INCREASED RHINACANTHIN PRODUCTION IN *RHINACANTHUS NASUTUS* HYDROPONICS BY DUAL ELICITATION USING CHITOSAN AND *TRICHODERMA HARZIANUM*

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Abstract

A highly efficient hydroponic cultivation system was developed to enhance rhinacanthin production in *Rhinacanthus nasutus*. Within this framework, dual elicitation using chitosan (CHI) and *Trichoderma harzianum* extract (THE) was identified as a promising technique, strategically employed to increase the overall yield of rhinacanthins. *R. nasutus* hydroponics were treated with CHI (0.15 mg ml^{-1}) and THE (1.0 mg ml^{-1}) either simultaneously or sequentially, with elicitor exposure periods ranging from 24 to 96 h. The total rhinacanthin content (rhinacanthin-C, -D, and -N) in roots and leaves was analyzed using HPLC. Simultaneous dual elicitation with CHI and THE resulted in lower rhinacanthin levels in both leaves and roots compared to single elicitation with either agent. Among the treatments, the highest rhinacanthin contents in the leaves (7.05% w/w) and roots (9.65% w/w) were observed with sequential dual elicitation, in which 0.15 mg ml^{-1} CHI was applied for 48 h, followed by 1.0 mg ml^{-1} THE for 24 h and 72 h, respectively. In contrast, a reversed sequential dual elicitation, in which 1.0 mg ml^{-1} THE was applied for 24 h followed by 0.15 mg ml^{-1} CHI for 72 h and 48 h resulted in rhinacanthin contents of 7.34% and 7.32% w/w in the leaves and roots, respectively. The present study demonstrates an effective approach for enhancing rhinacanthin content in hydroponically cultivated *R. nasutus* through sequential dual elicitation using CHI and THE.

Keywords: **Chitosan; Elicitation; Rhinacanthins; Sequential elicitation; Simultaneous elicitation; *Trichoderma harzianum***

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Introduction

Roots and leaves are the medical parts used for *Rhinacanthus nasutus* (L.) Kurz due to their high accumulation of the bioactive compounds, rhinacanthins (Suksawat and Panichayupakaranant, 2023). The major rhinacanthins (Figure 1), namely rhinacanthin-C (Rn-C), rhinacanthin-D (Rn-D), and rhinacanthin-N (Rn-N) have been reported to exhibit anti-Parkinson's (Saleem *et al.*, 2021), hypoglycemic and hypolipidemic (Shah *et al.*, 2017; Rosdah *et al.*, 2019; Shah *et al.*, 2019), anti-tumor (Boueroy *et al.*, 2018), anti-Alzheimer (Chuang *et al.*, 2017), antibacterial (Puttarak *et al.*, 2010), antifungal (Panichayupakaranant *et al.*, 2009), anti-allergic (Tewtrakul *et al.*, 2009b), and anti-inflammatory activities (Tewtrakul *et al.*, 2009a). The industrial-scale development of *R. nasutus*-based herbal products requires a substantial supply of high-quality root and leaf raw materials. Recently, hydroponic cultivation has been applied to facilitate high-quality plant production due to its easily controlled environment and harvesting process.

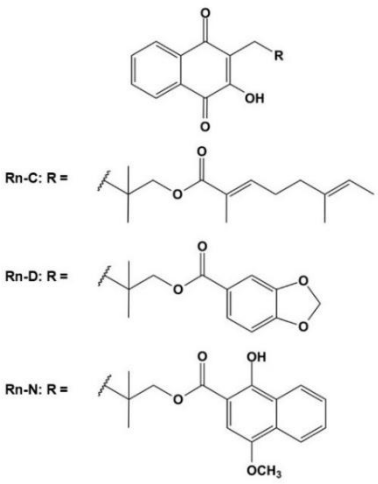

Figure 1. Chemical structures of rhinacanthin-C (Rn-C), rhinacanthin-D (Rn-D), and rhinacanthin-N (Rn-N)

Our previous study demonstrated that the hydroponics of *R. nasutus* could be used as an alternative source of its high-quality leaves and roots. Moreover, CHI (0.15 mg ml^{-1}) or THE (1.0 mg ml^{-1}) effectively stimulated rhinacanthin production in the hydroponics leaves and roots up to 6.7 and 6.1% w/w, respectively, which were higher than those obtained from the natural sources, and made them easier to be harvested in a short period of three months (Suksawat and Panichayupakaranant, 2024). Recent reports have shown that dual elicitation techniques, applied either simultaneously or sequentially enhanced naphthoquinone accumulation in cultured roots of *Plumbago indica* (Jaisi and Panichayupakaranant, 2020) as well as anthraquinone production in *Cassia tora* root cultures (Teptat *et al.*, 2020), compared to the use of single elicitors alone. Therefore, this study aimed to optimize dual elicitation using CHI and THE by evaluating the mode of application (simultaneous vs. sequential), the sequence of the elicitor treatment, and the elicitor exposure period, in order to increase production of rhinacanthins in leaves and roots of the hydroponically cultivated *R. nasutus*.

Materials and Methods

Chemicals

Basal medium, namely Murashige and Skoog (MS) was purchased from Phytotechlab (KS, USA). The standard rhinacanthins were prepared using the method described by Shah et al. (2019). Methanol (HPLC grade), ethyl acetate (analytical grade), were obtained from Labscan Asia (Bangkok, Thailand). Chitosan (CHI) was an analytical grade (Toronto Research Chemicals, Canada). *T. harzianum* extract (THE) was purchased from iLab SV Biotech (Bangkok, Thailand). Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were analytical grade and obtained from MERCK (Darmstadt, Germany) or Sigma (St. Louis, MO).

Plant materials

The shoots of *R. nasutus* were collected from the botanical garden and their voucher specimen (no. 001 18 14) was kept in the herbarium at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Hydroponic system of *R. nasutus*

A hydroponic system of *R. nasutus* was prepared according to the method previously described and maintained in a liquid medium of 20% MS, supplied with aeration (4.5 l min^{-1}), at 25 ± 2 °C under light (230 µmol/s/m^2, 16 h/day). The liquid medium was added every one month (Suksawat and Panichayupakaranant, 2024).

Simultaneous dual elicitation

Twelve-week-old hydroponically grown *R. nasutus* were simultaneously treated with CHI (final concentration of 0.15 mg ml^{-1}) and THE (final

concentration of 1.0 mg ml^{-1}) for 24, 48, 72, and 96 h. The negative control groups were treated with an equal volume of deionized water. Based on our previous study (Suksawat and Panichayupakaranant, 2024), the ordinary single elicitation groups were treated with the same concentration of CHI and THE for 48 and 24 h, respectively. After the treatments, the leaves and roots were harvested and immediately subjected to determination of rhinacanthin content via an HPLC method.

Sequential dual elicitation

Twelve-week-old hydroponically grown *R. nasutus* were initially treated with either CHI (0.15 mg ml^{-1}) for 48 h or THE (1.0 mg ml^{-1}) for 24 h. After the first elicitation, the medium solution was removed, and the fresh medium with the second elicitor (1.0 mg ml^{-1} of THE or 0.15 mg $ml^{-1}$of CHI) was added and further cultivated for 24, 48, 72, and 96 h. After the treatments, the leaves and roots were harvested and immediately subjected to a quantitative HPLC determination of total content of rhinacanthins.

Sample preparation

Sample preparation was performed using the method previously described (Suksawat and Panichayupakaranant, 2024). Briefly, the harvested leaves and roots were washed with deionized water and subsequently dried in a hot air oven at 60 °C for 24 h. The dry weights were then recorded. The plant samples were ground and passed through a no. 20 sieve. The plant powders (100 mg) were extracted with ethyl acetate (50 mL) via a microwave-assisted extraction (2,450 MHz and 450 watts, at 72 ± 2 °C, for 3 cycles, 1 cycle: 45 s power-on and 30 s power-off). The filtrate was subsequently evaporated to obtain a dry extract. The dried extract was kept in a well closed container protected from light at 4 °C.

HPLC analysis

A quantitative HPLC determination was performed via the method described by Panichayupakaranant *et al.* (2009) with slight modifications. In brief, the method was carried out on a Shimadzu HPLC system (Model LC-20, Shimadzu, Tokyo, Japan) using a Phenomenex ODS column (150 × 4.6 mm), a mobile phase of methanol and 5% v/v acetic acid (80:20, v/v), a flow rate of 1 ml min^{-1}, and a detection wavelength of 254 nm.

The calibration curves were constructed using the standard compounds, namely Rn-C, Rn-D, and Rn-N. The calibration curves of Rn-C, Rn-D, and Rn-N were calculated as the linear equations of Y = 32731 X - 11169 (r^2 = 0.9999), Y = 99627 X - 3918.2 (r^2 = 0.9999), and Y = 117918 X - 18187 (r^2 = 0.9999), respectively.

The plant extracts were dissolved in methanol and the volume adjusted to10 ml and then filtered through a 0.45 m membrane filter. All experiments were conducted in triplicate.

Statistical method

The results were calculated as mean ± standard error (SE). Significant differences were determined through one-way ANOVA and followed by Tukey's analysis. Values are considered statistically different when a p value was less than 0.05.

Results and Discussion

Concentrations of CHI and THE as well as the elicitor exposure periods used for the ordinary single elicitations have been previously optimized. Elicitor exposure periods usually play a critical role in plant growth and phytoalexin production. Therefore, the elicitor exposure periods (24 - 96 h) for the second elicitors were determined for the dual elicitation. Based on the dual elicitation methods used in this study, all treatments had no effect on the plant growth or root and leaf biomass compared to the control groups (Tables 1 - 6).

When either CHI or THE was used alone, it significantly increased the production of total rhinacanthins in the roots (6.75% and 6.84%, respectively) and the leaves (6.53% and 7.06%, respectively) of hydroponically cultivated *R. nasutus* (Tables 1 and 2). Unfortunately, although simultaneous dual elicitation led to significantly higher rhinacanthin production in both the roots (Table 1) and the leaves (Table 2) compared to the untreated group, it resulted in a lower rhinacanthin level than that achieved by individual elicitors. Elicitors function as signaling molecules, initiating the elicitation process when recognized by specific receptors on plant cells. Subsequently, this event triggers a signal transduction cascade, ultimately modulating the expression levels of various regulatory transcription factors as well as rate-limiting genes within the biosynthetic pathways of secondary metabolites (Kandoudi and Németh-Zámboriné, 2022). This orchestrated series of molecular events ultimately enhances the synthesis and accumulation of secondary metabolites. Various elicitors, such as CHI and THE, have been substantiated for their ability to increase rhinacanthin production in hydroponically grown *R. nasutus*. Different plant cultures respond to distinct elicitors, and in many cases, researchers have explored dual elicitation strategies. One such approach involves combining abiotic elicitors,

whether chemical or physical, with fungal elicitors, resulting in a synergistic effect on secondary metabolite production. Studies have also investigated a combined approach using fungal elicitation and precursor feeding, demonstrating its effectiveness in increasing the production of sanguinarine in *Papaver somniferum* suspension cultures (Verma *et al.*, 2014). Notably, the filtrate of *T. harzianum* cultures has been found to increase sanguinarine production. However, when *T. harzianum* filtrate was used in combination with shikimate, the highest increase in sanguinarine production was observed (Verma *et al.*, 2014). This enhancement may be attributed to shikimate's ability to redirect metabolic flux towards enhanced sanguinarine biosynthesis. Hence, the extract of *T. harzianum* might serve as a supportive agent in CHI elicitation for enhancing rhinacanthin production.

In contrast, sequential dual elicitation with CHI followed by THE resulted in the highest content of total rhinacanthins in the roots (9.65% w/w), which was markedly higher that achieved through individual elicitation (Table 3). However, this stimulating effect required a longer exposure period to THE of 72 h. Extending the elicitor exposure period beyond 96 h appeared to result in a decrease in rhinacanthin production in the roots. In contrast, this dual stimulation produced the highest content of total rhinacanthins in the leaves (7.05% w/w) when a contact period of 24 h was used for the second elicitor, but it was not significantly different from single elicitation with THE (Table 4). The effect of simultaneous dual elicitation observed

Table 1. Effect of simultaneous dual elicitation of CHI and THE on dried biomass and rhinacanthin production of the hydroponic roots

Treatments	Root biomass (mg per plant)	Rhinacanthin content (% w/w)			
		Rn-C	Rn-C	Rn-C	Rn-C
Untreated group	254.42 ± 2.76^{a}	3.23 ± 0.01^{a}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	3.43 ± 0.01^{a}
CHI (48 h)	244.59 ± 13.80^{a}	6.29 ± 0.05^{b}	0.23 ± 0.01^{b}	0.22 ± 0.01^{b}	6.75 ± 0.06^{b}
THE (24 h)	250.81 ± 15.17^{a}	6.37 ± 0.07^{b}	0.24 ± 0.01^{b}	0.23 ± 0.01^{b}	6.84 ± 0.07^{b}
CHI + THE					
24 h	244.85 ± 13.73^{a}	3.90 ± 0.02^{c}	0.14 ± 0.01^{c}	0.12 ± 0.01^{ac}	4.16 ± 0.02^{c}
48 h	240.40 ± 24.46^{a}	3.94 ± 0.09^{c}	0.14 ± 0.01^{c}	0.13 ± 0.01^{c}	4.20 ± 0.09^{c}
72 h	226.34 ± 1.59^{a}	4.12 ± 0.17^{cd}	0.14 ± 0.01^{c}	0.13 ± 0.01^{c}	4.39 ± 0.17^{c}
96 h	228.89 ± 8.64^{a}	4.45 ± 0.07^{d}	0.14 ± 0.01^{c}	0.13 ± 0.01^{c}	4.71 ± 0.08^{d}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Table 2. Effect of simultaneous dual elicitation of CHI and THE on dried biomass and rhinacanthin production in the hydroponic leaves

Treatments	Leaf biomass (mg per plant)	Rhinacanthin content (% w/w)			
		Rn-C	Rn-D	Rn-N	Total
Untreated group	299.94 ± 11.54^{a}	4.48 ± 0.05^{a}	0.23 ± 0.01^{a}	0.34 ± 0.01^{a}	5.05 ± 0.05^{a}
CHI (48 h)	301.34 ± 15.10^{a}	5.95 ± 0.07^{b}	0.16 ± 0.01^{b}	0.42 ± 0.01^{b}	6.53 ± 0.07^{b}
THE (24 h)	305.50 ± 14.87^{a}	6.18 ± 0.05^{c}	0.36 ± 0.01^{c}	0.47 ± 0.01^{b}	7.06 ± 0.05^{c}
CHI + THE					
24 h	303.32 ± 16.63^{a}	5.14 ± 0.07^{d}	0.26 ± 0.01^{d}	0.36 ± 0.01^{a}	5.76 ±0.08^{d}
48 h	281.17 ± 6.50^{a}	5.03 ± 0.14^{d}	0.25 ± 0.01^{d}	0.34 ± 0.01^{a}	5.61 ± 0.14^{d}
72 h	301.18 ± 13.72^{a}	5.04 ± 0.24^{d}	0.25 ± 0.01^{d}	0.34 ± 0.01^{a}	5.63 ± 0.24^{d}
96 h	300.31 ± 15.07^{a}	4.46 ± 0.04^{a}	0.23 ± 0.01^{a}	0.31 ± 0.01^{c}	5.00 ± 0.04^{a}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Table 3. Effect of sequential dual elicitation of CHI followed by THE on dried biomass and rhinacanthin production in the hydroponic roots

Treatments	Root biomass (mg per plant)	Rhinacanthin content (% w/w)			
		Rn-C	Rn-D	Rn-N	Total
Pretreatment with CHI (48 h) followed by THE					
24 h	248.78 ± 14.69^{a}	6.39 ± 0.12^{a}	0.21 ± 0.01^{a}	0.20 ± 0.01^{a}	6.81 ± 0.12^{a}
48 h	242.24 ± 10.34^{a}	7.05 ± 0.22^{b}	0.23 ± 0.01^{a}	0.21 ± 0.01^{a}	7.49 ± 0.22^{b}
72 h	236.32 ± 13.10^{a}	9.04 ± 0.22^{c}	0.33 ± 0.01^{b}	0.28 ± 0.01^{b}	9.65 ± 0.22^{c}
96 h	246.79 ± 6.40^{a}	8.89 ± 0.15^{c}	0.32 ± 0.01^{b}	0.27 ± 0.01^{b}	9.48 ± 0.15^{c}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

in this study contradicts previous reports on enhanced naphthoquinone and anthraquinone production through simultaneous dual elicitation using CHI and another elicitor in *P. indica* root cultures (Jaisi and Panichayupakaranant, 2020) and *C. tora* (Teptat *et al.*, 2020), respectively. The reasons behind this phenomenon remain unclear. The effects of elicitor applied in combination may be due to their toxicity, compatibility, and specificity (Halder *et al.*, 2019). A reduction in the stimulatory effect of simultaneous dual treatment with CHI and THE might result from chemical interaction, incompatibility, or instability between CHI and THE. Therefore, additional research is required to identify the composition of THE as well as the mechanisms underlying elicitation and chemical interaction between CHI and THE.

On the other hand, the sequential treatment of THE followed by CHI significantly increased total rhinacanthin content in both roots and leaves to 7.32% w/w (Table 5) and 7.34% (Table 6), respectively. Notably, the stimulating effect in the leaves required a longer CHI exposure period of 72 h, whereas the roots responded to dual elicitation with the same exposure duration (48 h) as in single elicitation. This difference might be due to the direct contact of the elicitor with the roots. The CHI-enhanced permeability of plant cells might be one of the mechanisms accelerating THE intracellular integration (Hosseini *et al.*, 2018), leading to increased rhinacanthin production. In contrast, pretreatment with CHI had less effect on rhinacanthin production in the leaves because CHI did not directly contact the leaves and therefore did not enhance cell permeability to THE. The mechanism underlying the elicitation effect of *T. harzianum* might involve trichokonins, one of the peptides produced by *Trichoderma* that have been suggested to act as permeabilizers and plant immune inducers (Keswani *et al.*, 2014; Kappel *et al.*, 2020). The potential applications of CHI in agriculture are diverse, encompassing its use as a biotic elicitor, plant growth stimulant, protective agent, and storage stabilizer for plant materials. CHI, characterized by its notable properties such as the induction of plant

Table 4. Effect of sequential dual elicitation of CHI followed by THE on dried biomass and rhinacanthin production in the hydroponic leaves

Treatments	Leaf biomass (mg per plant)	Rhinacanthin content (% w/w)			
		Rn-C	Rn-D	Rn-N	Total
Pretreatment with CHI (48 h) followed by THE					
24 h	295.49 ± 9.00^{a}	6.30 ± 0.05^{a}	0.37 ± 0.01^{a}	0.37 ± 0.01^{a}	7.05 ± 0.06^{a}
48 h	300.27 ± 16.43^{a}	6.27 ± 0.10^{a}	0.31 ± 0.01^{b}	0.44 ± 0.01^{b}	7.03 ± 0.10^{a}
72 h	299.96 ± 15.62^{a}	5.93 ± 0.07^{b}	0.31 ± 0.01^{b}	0.38 ± 0.02^{a}	6.63 ± 0.11^{b}
96 h	297.91 ± 13.69^{a}	5.07 ± 0.11^{c}	0.26 ± 0.01^{c}	0.35 ± 0.01^{a}	5.69 ± 0.11^{c}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Table 5. Effect of sequential dual elicitation of THE followed by CHI on dried biomass and rhinacanthin production in the hydroponic roots

Treatments	Root biomass (mg per plant)	Rhinacanthin content* (% w/w)			
		Rn-C	Rn-D	Rn-N	Total
Pretreatment with THE (24 h) followed by CHI					
24 h	247.43 ± 13.80^{a}	6.47 ± 0.10^{a}	0.28 ± 0.01^{a}	0.23 ± 0.01^{a}	7.03 ± 0.10^{a}
48 h	236.16 ± 13.32^{a}	6.86 ± 0.05^{b}	0.24 ± 0.01^{b}	0.22 ± 0.01^{a}	7.32 ± 0.05^{b}
72 h	247.58 ± 22.30^{a}	5.87 ± 0.07^{bc}	0.20 ± 0.01^{c}	0.20 ± 0.01^{a}	6.26 ± 0.07^{c}
96 h	239.08 ± 20.63^{a}	5.73 ± 0.09^{c}	0.20 ± 0.01^{c}	0.19 ± 0.01^{a}	6.12 ± 0.09^{c}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Table 6. Effect of sequential dual elicitation of THE followed by CHI on dried biomass and rhinacanthin production in the hydroponic leaves

Treatments	Leaf biomass (mg per plant)	Rhinacanthin content* (% w/w)			
		Rn-C	Rn-D	Rn-N	Total
Pretreatment with THE (24 h) followed by CHI					
24 h	289.57 ± 13.60^{a}	6.34 ± 0.10^{a}	0.31 ± 0.02^{a}	0.45 ± 0.01^{a}	7.10 ± 0.11^{a}
48 h	307.08 ± 16.20^{a}	6.40 ± 0.13^{a}	0.33 ± 0.01^{a}	0.43 ± 0.03^{a}	7.16 ± 0.14^{a}
72 h	301.04 ± 11.56^{a}	6.57 ± 0.08^{ab}	0.32 ± 0.01^{a}	0.45 ± 0.01^{b}	7.34 ± 0.08^{ab}
96 h	298.46 ± 15.38^{a}	6.79 ± 0.13^{c}	0.34 ± 0.01^{a}	0.47 ± 0.02^{a}	7.59 ± 0.15^{b}

Rhinacanthin content was calculated based on the root dry weight. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

defense mechanisms and the regulation of metabolic processes, is highly significant in agriculture. Furthermore, it exhibits antifungal, antibacterial, antiviral, and antioxidant activities, contributing to its multifaceted utility (Stasińska-Jakubas and Hawrylak-Nowak, 2022). Despite these advantageous attributes, the limited solubility of CHI remains a primary limitation. Nevertheless, CHI is widely recognized for its practical applications, being recognized as a cost-effective substance with considerable potential in various agricultural contexts.

Microorganisms from the genus *Trichoderma* are extensively employed and researched as biocontrol agents, owing to their diverse biocontrol traits. These traits encompass parasitism, antibiosis, the production of secondary metabolites, and the induction of plant defense systems. Notably, *Trichoderma* sp. has been widely utilized in agriculture through innovative bioformulations, with *T. harzianum* being one of the most frequently employed species (Guzmán-Guzmán *et al.*, 2023). *T. harzianum*, as a biocontrol agent, demonstrates efficacy in suppressing plant pathogens. This effectiveness is attributed to mechanisms such as competition, antibiosis, and the elicitation of plant defense responses. Moreover, *T. harzianum* serves as a biocontrol agent against pests like aphids by activating the plant defense system in response to their presence (Coppola *et al.*, 2017). The subsequent section provides illustrative examples of both direct and indirect biocontrol mechanisms employed by *T. harzianum*. Thus, CHI and THE may serve purposes beyond their role as enhancers of rhinacanthin production, potentially offering protective effects in the hydroponic cultivation of *R. nasutus*.

Conclusions

The present study successfully established a sequential dual elicitation method using CHI and THE to enhance rhinacanthin production in *R. nasutus* hydroponics. This innovation approach achieved the highest rhinacanthin yields in both the leaves and roots of *R. nasutus*. The optimal dual elicitation methods were as follows:

- For leaves: Sequential treatment with 0.15 mg ml^{-1} CHI for 48 h, followed by 1.0 mg ml^{-1} THE for 24 h, yielding a maximum rhinacanthin content of 7.05% w/w.
- For roots: Sequential treatment with 0.15 mg ml^{-1} CHI for 48 h, followed by 1.0 mg ml^{-1} THE for 24 h, achieving 9.65% w/w rhinacanthin content.
- An alternative method involved 1.0 mg ml^{-1} THE for 24 h, followed by 0.15 mg ml^{-1} CHI for 72 h (leaves) or 48 h (roots), which resulted in rhinacanthin levels of 7.34% w/w in leaves and 7.32% w/w in roots.

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