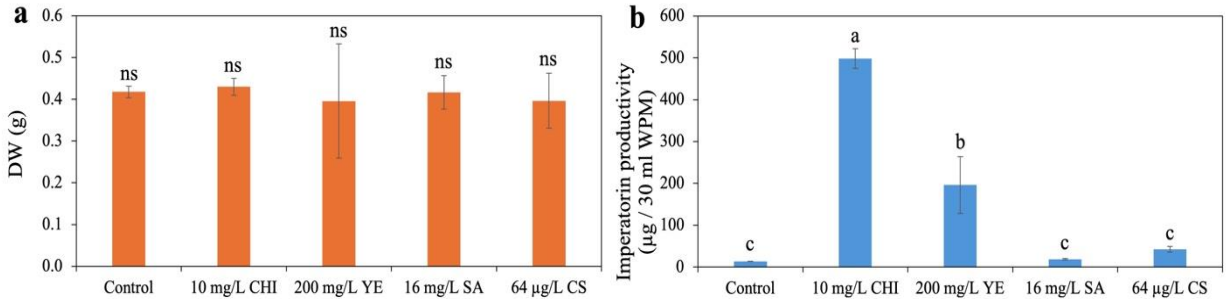

Figure 10: A comparison between the greatest concentrations of each elicitor to the growth and imperatorin productivity of *U. lobata* HR. (a) dry weight, (b) imperatorin productivity. The values in a line marked with different lower-case letters denote significant differences between samples at $p \leq 0.05$ (DMRT)

YE is rich in nucleotides, proteins, amino acids, sugars and various trace elements essential for robust biological metabolism. Additionally, yeast cell walls feature key components like chitin and glucans, common in fungal pathogens[9]. Plants perceive these components as signals of pathogen attack, triggering enhanced oxidative reactions, increasing the transcription of genes encoding enzymes involved in the conversion of plant carbon sources from primary to secondary sources such as phenylalanine ammonia lyase (PAL), an important enzyme in the biosynthesis of phenolic compounds. Therefore, YE is also an elicitor that has been applied in plant culture to produce secondary metabolites.

Rhee et al[23] added 2 mg L^{-1} YE increasing decursinol angelate approximately 3.0-fold compared to the control and showed signs of gradually decreasing decursinol angelate content when increasing the YE dose to 5 and 10 mg L^{-1} in *Angelica gigas* root culture. Younesikelaki et al[36] also added 100 mg L^{-1} YE in *Althaea officinalis* cell culture with the result that scopoletin content increased 3.08-fold. Coumaric acid increased 2.95-fold compared to the control, while continuing to increase YE concentration increased to 150 mg L^{-1}, the content of scopoletin and coumaric acid decreased. The results of this experiment show that 200 mg L^{-1} YE is a suitable treatment for increasing imperatorin accumulation.

A comparison between using elicitors on growth and imperatorin productivity of the *U. lobata* hairy roots: In this study, while elicitors caused no difference in the growth of *U. lobata* HR, the imperatorin production was significantly improved by the effect of elicitors (Fig. 10). The abiotic elicitors, SA and CUS, increased but not significantly, the accumulation of imperatorin. The biotic elicitors, CTS and YE both strongly stimulated imperatorin accumulation. Imperatorin production by biotic elicitors was 4.64 to 27.24-fold higher than that by abiotic elicitors. The superiority of biotic elicitors (CTS, YE) over abiotic elicitors (SA, CUS) could stem from variances in signaling pathways within secondary metabolism.

SA is a phytohormone, acts as a secondary signal, regulating numerous physiological processes in plants. CUS is also an essential element required and involved in intrinsic signaling pathways of plants. Meanwhile, biotic elicitors (CTS, YE) act as primary signals requiring initial recognition receptors and trigger a cascade of signaling pathways involving calcium ion, nitric oxide, jasmonic acid, abscisic acid and salicylic acid[21]. Moreover, CTS is a smaller, more defined molecule compared to YE, which is a complex mixture composed of numerous diverse components. So, biotic elicitors stimulate a broader spectrum of signals than abiotic elicitors and CTS is more effective than YE.

Conclusion

Precursor or elicitor treatment has changed the production of imperatorin in *U. lobata* HR clones. Especially, the use of CS required less time and obtained a higher yield of

imperatorin whereas the use of precursors fell short of delivering the desired efficiency in our treatments.

Specifically in this study, when eliciting with 10 mg L^{-1} CTS, the greatest yield of imperatorin (498.29 µg) was obtained after 32 days in HR cultures. Hence, there is a high potential for using elicitors to produce imperatorin from HR as an alternative to the natural imperatorin source.

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