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Original Article

Enhanced Rhinacanthin Production in *Rhinacanthus nasutus* Roots Using a Hydroponics and Elicitation System

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ABSTRACT

Background: A proficient hydroponic system for the cultivation of *Rhinacanthus nasutus* has been implemented with the aim of augmenting rhinacanthin production. Elicitation stands out as a viable technique for bolstering the yield of rhinacanthin. **Materials and Methods:** A hydroponics system of *R. nasutus* was treated with six elicitors, namely chitosan, *Trichoderma harzianum* extract, sodium alginate, lawsone, methyl jasmonate, and salicylic acid at three concentrations. A suitable elicitation period for the selected elicitor was also determined. The contents of rhinacanthin-C, -D, and -N accumulated were determined using an HPLC method. **Results:** Amongst these, chitosan (0.15 mg/mL) and *T. harzianum* extract (1.0 mg/mL) significantly enhanced rhinacanthin production and gave the highest total rhinacanthin content, up to 6.0-6.1% w/w, which was 2.2-fold higher than the untreated roots (2.7% w/w). In addition, lawsone (3.0 µM) and salicylic acid (100 µM) significantly increased rhinacanthin production up to 4.6% and 3.4% w/w, respectively, while neither methyl jasmonate nor sodium alginate increased rhinacanthin production. The suitable elicitor-contact periods for chitosan, *T. harzianum* extract, and lawsone were 48, 24, and 72 h, respectively. Moreover, the leaves harvested from these optimized conditions also contained markedly high contents of rhinacanthins, up to 5.4%, 6.7%, and 5.1% w/w, respectively. **Conclusion:** The present study established the optimized elicitation methods for the *R. nasutus* hydroponics system using chitosan, *T. harzianum* extract, and lawsone as elicitors to obtain the high-rhinacanthin-producing roots and leaves. These hydroponics and elicitation systems might be alternative sources of the high-quality *R. nasutus* roots and leaves.

Keywords: Chitosan, Elicitation, Hydroponics, Lawsone, Rhinacanthin, Salicylic acid, *Trichoderma harzianum*.

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INTRODUCTION

Plants contain a wide range of secondary metabolites, such as alkaloids, terpenoids, sterols, flavonoids, coumarin, and quinones, which usually possess therapeutic properties against the causes of fatal ailments such as diabetes, cancer, infectious, musculoskeletal, and cardiovascular diseases. However, the production of secondary metabolites in plants is definitely influenced by environmental variations, which markedly affect the consistency of therapeutic efficacy of herbal medicines. Rhinacanthins (Figure 1), namely rhinacanthin-C (RC), -D (RD), and -N (RN) are the major bioactive naphthoquinone esters mainly accumulated in the roots and leaves of *Rhinacanthus nasutus* (L.) Kurz.[1] These naphthoquinones have been reported as the active constituents of *R. nasutus* for the treatments of various diseases associated with their antifungal,[2] antibacterial,[3] anti-inflammatory,[4] anti-allergic,[5] anti-tumor,[6] anti-Alzheimer,[7] anti-Parkinson,[8] hypoglycemic and hypolipidemic[9-12] activities. Although *R. nasutus* roots and leaves are the major sources of rhinacanthins, most of the raw materials commercially supplied for *R. nasutus*-based herbal products are the whole aerial parts, which contain a low content of rhinacanthins, thus leading to low-quality products.

Several efforts are being made toward the establishment of an in-house cultivation system, i.e., a hydroponics system, in which the growth conditions can be easily controlled, in order to enhance secondary metabolite production in plants. Recently, a hydroponics system for *R. nasutus* has been established to overcome the limitations of commercially available *R. nasutus* roots. This hydroponics system produced high dried root biomass (0.25 g/plant) with a high rhinacanthin content (3.3% w/w) in a short period of cultivation. However, the content of rhinacanthin was rather lower than that of the natural roots (3.4-4.1% w/w). The quality of *R. nasutus* roots certainly depends on the content

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of rhinacanthins. Fortunately, an elicitation technique could be easily applied with a hydroponics system to enhance phytoalexin production. Therefore, the present study puts an emphasis on increasing rhinacanthin production in the hydroponics system of *R. nasutus* using an elicitation technique. Both biotic and biotic elicitors, including chitosan, *T. harzianum* extract, sodium alginate, lawsone, methyl jasmonate, and salicylic acid, were determined for their biosynthetic-stimulating properties at different concentrations. The selected elicitors were subjected to further optimization for the periods of the elicitor contract to enhance rhinacanthin production in the hydroponics system of *R. nasutus*.

MATERIALS AND METHODS

Chemicals

Murashige and Skoog (MS) Basal Salt Mixture M524 was obtained from Phytotechlab (KS, USA). The authentic rhinacanthins (RC, RD, and RN) were previously purified.[12] Methanol (HPLC grade), ethyl acetate (analytical grade), and ethanol (analytical grade) were purchased from Labscan Asia (Bangkok, Thailand). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Chitosan (Toronto Research Chemicals, Canada), methyl jasmonate, sodium alginate, salicylic acid, and lawsone (Sigma-Aldrich, USA) were analytical grade. *Trichoderma harzianum* extract was obtained from iLab SV Biotech (Bangkok, Thailand). Other chemicals used were analytical grade and purchased from Sigma (St. Louis, MO) or MERCK (Darmstadt, Germany).

Plant materials

R. nasutus shoots were collected from two-year-old plants grown in the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, in 2021. The plant was identified by Pharkphoom Panichayupakaranant, and the voucher specimen (no. 001 18 14) was deposited at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Establishment and maintenance of the hydroponics system

The shoots of *R. nasutus* were washed with tap water and subsequently soaked in a 2% sodium hypochlorite solution (15 min), and then washed with deionized water. The shoots were subsequently transferred to a growing container for root initiation (20 plants/container). The roots were initiated in deionized water (8 L) to produce the plantlets. The hydroponics conditions of *R. nasutus* were as follows: the plantlets were grown in 20% MS liquid medium (8 L) supplied with aeration (4.5 L/min) and was cultivated at 25±2°C under LED white light (230 µmol/s/m^2) for 16 h/day. The solution of 20% MS medium was periodically added every 4 weeks. Root and leaf harvesting could be performed every 12 weeks.

Harvesting and extraction

After the treatments, the roots or leaves (in some cases) were cut and washed with tap water and deionized water, respectively. The plant samples were dried in a hot air oven at 60°C for 24 h, then ground and passed through a no. 20 sieve. The sample powders (100 mg) were extracted with 50 mL of ethyl acetate using a microwave-assisted extraction. The extraction conditions were as follows: microwave frequency and power of 2,450 MHz and 450 watts, respectively; 3 irradiation cycles (1 cycle=45 sec power-on and 30 sec power-off); a final extraction temperature of 72±2°C. The extracts were filtered and evaporated to dryness using a rotary evaporator at 40°C.

Quantitative HPLC method

HPLC conditions

Quantitative HPLC analysis was carried out using the method previously described[2] with minor modifications. Briefly, the method was performed using a Shimadzu HPLC system (Model LC-20, Shimadzu, Tokyo, Japan) equipped with a LC-20AD pump, an SPD-M20A photodiode-array detector, and an SIL-20A autosampler. A 150 mm x 4.6 mm Phenomenex ODS column was eluted with a mobile phase of methanol and 5% aqueous acetic acid (80:20, v/v) at a flow rate of 1 mL/min. The quantitative UV detection was set at 254 nm.

Calibration curve

The calibration curves were established from the authentic RC (12.5-200 µg/mL), RD (3.12-50 µg/mL), and RN (3.12-50 µg/mL), respectively. The linear equations of y=37966x + 21418 (r^2=0.9999), and y=95934x-33213 (r^2=0.9999), and y=150297x-86082 (r^2=0.9997) were established as the calibration curves of RC, RD, and RN, respectively.

Sample preparation

The dried extracts were reconstituted with methanol (10 mL) in a volumetric flask and then filtered through a 0.45 µm membrane filter. All experiments were carried out in triplicate.

Preparation of elicitors

The stock solutions of elicitors, including chitosan, *T. harzianum* extract, sodium alginate, and lawsone, were prepared in deionized water, while those of methyl jasmonate and salicylic acid were prepared in 50% v/v ethanol.

Effect of elicitor type and concentration

R. nasutus hydroponics plants (12-week-old) were treated with each elicitor at three different concentrations (Table 1) for 48 h. The control groups were treated with an equal amount of the solvent used for each elicitor (water or ethanol). After the treatments, the roots were harvested and subjected to the drying

process. The dry weights of roots were determined using an analytical balance, and their HPLC determinations of RC, RD, and RN contents were carried out.

Effect of elicitor contact time

The hydroponics plants (12 weeks old) were treated with chitosan (0.15 mg/mL), *T. harzianum* extract (1 mg/mL), and lawsone (6 μM) for 24, 48, and 72 h. Subsequently, the roots were harvested and subjected to the drying process to determine the dry biomass weights. The contents of RC, RD, and RN were determined using the HPLC method.

Statistical analysis

The data was presented as the Mean±Standard Error (SE). Statistical significance was analyzed using a one-way ANOVA, followed by Tukey's test. By convention, results are considered statistically significant when $p<0.05$.

RESULTS

Types and concentrations of elicitors, as well as the elicitor contact periods, usually affect the growth and production of phytoalexins in plant tissues.[13] Therefore, the present study determined the effects of various elicitors, including chitosan, *T. harzianum* extract, sodium alginate, lawsone, methyl jasmonate, and salicylic acid at three concentrations, on root biomass and rhinacanthin (RC, RD, and RN) production in *R. nasutus* hydroponics. The 12 weeks old hydroponics plants were used to treat with the elicitors because they were in the linear growth phase, which actively responded to the elicitor stimulation (result not shown). None of elicitor at all concentrations used in this study exhibited detrimental effects on plant growth and root biomass production compared to the control groups (Table 1). Regarding rhinacanthin production, four elicitors, including chitosan, *T. harzianum*, lawsone, and salicylic acid, but not methyl jasmonate and sodium alginate, significantly increased rhinacanthin production in the hydroponics plants. In addition, chitosan and *T. harzianum* extract gave the highest rhinacanthin content up to 6.0-6.1% w/w, followed by lawsone (up to 4.6% w/w) and salicylic acid (up to 3.4% w/w), respectively (Table 1).

Rhinacanthin production was changed by the amounts of chitosan, *T. harzianum* extract, and lawsone, but not by salicylic acid. The highest amounts of total rhinacanthins were found in chitosan (0.15 mg/mL) and *T. harzianum* extract (1.0 mg/mL) (Table 1). The results show that chitosan and *T. harzianum* extract are the best elicitors for increasing rhinacanthin production in *R. nasutus* hydroponics roots. This is because both elicitors led to 2.2 times more rhinacanthin production than the roots that were not treated (2.7% w/w). Notably, *T. harzianum* extract exhibited more specifically increased rhinacanthin-C production (5.5% w/w) than the others. This finding is the first report of *T. harzianum* extract eliciting naphthoquinone production in plants.

The elicitor contact period plays a significant role in phytoalexin biosynthesis. Extending the elicitor contact period might result in either increased metabolic production or physiological damage. As a result, determining an optimal contact period for any elicitor was needed to maximize rhinacanthin production. The top three elicitors that gave the highest boosting power of rhinacanthin production, including chitosan, *T. harzianum* extract, and lawsone were selected to determine the effect of elicitor contact periods (24, 48, and 72 h) on rhinacanthin production in the roots. The results revealed that an increase in elicitor contact periods up to 72 h did not significantly affect the growth and root biomass production of *R. nasutus* hydroponics when compared to the control (Table 2). In contrast, the elicitor-contact periods exhibited different eliciting powers on the rhinacanthin production of the hydroponics plant. An increase in the contact period for chitosan and *T. harzianum* extract elicitations to 72 h resulted in a decrease in rhinacanthin production (Table 2). However, the optimal contact periods that gave the highest content of rhinacanthins for chitosan and *T. harzianum* extract elicitations were 48 and 24 h, respectively. In contrast, a significant increase in rhinacanthin production was observed when the contact period of lawsone was increased to 72 h. The results indicated that each elicitor needed their own optimal elicitor contact periods to stimulate secondary metabolite production. Therefore, the suitable elicitation periods

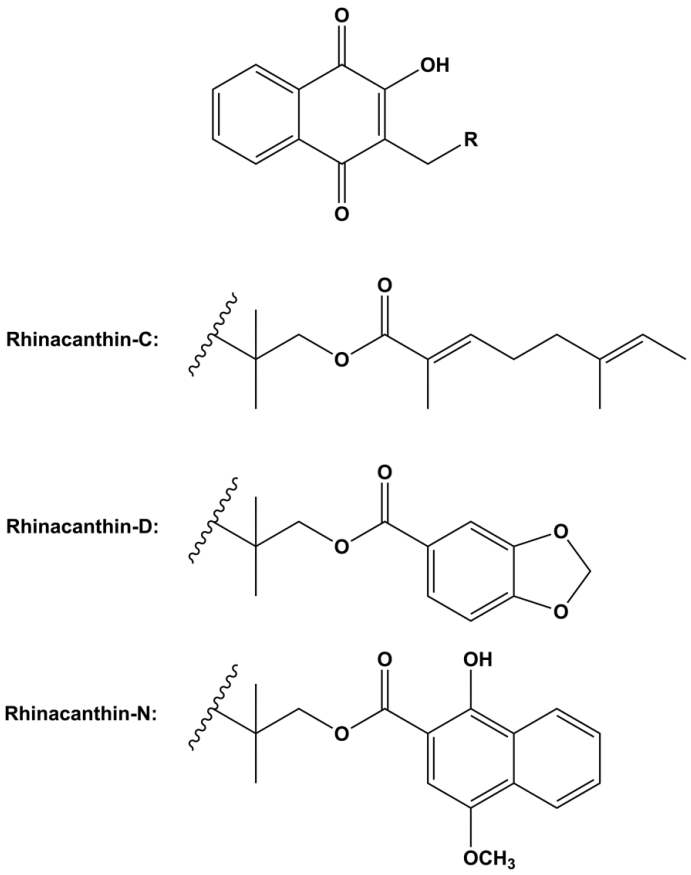

Figure 1: Chemical structures of rhinacanthin-C, rhinacanthin-D, and rhinacanthin-N.

for chitosan, *T. harzianum* extract, and lawsone to increase rhinacanthin production in the roots were 48, 24, and 72 h, respectively, which maximized rhinacanthin production up to 6.1%, 6.1%, and 4.6% w/w, respectively.

During the determination of elicitor contact periods, the leaves of *R. nasutus* hydroponics plants were also harvested and subjected to the determination of the dried leaf biomass and rhinacanthin content. The results are shown in Table 3. Treatment with chitosan for 24 h gave the highest content of rhinacanthin in the leaves, up to 5.4% w/w, which was significantly higher than the control leaves (4.6% w/w). Therefore, the leaves should be harvested after 24 h of chitosan elicitation, but the roots should be harvested after 48 h of treatment. In contrast, variation of the contact periods between 24 and 72 h did not significantly affect rhinacanthin production by *T. harzianum* extract elicitation. The highest content of rhinacanthins (6.7% w/w) was observed after 24 h of treatment. While a significant increase in rhinacanthin production (5.1% w/w) was also observed when the contact period of lawsone was increased to 72 h. Therefore, roots and leaves of the hydroponics plants could be harvested at the same time after the suitable contact periods of *T. harzianum* extract and lawsone.

DISCUSSION

The elicitation technique serves as a fundamental approach employed to augment or induce the production of secondary metabolites in plants. Distinct plant species, including *R. nasutus*, necessitate varying elicitation techniques tailored to elevate their secondary metabolite output.[14] Agents or stimuli utilized in elicitation are termed elicitors, and they can be categorized based

Table 1: Effect of elicitors and their concentrations on growth and rhinacanthin (RC, RD, and RN) production in the roots of *R. nasutus* hydroponics.

Elicitors and Concentrations	Dried root biomass (mg/plant)	Rhinacanthin content* (% w/w)			
		RC	RD	RN	Total
Control	125.26±4.04^{a}	2.18±0.16^{a}	0.24±0.02^{a}	0.30±0.01^{a}	2.72±0.20^{a}
Chitosan					
0.05 mg/mL	126.34±6.10^{a}	3.96±0.18^{b}	0.39±0.01^{b}	0.53±0.02^{b}	4.89±0.20^{b}
0.15 mg/mL	123.31±8.26^{a}	4.96±0.27^{c}	0.48±0.01^{c}	0.67±0.02^{c}	6.10±0.09^{c}
0.25 mg/mL	116.64±3.47^{a}	2.78±0.03^{d}	0.28±0.01^{ad}	0.38±0.01^{a}	3.44±0.03^{d}
T. harzianum extract					
1.0 mg/mL	134.45±6.78^{a}	5.53±0.10^{e}	0.29±0.03^{d}	0.22±0.01^{d}	6.04±0.12^{c}
2.0 mg/mL	123.39±3.28^{a}	4.10±0.11^{b}	0.20±0.01^{e}	0.18±0.01^{d}	4.48±0.12^{b}
3.0 mg/mL	124.32±4.65^{a}	4.16±0.63^{b}	0.18±0.03^{ef}	0.17±0.03^{d}	4.50±0.69^{b}
Lawsone					
1.5 µM	137.43±4.57^{a}	3.10±0.02^{g}	0.19±0.01^{e}	0.40±0.01^{bc}	3.69±0.02^{d}
3.0 µM	133.79±13.51^{a}	3.96±0.18^{b}	0.21±0.00^{ae}	0.46±0.02^{bc}	4.63±0.24^{b}
6.0 µM	126.14±7.32^{a}	3.82±0.25^{b}	0.22±0.03^{ae}	0.41±0.03^{c}	4.45±0.32^{b}
Salicylic acid					
50 µM	133.01±8.91^{a}	2.82±0.03^{d}	0.11±0.01^{f}	0.30±0.01^{a}	3.24±0.04^{d}
100 µM	132.70±9.39^{a}	3.31±0.02^{g}	0.06±0.01^{g}	0.08±0.01^{f}	3.45±0.02^{d}
150 µM	121.12±1.54^{a}	3.19±0.04^{g}	0.05±0.01^{g}	0.06±0.01^{f}	3.31±0.04^{d}
Sodium alginate					
0.8 mg/mL	132.04±7.41^{a}	2.33±0.16^{a}	0.15±0.02^{f}	0.12±0.01^{f}	2.61±0.01^{a}
1.5 mg/mL	136.58±8.11^{a}	2.34±0.27^{a}	0.09±0.02^{fg}	0.12±0.03^{f}	2.54±0.01^{a}
3.0 mg/mL	136.11±10.34^{a}	2.49±0.06^{a}	0.13±0.01^{f}	0.05±0.00^{f}	2.67±0.01^{a}
Methyl jasmonate					
100 µM	124.55±2.32^{a}	2.14±0.06^{a}	0.25±0.02^{ad}	0.21±0.01^{d}	2.60±0.08^{a}
200 µM	135.63±3.05^{a}	2.13±0.15^{a}	0.26±0.02^{ad}	0.21±0.01^{d}	2.60±0.16^{a}
400 µM	136.44±7.26^{a}	1.58±0.06^{f}	0.21±0.01^{ae}	0.17±0.01^{e}	1.96±0.06^{e}

Note: *Calculated based on the dry weight of the roots, RC=Rhinacanthin-C, RD=Rhinacanthin-D, and RN=Rhinacanthin-N. The values are mean±SEM (n=3). Statistical analysis was analyzed using a one-way ANOVA, followed by Tukey's test. The mean values within the same column labelled with different letters (a, b, c, etc.) are significantly different (p<0.05).

on their origins into abiotic and biotic elicitors.[15] The underlying mechanisms of elicitation across diverse elicitors predominantly involve the elicitors, substances, or factors acting as stressors that instigate defensive mechanisms, often resulting in the synthesis of secondary metabolites through intracellular signaling pathways.[16] The objective of employing elicitors in the hydroponic cultivation of *R. nasutus* is to facilitate their absorption through the roots, thereby instigating the synthesis of rhinacanthin *via* intracellular signaling pathways. The application of elicitation techniques to different plant species necessitates consideration of several key factors, including plant age, elicitor concentration, elicitation duration, and harvesting timing. It is discerned that both biotic and abiotic elicitors exhibit the potential to enhance secondary metabolite production in specific plants. Consequently, the implementation of elicitors, encompassing both biotic and abiotic types, in the root system of *R. nasutus* for the purpose of augmenting rhinacanthin production remains a formidable challenge. Notably, owing to their relatively low toxicity, elicitors such as chitosan, *T. harzianum* extract, lawsone, salicylic acid, sodium alginate, and methyl jasmonate emerge as promising candidates for effective employment in enhancing rhinacanthin production. Furthermore, the strategic manipulation of specific contact times may potentially contribute to further amplifying rhinacanthin production within the hydroponic cultivation of *R. nasutus*.

Chitosan is a nontoxic, natural, and biodegradable polymer that is well-known to stimulate plant growth as well as induce defensive mechanisms in plants, both of which necessitate changes in plant metabolite profiles.[17] Chitosan has been reported to activate jasmonic acid, a signal molecule involved in defensive gene regulation and Phospholipase C/protein Kinase C (PKC) cascades.[18] Furthermore, it has been reported to stimulate naphthoquinone productions in some plant tissue cultures, such as an increased plumbagin production in *Plumbago indica* root cultures[19] and an enhanced methylene-3,3-bilawsone biosynthesis in *Impatiens balsamina* root cultures.[20] The ability of chitosan to specifically increase naphthoquinone production might be due to mimicking a natural defense response or stimulating the enzymes involved in the biosynthesis of naphthoquinones. Although the mechanisms of chitosan for eliciting naphthoquinone biosynthesis have not yet been clarified, various mechanisms have been proposed, including stimulation of the antioxidant defense machinery, stimulation of nitrogen metabolism, increased uptake of water, and reduction of transpiration and essential nutrients through adjusting cell osmotic pressure.[17]

Trichoderma spp. are natural soil microorganisms that have been reported to boost the biosynthesis of various bioactive phytochemicals. *Trichoderma* spp. frequently leads to higher nutrient absorption, greater root growth and development, increased production, and improved tolerance to different pressures, including disease. The delivery of some effector chemicals from *Trichoderma* spp. during the interaction with plants may be involved in these effects. In addition, the biostimulant mechanisms of *Trichoderma* extracts may include the production of mitogen-activated proteins, auxin, phenylpropanoids, and phytoalexins, as well as the release of volatile and non-volatile substances that increase plant survival and nutrient uptake.[21,22]

Lawsone is a naturally occurring naphthoquinone, uniquely found in the leaves of *Lawsonia inermis* and *Impatiens balsamina*. The

Table 2: Effect of elicitor contact periods on growth and rhinacanthin (RC, RD, and RN) production in the roots of *R. nasutus* hydroponics.

Elicitors and Contact periods	Dried root biomass (mg/plant)	Rhinacanthin content* (% w/w)			
		RC	RD	RN	Total
Chitosan (0.15 mg/mL)					
24 h	125.88±3.01^{a}	3.95±0.03^{a}	0.41±0.01^{a}	0.52±0.01^{a}	4.89±0.03^{a}
48 h	123.31±8.26^{a}	4.96±0.07^{b}	0.48±0.01^{b}	0.67±0.02^{b}	6.12±0.09^{b}
72 h	116.64±7.43^{a}	4.58±0.29^{b}	0.43±0.02^{a}	0.59±0.04^{a}	5.61±0.35^{b}
T. harzianum extract (1.0 mg/mL)					
24 h	121.70±4.99^{a}	5.63±0.08^{c}	0.22±0.02^{c}	0.22±0.01^{c}	6.07±0.08^{b}
48 h	119.28±3.14^{a}	5.53±0.10^{c}	0.29±0.03^{c}	0.22±0.01^{c}	6.04±0.12^{b}
72 h	117.03±6.46^{a}	3.92±0.08^{a}	0.17±0.01^{d}	0.16±0.01^{d}	4.25±0.08^{c}
Lawsone (3.0 μM)					
24 h	131.60±3.35^{a}	3.56±0.03^{d}	0.13±0.01^{d}	0.36±0.01^{e}	4.15±0.03^{c}
48 h	121.01±14.35^{a}	3.59±0.05^{d}	0.22±0.01^{c}	0.41±0.01^{f}	4.22±0.05^{c}
72 h	128.82±12.92^{a}	3.86±0.02^{a}	0.25±0.01^{c}	0.46±0.01^{f}	4.57±0.02^{d}

Note: *Calculated based on the dry weight of the roots, RC=Rhinacanthin-C, RD=Rhinacanthin-D, and RN=Rhinacanthin-N. The values are mean±SEM (n=3). Statistical analysis was analyzed using a one-way ANOVA, followed by Tukey's test. The mean values within the same column labelled with different letters (a, b, c, etc.) are significantly different (p<0.05).

Table 3: Effect of elicitor contact periods on growth and rhinacanthin (RC, RD, and RN) production in the leaves of *R. nasutus* hydroponics.

Elicitors and Contact periods	Dried leaf biomass (mg/plant)	Rhinacanthin content* (% w/w)			
		RC	RD	RN	Total
Control	158.83±8.76[a]	3.94±0.03[a]	0.22±0.01[a]	0.42±0.01[a]	4.58±0.03[a]
Chitosan (0.15 mg/mL)					
24 h	168.90±11.41[a]	4.77±0.11[b]	0.24±0.01[a]	0.42±0.01[a]	5.42±0.11[b]
48 h	165.00±13.04[a]	4.15±0.03[a]	0.22±0.03[a]	0.47±0.01[b]	4.84±0.03[c]
72 h	173.86±15.62[a]	4.11±0.39[a]	0.20±0.02[a]	0.52±0.03[c]	4.82±0.42[ab]
T. harzianum extract (1.0 mg/mL)					
24 h	168.90±2.47[a]	5.72±0.19[c]	0.30±0.02[b]	0.64±0.01[d]	6.66±0.22[d]
48 h	165.00±16.27[a]	5.84±0.22[c]	0.23±0.01[a]	0.51±0.02[c]	6.58±0.26[d]
72 h	173.86±13.88[a]	4.96±0.02[a]	0.48±0.01[c]	0.62±0.03[d]	6.10±0.04[d]
Lawsone (3.0 μM)					
24 h	162.56±14.25[a]	3.88±0.01[a]	0.23±0.01[a]	0.50±0.01[c]	4.60±0.02[a]
48 h	168.99±13.47[a]	3.96±0.11[a]	0.25±0.01[a]	0.45±0.01[b]	4.66±0.11[a]
72 h	172.85±11.89[a]	4.38±0.07[d]	0.29±0.01[b]	0.46±0.01[b]	5.14±0.07[bc]

Note: *Calculated based on the dry weight of the roots, RC=Rhinacanthin-C, RD=Rhinacanthin-D, and RN=Rhinacanthin-N. The values are mean±SEM (n=3). Statistical analysis was analyzed using a one-way ANOVA, followed by Tukey's test. The mean values within the same column labelled with different letters (a, b, c, etc.) are significantly different (p<0.05).

present study is the first to demonstrate that lawsone is capable of increasing secondary metabolite, especially naphthoquinone production in plants. Up to date, the precise elicitation mechanism of lawsone has not been identified yet. However, the eliciting property of lawsone in rhinacanthin production might be related to its oxidative stress induction by increasing both H_2O_2 production and antioxidative enzyme activities in plant cells.[23] The redox homeostasis in plants is generally known as a key factor involved in auxin-mediated growth regulation. On the other hand, according to its 1,4-naphthoquinone core structure with an *ortho*-hydroxyl group substitution, which is a subunit of the rhinacanthin core structure, therefore, it might be an intermediate in the biosynthetic pathways of rhinacanthins. In addition, lawsone and rhinacanthins have been proposed to be biosynthesized *via* the shikimate pathways.[24] In this regard, the mechanism by which lawsone increases rhinacanthin production might be through the precursor feeding.

CONCLUSION

The present study established the practical elicitation methods to increase the production of rhinacanthins in the hydroponics system of *R. nasutus* using chitosan, *T. harzianum* extract, and lawsone as the elicitors. The optimized elicitation conditions made the hydroponics plant an alternative source of the high-rhinacanthin-producing roots and leaves of *R. nasutus*. The roots and leaves obtained from these cultivation systems contained a higher content of rhinacanthins than those obtained from natural sources and could be more easily harvested. Moreover, the established hydroponics systems might be a valuable model for exploring and elucidating the genes involved in the biosynthesis of rhinacanthins, as well as underlying the elicitation mechanism for increasing rhinacanthin production.

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CONFLICT OF INTEREST

The authors declare that there is no conflicts of interest.

ABBREVIATIONS

ANOVA: Analysis of variance; **HPLC:** High performance liquid chromatography; **LED:** Light-emitting diode; **MS:** Murashige and Skoog; **ODS:** Octadecyl-silica; **PKC:** Phospholipase C/ protein Kinase C; **RC:** Rhinacanthin-C; **RD:** Rhinacanthin-D; **RN:** Rhinacanthin-N.

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