

Evaluation of yeast extract, chitosan, and pectin as easy and cost-effective applications to increase indirubin and indigotin accumulation in *Isatis tinctoria* root cultures

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Abstract

This study was carried out to determine the effects of yeast extract, chitosan, and pectin as simple and cost-effective biotic elicitors on root growth and the accumulation of indigotin and indirubin in the root of *Isatis tinctoria*. For this purpose, different concentrations of yeast extract (1, 2, 3, and 4 g L⁻¹), chitosan (100, 150, 200, and 250 mg L⁻¹), and pectin (0.5, 1, 2, and 3%) were applied to 21 days old roots for 7 days. After harvest, roots were evaluated regarding fresh root weight, root growth index, dry root weight, and contents of indigotin and indirubin. As a result of the study, it was determined that yeast extract showed positive effects on root growth while chitosan inhibited. On the other hand, pectin had no positive or negative impact on root growth parameters. Yeast extract and chitosan increased indigotin accumulation in roots compared to control roots while indigotin amounts decreased with the pectin. Within yeast extract applications, the highest indirubin contents were obtained from the roots applied with 3 and 4 g L⁻¹ of yeast extract. All chitosan applications enhanced the indirubin accumulation compared to control. The effect of pectin on indirubin accumulation was changed depending on its concentrations. Pectin at 0.5 and 1% increased indirubin contents compared to control. In conclusion, 1 g L⁻¹ of yeast extract for root growth and 100 mg L⁻¹ of chitosan for indigotin and indirubin contents were selected as the most appropriate applications supplying the highest values.

Highlights

Yeast extract showed positive effects on root growth while chitosan inhibited

Pectin had no positive or negative impact on root growth parameters

Among the applications, 1 g/L of yeast extract for root growth was the most appropriate application

Yeast extract, chitosan, and pectin enhanced the indigotin and indirubin accumulation depending on their concentrations

The highest indigotin and indirubin contents were obtained from the roots applied with 100 mg/L of chitosan

Introduction

Plant secondary metabolites, essential components of the plant's defense system against various stress factors, have gained significant popularity in recent years due to their use in various fields such as medicine, pharmacy, cosmetics, perfumery, agriculture, food, and textiles (Fazili et al. 2023). The most common method used to obtain these valuable metabolites synthesized in plants is the collection of plants from nature (Selwal, et al. 2023). However, meeting the demand by collecting from nature poses a great danger to the survival of endemic and over-collected plant species, especially those with root-derived metabolites (Wawrosch and Zotchev 2021). In addition, the emergence of quality and quantity differences due to climatic and geographical conditions in the production of secondary metabolites, the

destruction of plants due to disease, pests, or unfavourable environmental conditions, the necessity of harvesting at certain times of the year, and unsuitable geographical conditions constitute other negative aspects of this metabolite production technique performed by traditional methods (Fazili et al. 2022). As a result of the search for alternative production techniques that eliminate these drawbacks and ensure standardized quantity and quality production throughout the year, it has been found that in vitro techniques present significant potential for this purpose (Fazili et al. 2022). In addition, metabolite yield can be increased to much higher levels by applying different treatments to plants, cells, and tissues under in vitro conditions that are not possible in nature (Ramawat 2007). Changes in the composition of nutrient media along with adjustments in cultural conditions such as temperature and light, two-phase culture systems, genetic transformation, immobilization, permeabilization, and the use of precursors or intermediates, as well as elicitor applications stimulating enzymes and genes in secondary metabolite synthesis pathways, are among the methods that can be applied to increase secondary metabolite production under in vitro conditions (Zhao et al. 2005). Elicitors are biotic or abiotic factors that induce chemical reactions known as elicitation, which stimulate the plant's stress response. And when applied at appropriate concentrations and durations, they enhance the production of secondary compounds in in vitro cultures.

Elicitors can be either abiotic, such as UV, heavy metals, methyl jasmonate, and salicylic acid, or biotic, such as polysaccharides derived from plant cell walls and components of microbial cell walls (Namdeo 2007). Jasmonic acid, salicylic acid, and their derivatives are among the most widely used elicitors in in vitro cultures. However, the high cost of these elicitors constitutes an important obstacle for commercial applications (Giri and Zaheer 2016). To reduce costs and increase their commercial use, it is crucial to select cost-effective, non-toxic, and easy-to-apply elicitors for in vitro secondary metabolite production. Yeast extract, chitosan, and pectin are among the biotic elicitors that can be considered in this context.

Isatis tinctoria L., a species belonging to the Brassicaceae family, represented by a total of 338 genera and 3350 species and encompasses many important medicinal plants, is an important dye plant due to containing a blue dye substance called indigotin. Indigotin, estimated to have an annual production worldwide of 16.000 tons (Çömlekçioğlu 2011), is considered one of the most important natural dyes due to its extensive industrial use and rarity in nature for its blue colour (Kızıl and Arslan 2001). Because of the carcinogenic effects of synthetic dyes, most of which are benzene and benzene-derived aromatic hydrocarbons, and their negative impact on the environment, interest in natural and environmentally friendly dyes is increasing day by day.

Therefore, as an alternative to synthetic dyes, significant efforts have been made in the past 20 years by the EU to support various projects aimed at the natural extraction of indigotin from *Isatis* species. In 2001, the SPINDIGO (The Sustainable Production of Plant-Derived Indigo) project received approximately 3.5 million Euros in funding. Additionally, several European countries have supported various projects on the cultivation of *Isatis* and *Polygonum* species for production, extraction, and use in the textile industry of indigotin (CORDIS 2020).

In addition to their use as dye plants, *Isatis* species have been used for medicinal purposes since ancient times with their indole alkaloids and phenylpropanoids (Çömlekçioğlu et al. 2017). Indeed, they have been found to possess antibacterial, antiseptic, antiviral activities, as well as preventive effects against pharyngitis, laryngitis, erysipelas, carbuncle, hepatitis A, meningitis, cancer, and inflammation (Bown 1995; Han et al. 2011; Taviano et al. 2018; Speranza et al. 2020). The dried roots of *Isatis tinctoria* called "Banlangen" in Traditional Chinese Medicine, have been successfully used in the treatment of severe acute respiratory syndrome (SARS) and H1N1 diseases due to their anti-influenza activities (Xiao et al. 2015). Due to these pharmacological effects, *Isatis tinctoria* is one of the best-selling medicinal plants in East Asia and the United States (Ni et al. 2012). Despite being an important plant both as a dye and medicinal herb, it is observed that there are very limited studies on metabolite production under in vitro conditions in *Isatis tinctoria*. Majority of these studies, particularly intensified in recent years, have focused on flavonoid accumulation (Gai et al. 2015a; Jiao et al. 2018a, Jiao et al. 2018b; Gai et al. 2019), with fewer studies targeting alkaloid accumulation (Gai et al. 2015b; Gai et al. 2019).

This study was conducted to investigate the effects of biotic elicitors consisting of yeast extract, chitosan, and pectin, which were selected due to their simplicity, availability, and cost-effectiveness, on root growth and the accumulation of the plant's primary indole alkaloids, indigotin, and indirubin in *Isatis tinctoria* root cultures. The aim was to determine the most suitable elicitor application.

Material and Methods

Plant material In this study, roots obtained by in vitro germination of *Isatis tinctoria* L. seeds sourced from the Department of Field Crops at Isparta University of Applied Sciences, Faculty of Agriculture, were used as the plant material.

Sterilization and germination of seeds For surface sterilization, *Isatis tinctoria* seeds were first kept in 70% ethanol for 60 seconds and washed with sterile distilled water, followed by agitation in a 15% sodium hypochlorite solution for 10 minutes. Afterward, the seeds were rinsed five times with sterile distilled water and transferred to MS nutrient medium (Murashige and Skoog 1962) containing 6 g L⁻¹ agar and 30 g L⁻¹ sucrose for germination. The seeds were cultured in climate rooms with a temperature of 25°C under 16 hours of light (50 µmol m⁻²s⁻¹ light intensity) and 8 hours of darkness. The root parts of the seedlings were cultured in these media for 2 weeks and became to 3–4 true leaves were used to obtain root cultures.

Propagation of roots in liquid media In the study, the roots obtained by germinating the seeds were separated into 1–2 mm long pieces and cultured in 100 mL flasks containing 30 mL of liquid ½ MS nutrient medium with 30 g L⁻¹ sucrose added at an inoculation density of 10 g L⁻¹. Roots were then cultured for 3 weeks at 90 rpm shaking speed in dark climate chambers with a temperature of 25°C ± 1. The roots were multiplied for use in elicitor applications by subculturing them twice in nutrient media with the same composition.

Application of biotic elicitors to root cultures Yeast extract, chitosan, and pectin were applied to root cultures to determine their effects on root growth and indole alkaloid accumulation. For this purpose, 0.3 g of roots were cultured in 30 mL of liquid $\frac{1}{2}$ MS nutrient medium containing 30 g L^{-1} sucrose for 21 days. Then, the roots were transferred to freshly prepared nutrient media of the same content containing different concentrations of biotic elicitors. Yeast extract at 1, 2, 3, and 4 g L^{-1} ; chitosan at 100, 150, 200, and 250 mg L^{-1} ; and pectin at 0.5%, 1%, 2%, and 3% concentrations were added to culture media as biotic elicitors. The stock solutions of yeast extract and chitosan were filter sterilized and added to the sterilized media while pectin was added to the nutrient media before sterilization. Sterile distilled water was added to the control group. The root cultures were maintained at 25°C in climate chambers under dark conditions with shaking at 90 rpm for 7 days. The experiment was laid out in a randomized experimental design with 3 replicates and 5 flasks in each replicate. On the 7th day following the treatments, the roots were harvested and washed with sterile distilled water.

Determination of root growth parameters To determine the effects of biotic elicitors on root growth, harvested roots were evaluated in terms of fresh and dry root weights and root growth index. Fresh root weight was obtained by weighing freshly harvested roots and dry root weight was determined by weighing roots dried to constant weight at 40°C using an analytical balance. Values were calculated as $\text{g } 100 \text{ mL}^{-1}$. The root growth index was determined according to the following formula:

Growth index = (harvested fresh root weight (g)-inoculated fresh root weight (g))/inoculated fresh root weight (g)

Determination of indigotin and indirubin amounts in roots by HPLC The extraction process for alkaloid analysis was carried out by soaking 0.1 g of dried and powdered root in 20 mL of 80% methanol solution in an ultrasonic water bath under infrared light for 30 min. The samples were then filtered through filter paper and kept under vacuum at 45°C with a rotary evaporator to obtain dry extracts. The dried extract was subsequently dissolved with HPLC-grade methanol and used in the analyses.

After elicitor treatments, the amounts of indigotin and indirubin were determined in the extracts obtained from the roots using HPLC (SHIMADZU, Japan) and DAD detector. The extracts passed through a $0.45 \mu\text{m}$ filter were analyzed at room temperature using an Agilent TC-C18 column ($5 \mu\text{m}$, $250 \text{ mm} \times 4.60 \text{ mm}$). In HPLC analysis, indigotin and indirubin were read at 289 nm wavelength. HPLC was performed isocratically using HPLC-grade methanol and ultrapure water (80:20, v/v) as the mobile phase. The flow rate was set to 1 mL min^{-1} and the injection volume to $20 \mu\text{L}$ (Zhang et al. 2012). Standards of these alkaloids were prepared with methanol at different concentrations ($\mu\text{g mL}^{-1}$) and calibrated by HPLC. Amounts of indigotin and indirubin were expressed as $\mu\text{g g}^{-1}$ DW. All samples were analyzed in duplicate.

A schematic view of the steps performed in the study is presented in Fig. 1.

Statistical analyses Each experiment included three independent biological replicates, and the results were performed using SPSS 26.0 statistical program. The differences between the treatments were determined by Duncan Multiple Comparison Test ($p \leq 0.05$). Variables were presented as mean and standard deviation. Heat map analysis using the averages of the data and grouping of the data was performed with the ClustVis web tool as described by Metsalu and Vilo (2015).

Results

The effects of biotic elicitor applications on root growth To determine the effects of different elicitors and their concentrations on root growth, roots were evaluated in terms of fresh root weight, root growth index, and dry root weight. In roots treated with yeast extract and chitosan, the root growth parameters changed significantly depending on the concentrations ($p \leq 0.05$) while pectin had no significant effect on root growth parameters. Yeast extract treatments promoted root growth at all concentrations compared to the control (Fig. 2). The highest values in all root growth parameters were obtained with yeast extract applied at a concentration of 1 g L^{-1} while increases in concentration reduced the stimulatory effect of yeast extract on root growth. The study revealed that increasing chitosan concentration to 150 mg L^{-1} led to a decrease in root fresh weight and root growth index, with values being lower compared to the control (Fig. 3). Among the chitosan applications, the lowest root dry weight was observed in roots treated with the highest concentration of 250 mg L^{-1} . The values for root fresh weight, root growth index, and root dry weight with pectin applications ranged from 4.18 to $4.15 \text{ g } 100 \text{ mL}^{-1}$, 3.38 to 3.15 , and 0.32 to $0.31 \text{ g } 100 \text{ mL}^{-1}$, respectively (Fig. 4).

The effects of biotic elicitor applications on indigotin accumulation in root cultures

The biotic elicitors used to increase the accumulation of indigotin, which is one of the most important components of *Isatis tinctoria* and has great importance as a natural blue colour source, significantly changed the indigotin accumulation in the roots ($p \leq 0.05$). In yeast extract-treated roots, the highest values of $1258.44 \mu\text{g g}^{-1}$ and $1140.52 \mu\text{g g}^{-1}$ were obtained at concentrations of 2 g L^{-1} and 1 g L^{-1} , respectively (Fig. 5A). However, it was observed that the indigotin content began to decrease when the yeast extract concentration was increased to 3 g L^{-1} , and this decrease continued with 4.0 g L^{-1} . Despite variations in yeast extract concentrations, yeast extract treatments increased indigotin accumulation in the roots compared to the control group, where the lowest indigotin content of $442.60 \mu\text{g g}^{-1}$ was recorded. In the roots treated with chitosan, the concentration of 100 mg L^{-1} was found to be the most effective for increasing indigotin accumulation resulting in $1999.472 \mu\text{g g}^{-1}$ (Fig. 5B). However, concentrations above 100 mg L^{-1} caused a decrease in indigotin accumulation. Chitosan at all concentrations increased the amount of indigotin compared to the control and the lowest amount of indigotin was obtained from the control roots. However, indigotin accumulation decreased with pectin

treatments (Fig. 5C). The inhibitory effect of pectin on indigotin accumulation increased in parallel with the rise in concentration, and the lowest value was obtained from the highest pectin concentration of 3%.

The effects of biotic elicitor applications on indirubin accumulation in root cultures Treatment of *Isatis tinctoria* root cultures with different concentrations of yeast extract, chitosan, and pectin significantly changed the amount of indirubin in roots ($p \leq 0.05$). In roots treated with yeast extract, the accumulation of indirubin increased with higher concentrations. The greatest values of $2.15 \mu\text{g g}^{-1}$ and $1.92 \mu\text{g g}^{-1}$ were obtained from yeast extract applications of 4 g L^{-1} and 3 g L^{-1} , respectively, while the lowest value of $1.56 \mu\text{g g}^{-1}$ was obtained from the 1 g L^{-1} yeast extract application (Fig. 6A). Chitosan, which was effective in increasing the amount of indirubin compared to the control group, provided the highest indirubin accumulation at concentrations of 100, 150, and 200 mg L^{-1} , while the amount of indirubin decreased as the concentration increased to 250 mg L^{-1} (Fig. 6B). The lowest indirubin amount was obtained from the control roots, with $1.81 \mu\text{g L}^{-1}$. In pectin-treated roots, it was found that as the concentration of pectin increased from 0.5–1%, the amount of indirubin in the roots increased and reached a maximum (Fig. 6C). However, with the increase of pectin concentration to 2% and 3%, the stimulatory effect of pectin on indirubin accumulation decreased.

To better understand the effects of biotic elicitors at different concentrations on root growth and the accumulation of indigotin and indirubin, the data were visualized by the heat map method and presented in Fig. 7. According to these results, all parameters were affected depending on the applications. From the densities of root growth parameters in the heat map, it is clearly seen that the highest root growth parameters were obtained from the roots treated with yeast extract, especially at 1 g L^{-1} . On the other hand, chitosan was the most appropriate biotic elicitor for the accumulation of indigotin and indirubin. The highest indigotin contents were obtained from the roots treated with 100 mg L^{-1} of chitosan, followed by 150 mg L^{-1} chitosan. When only indirubin was evaluated, it was observed that the most effective treatments in the heat maps were 100 mg L^{-1} , 200 mg L^{-1} , and 150 mg L^{-1} chitosan, respectively. The lowest metabolite accumulations in the heat map were observed in pectin applications depending on their concentrations.

Discussion

In this study investigating the effects of yeast extract, chitosan, and pectin as simple, available and cost-effective biotic elicitors on root growth and the accumulation of indigotin and indirubin, roots obtained from the germination of *Isatis tinctoria* seeds were used as plant material. Root cultures are particularly favored for obtaining root-derived metabolites due to their rapid growth rates and stable metabolite production (Carvalho and Curtis 1998). Indeed, previous studies showed that root culture was a successful culture technique for the *in vitro* production of many metabolites (Demirci et al. 2021; Demirci et al. 2022).

Plants stimulate secondary metabolism to synthesize essential compounds as part of the struggle for survival against different biotic and abiotic factors, known as elicitation (Gorelick and Bernstein 2014). In this study, as an alternative to high-cost abiotic elicitors such as brassinosteroid, jasmonic acid, salicylic acid, and their derivatives for enhancing metabolite production under in vitro conditions, biological elicitors consisting of yeast extract, chitosan, and pectin, which stand out with their practical and economical properties, were used. This study revealed that biotic elicitors had different effects on root growth. In roots treated with yeast extract, the highest values in terms of all root growth criteria were obtained at a concentration of 1 g L^{-1} , but this stimulatory effect decreased with increasing concentrations. Similarly, the highest root growth in eggplant was observed with a 1 g L^{-1} yeast extract application in hairy root cultures (Jain and Singh 2015). While the yeast extract applied at low concentrations did not create any difference in root growth in *Azadirachta indica* hairy roots, it was found that increasing the concentration to 100 mg L^{-1} significantly enhances root growth. The growth-promoting effect of yeast extract on biomass was attributed to its complex nitrogenous compounds containing specific amino acids, vitamins, and minerals (Srivastava and Srivastava 2014). Conversely, yeast extract did not have a significant effect on root growth in *Calotropis gigantea* hairy roots (Sun et al. 2012). These results indicated that the stimulatory effect of yeast extract on biomass varied depending on the concentration of yeast extract as well as the plant species.

In the present study, chitosan, another biotic elicitor, was found to suppress root growth. Similar to the results chitosan applied at concentrations of 50, 100, 250, and 500 mg L^{-1} significantly reduced root growth in the hairy roots of *Azadirachta indica*, especially in increasing concentrations compared to the control (Srivastava and Srivastava 2014). Additionally, chitosan also reduced root growth in *Polygonum tinctorium* hairy roots compared to the control (Young-Am et al. 2000). Pectin treatments did not have a promoting or inhibitory effect on root growth in this study. Baque et al. (2012) found that combined applications of chitosan and pectin inhibited root growth in adventitious root cultures of *Morinda citrifolia*, attributing this inhibitory effect to a strong negative impact on cell viability. In the same study, it was observed that dual applications at a dose of 0.8 mg mL^{-1} caused 79% cell death. However, Dornenburg (1993) noted that pectin had protective effects on cell viability in *Morinda citrifolia* cell suspension cultures, and this protective effect was also observed when used in combination with chitosan. Similarly, in *Chenopodium rubrum* cell cultures treated with chitosan, the addition of pectin promoted cell growth, but chitosan applications above $400 \text{ } \mu\text{g mL}^{-1}$ had a lethal effect on the cells. In the same study, it was also found that increasing chitosan concentrations caused increased pigment release associated with cell death (Dornenburg 1993). Cell deaths were linked to the malfunction of membrane ion pumps, loss of membrane integrity, and increased intercellular Ca^{2+} , which initiated various degradation processes leading to cytoplasmic swelling and ultimately cell death (Cobb et al. 1996). All these results showed that biomass increase varied significantly depending on the elicitor, concentration, culture type, and plant genotype.

Yeast extract is one of the most used biotic elicitors in in vitro cultures to stimulate secondary metabolite production (Jain and Singh 2015; Kochan et al. 2017). Yeast extract is a rich source of B

vitamin complexes. Additionally, yeast extract contains various compounds involved in plant defense responses, including chitin, β -glucan, N-acetyl-glucosamine oligomers, glycopeptides, and ergosterol (Boller 1995; Putalun et al. 2007; Cai et al. 2012). In this study, it was determined that yeast extract significantly altered the synthesis of indirubin and indigotin in *Isatis tinctoria* roots, depending on their concentration. Lower concentrations of yeast extract, such as 1 g L^{-1} and 2 g L^{-1} , were more suitable for achieving the highest accumulation of indigotin, while higher concentrations, such as 3 g L^{-1} and 4 g L^{-1} , were required for indirubin accumulation. Shinde et al. (2009) reported that, like other elicitors, the concentration of yeast extract in the culture medium was a significant factor in metabolite biosynthesis and that the optimal concentration varied for each plant species. Indeed, when applied at appropriate concentrations, yeast extract was identified as a compound successfully used to enhance the production of various metabolites under in vitro conditions, including rosmarinic acid (Gonçalves et al. 2019), isoflavonoids (Rani et al. 2020), plumbagin (Singh et al. 2020), artemisinin (Putalun et al. 2007), tropane alkaloids (Pitta-Alvarez et al. 2000) and ginsenoside (Kochan et al. 2017). The contribution of yeast extract to secondary metabolite production was attributed to the presence of some cations in the yeast extract such as zinc, calcium, and cobalt, which can act as abiotic elicitors (Suzuki et al. 1985; Srivastava and Srivastava 2014).

Chitosan is a natural, low-cost, and non-toxic signalling molecule that can increase secondary metabolite production by triggering plant defense responses (Jiao et al. 2018c). Indeed chitosan has been found to significantly enhance the accumulation of withanolides in *Withania somnifera* (Sivanandhan et al. 2012), artemisinin in *Artemisia annua* (Lei et al. 2011), curcumin in *Curcuma longa* (Sathiyabama et al. 2016), echinacoside in *Scrophularia striata* (Kamalipourazad et al. 2016), isoflavonoids in *Pueraria candollei* (Udomsuk et al. 2011), xanthones in *Hypericum perforatum* (Tocci et al. 2010), and stevioside in *Stevia rebaudiana* (Bayraktar et al. 2016). In this study, it was determined that chitosan applied to *Isatis tinctoria* root cultures increased both indigotin and indirubin synthesis compared to the control. The highest indigotin accumulation was obtained from the application of 100 mg L^{-1} chitosan while the maximum indirubin accumulation was detected in roots applied with 100, 150, and 200 mg L^{-1} . The effect of chitosan on metabolite yield was highly dose-dependent (Sauerwein et al. (1991; Srivastava and Srivastava 2014). Indeed Young-Am et al. (2000) determined that chitosan applied at concentrations of 100, 200, and 300 mg L^{-1} increased the amount of indigotin in *Polygonum tinctorium* hairy roots, while lower and higher concentrations inhibited the amount of indigotin. Additionally, applying 50 mg L^{-1} chitosan to the adventitious roots of *Calotropis gigantea* for 20 days resulted in the highest accumulation of cardenolides; however, exceeding this concentration led to a decrease in cardenolide accumulation (Sun et al. 2012).

The potential of elicitors to enhance metabolite productivity varied depending on the metabolite. Indeed, the production of cryptotanshinone in *Salvia miltiorrhiza* cultures reached its highest level with the application of yeast extract, while the production of tanshinone I was maximized with chitosan treatments (Zhao et al. 2010). Additionally, chitosan was found to be more effective than yeast extract in increasing the xanthone content in the hairy root cultures of *Gentiana dinarica* (Krstić-Milošević et al.

2017). The promoting effect of chitosan on metabolite accumulation was thought to be related to plant defense responses triggered by chitosan (Iriti and Faoro 2007). In general, moderate elicitor applications induce a beneficial type of stress that contributes to the activation of defense-related secondary metabolite production in plants. However, when the tolerance thresholds are exceeded, they can lead to harmful stress characterized by metabolic damage or cell death, resulting in a decrease in secondary metabolite production (Kranner et al. 2010). Plants also have a certain limit for the secondary metabolites they can synthesize in their tissues. When this limit is exceeded, plants are triggered to produce the necessary enzymes for the degradation of the excess synthesized metabolite through feedback regulation (Malik et al. 2013). The brown coloration of chitosan-treated *Isatis tinctoria* hairy roots compared to the control was reported to be an indicator of increased oxidative stress (Jiao et al. 2018c). Indeed, it was reported that chitosan treatments could induce excessive ROS production in *Scrophularia striata* cell cultures, disrupt intracellular redox balance, and ultimately lead to oxidative stress (Kamalipourazad et al. 2016). Chitosan can be recognized as a fungal pathogen attack by immune receptors localized in plant cell membranes, which triggers various signaling molecules and activates genes associated with phytoalexin biosynthesis, leading to the emergence of the plant's defense responses (Bueter et al. 2013; Hadwiger 2013). Therefore, chitosan was found to be an easy, cost-effective, and successful biotic elicitor that, when used at appropriate concentrations, enhances the production of secondary metabolites *under in vitro* conditions (Jiao et al. 2018c).

Pectin, a compound rich in galacturonic acid, is another biotic elicitor frequently used to obtain plant secondary metabolites. In this study, it was determined that compared to the control, pectin treatments decreased indigotin accumulation while promoting indirubin accumulation at low concentrations. The highest indirubin accumulation was obtained from the application at a concentration of 1%. However, indirubin decreased as the pectin concentration increased above 1%. Similarly, the highest amount of solasodin in eggplant was obtained from the application of 1% pectin (Jain and Singh 2015). Noting that pectin increases the production of metabolite synthesis as a result of its ability to mimic stress conditions, Gadzovska et al. (2014) revealed a significant correlation between increased POD (peroxidase) activity and metabolite content, indicating that polysaccharides such as pectin trigger a strong disruption in the cellular redox system and activation of defense reactions. Similarly, pectin was found to increase the accumulation of oleanolic acid in *Calendula officinalis* cell suspension cultures (Wiktorowska et al. 2010) and the amount of anthocyanins and phenolics in grapevine cell suspension cultures (Cai et al. 2012). Additionally, it was also found that pectin had positive effects on amaranthine accumulation in chitosan-treated *Chenopodium rubrum* cell cultures (Dornenburg 1993). All these results show that pectin treatments affect metabolite yield differently depending on plant species and variety, pectin concentration, metabolite, and other elicitors used together.

Elicitors are compounds that stimulate the synthesis of reactive oxygen species (ROS), phytoalexins, secondary metabolites with antimicrobial properties, and other compounds that form the defense system (Montesano et al. 2003).

Conclusion

This study aims to investigate the effects of biotic elicitors applied to the root cultures of *Isatis tinctoria*, a plant of medical and dye significance due to its indole alkaloid content, on root growth and the accumulation of indole alkaloids. For this purpose, it was determined that among the applications of yeast extract, chitosan, and pectin at different concentrations, 1 g L⁻¹ yeast extract was the most suitable for root development, while 100 mg L⁻¹ chitosan was optimal for the accumulation of indigo and indirubin. Identifying inexpensive, practical, and effective applications to enhance metabolite yield under *in vitro* conditions is of great importance for meeting the industrial demand for these metabolites. This study particularly demonstrated that when applied at appropriate concentrations, both yeast extract and chitosan have significant potential for biomass and metabolite production. Ultimately, the findings from this research will contribute to the establishment of sustainable and commercially viable production methods for valuable plant-derived compounds.

Declarations

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

AC: Conceptualization, Methodology, HPLC analysis, Writing – original draft. NGB: Conceptualization, Design, Formal analysis, Data curation, Writing – review & editing, Supervision. All authors contributed to the writing and approved the final version of the manuscript.

Data availability

Data will be made available on request.

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Figures

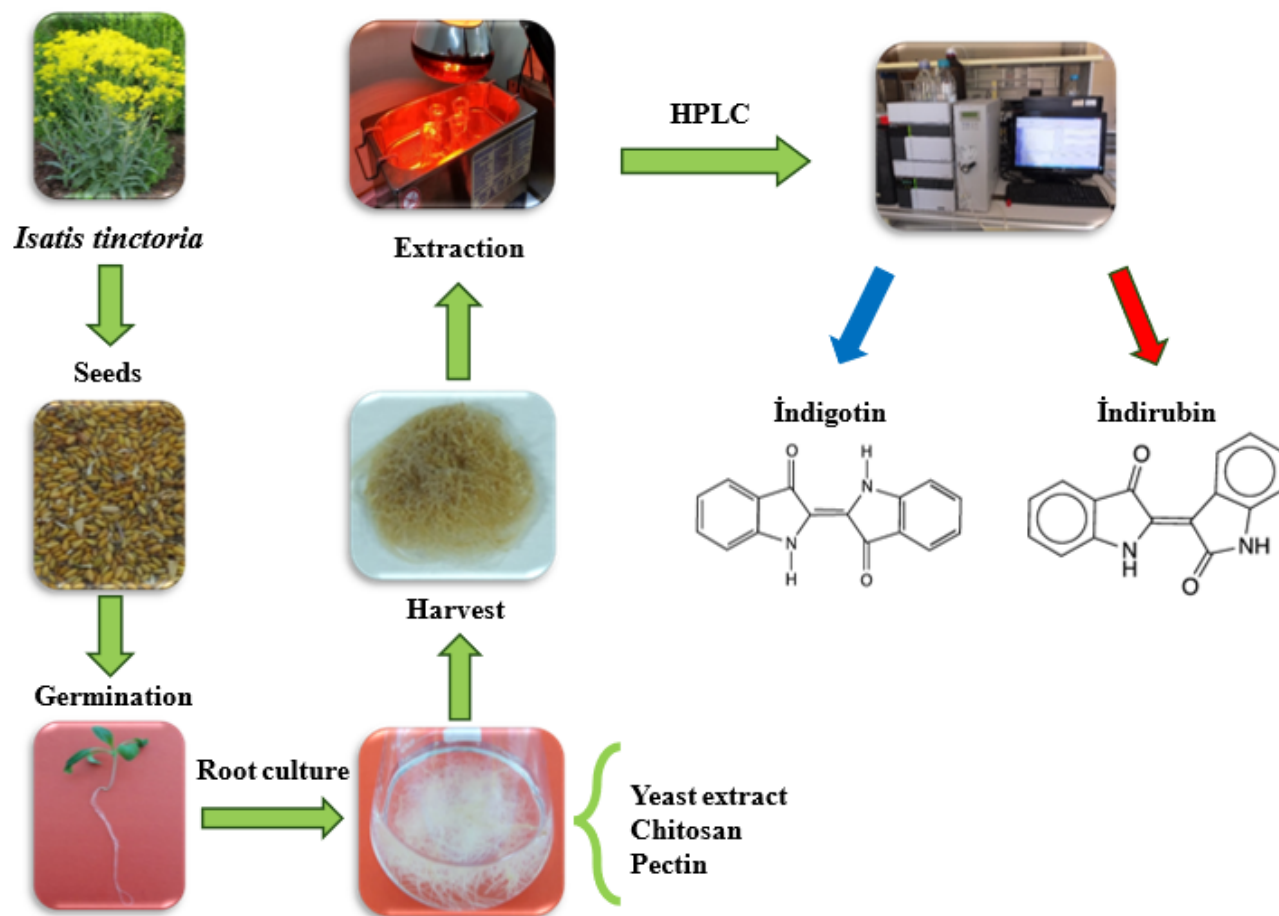
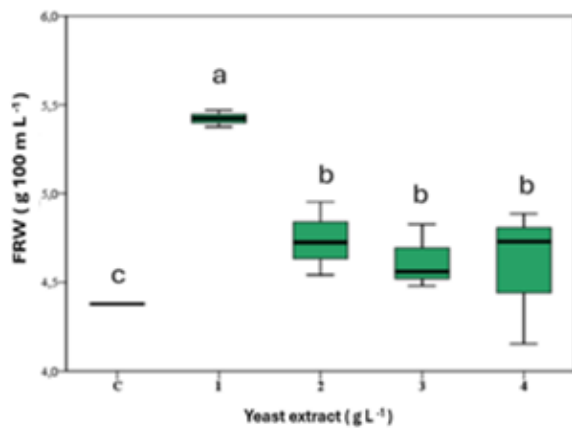
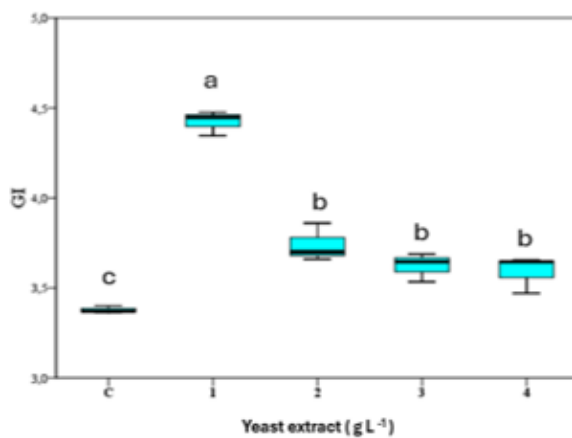


Figure 1

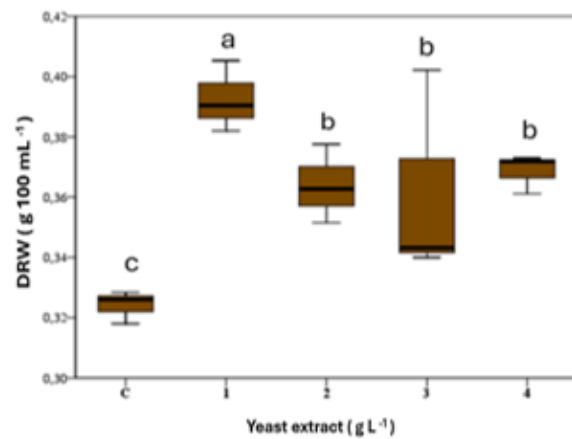
Schematic view of the steps of the study



A



B

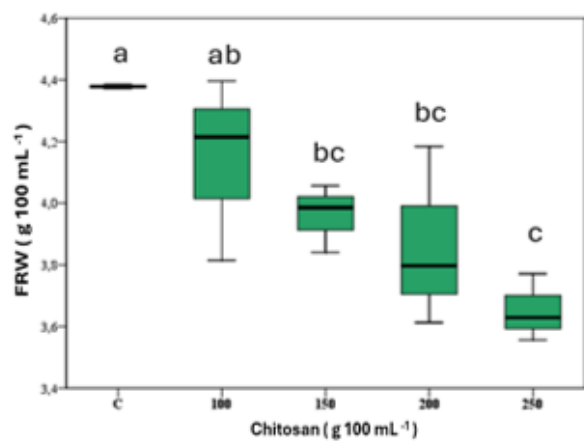


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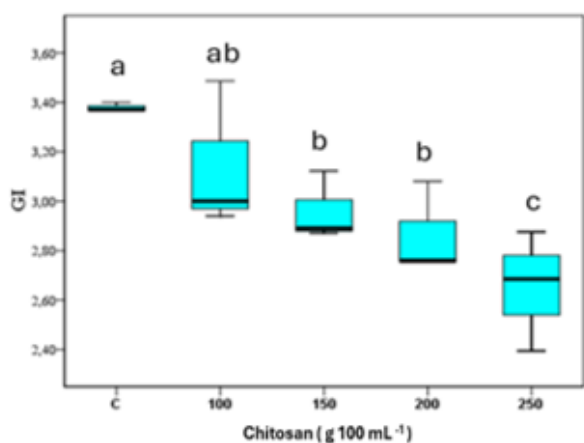
Figure 2

Effects of yeast extract on fresh root weight (A), root growth index (B), and dry root weight (C) in *Isatis tinctoria*

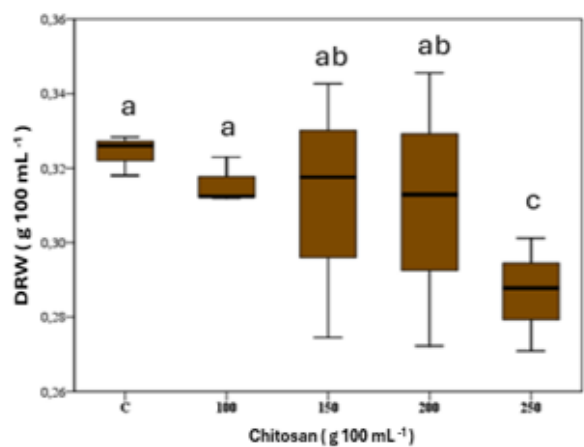
*Mean values with the same letter are not statistically significant ($p \leq 0.05$).



A



B

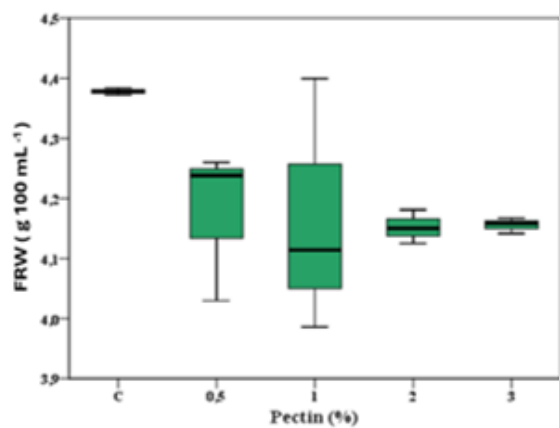


C

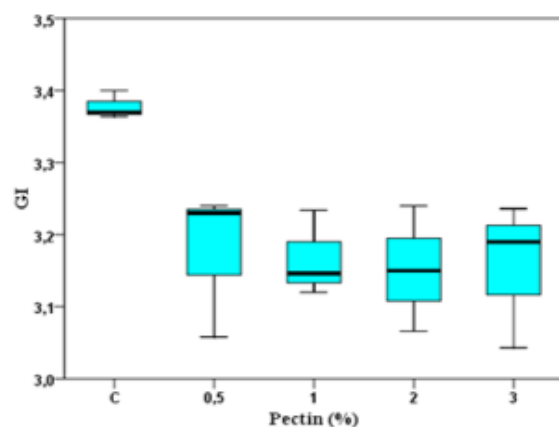
Figure 3

Effects of chitosan on fresh root weight (A), root growth index (B), and dry root weight (C) in *Isatis tinctoria*

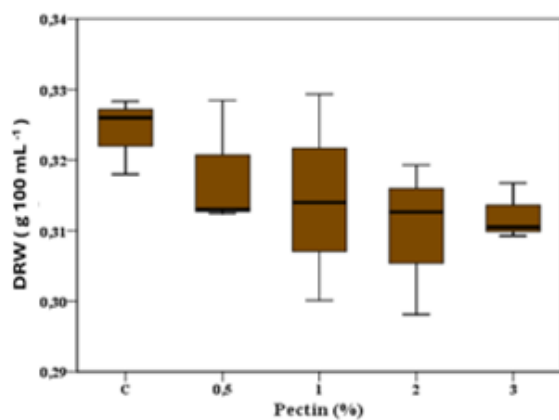
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A



B

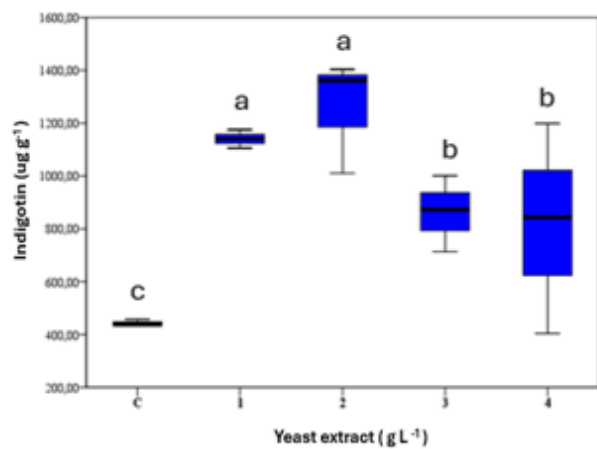


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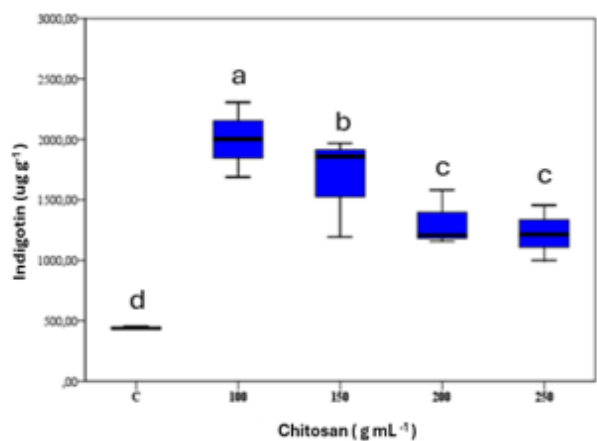
Figure 4

Effects of pectin on fresh root weight (A), root growth index (B), and dry root weight (C) in *Isatis tinctoria*

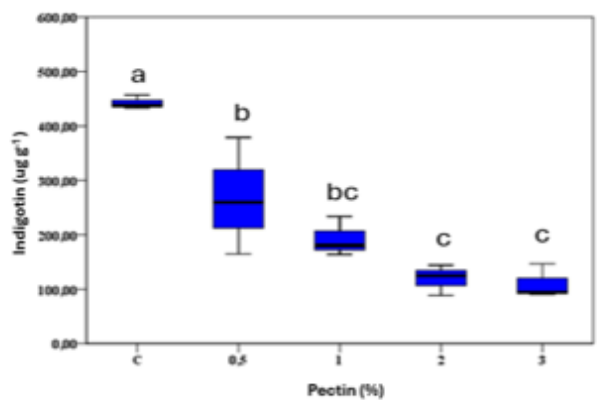
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A



B

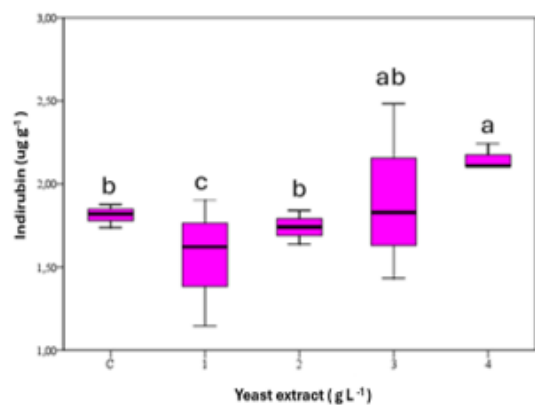


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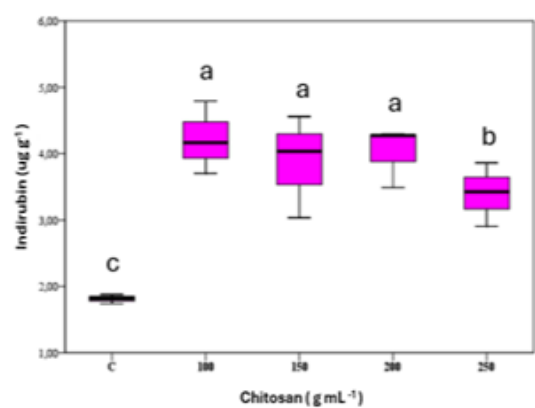
Figure 5

Effects of yeast extract (A), chitosan (B), and pectin (C) on indigotin accumulation in *Isatis tinctoria* roots

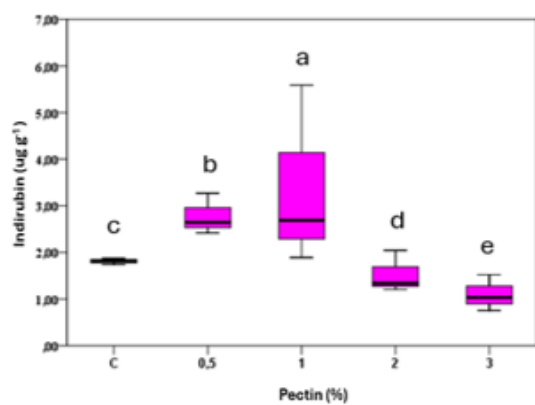
*Mean values with the same letter are not statistically significant ($p \leq 0.05$).



A



B



C

Figure 6

Effects of yeast extract (A), chitosan (B), and pectin (C) on indirubin accumulation in *Isatis tinctoria* roots

*Mean values with the same letter are not statistically significant ($p \leq 0.05$).

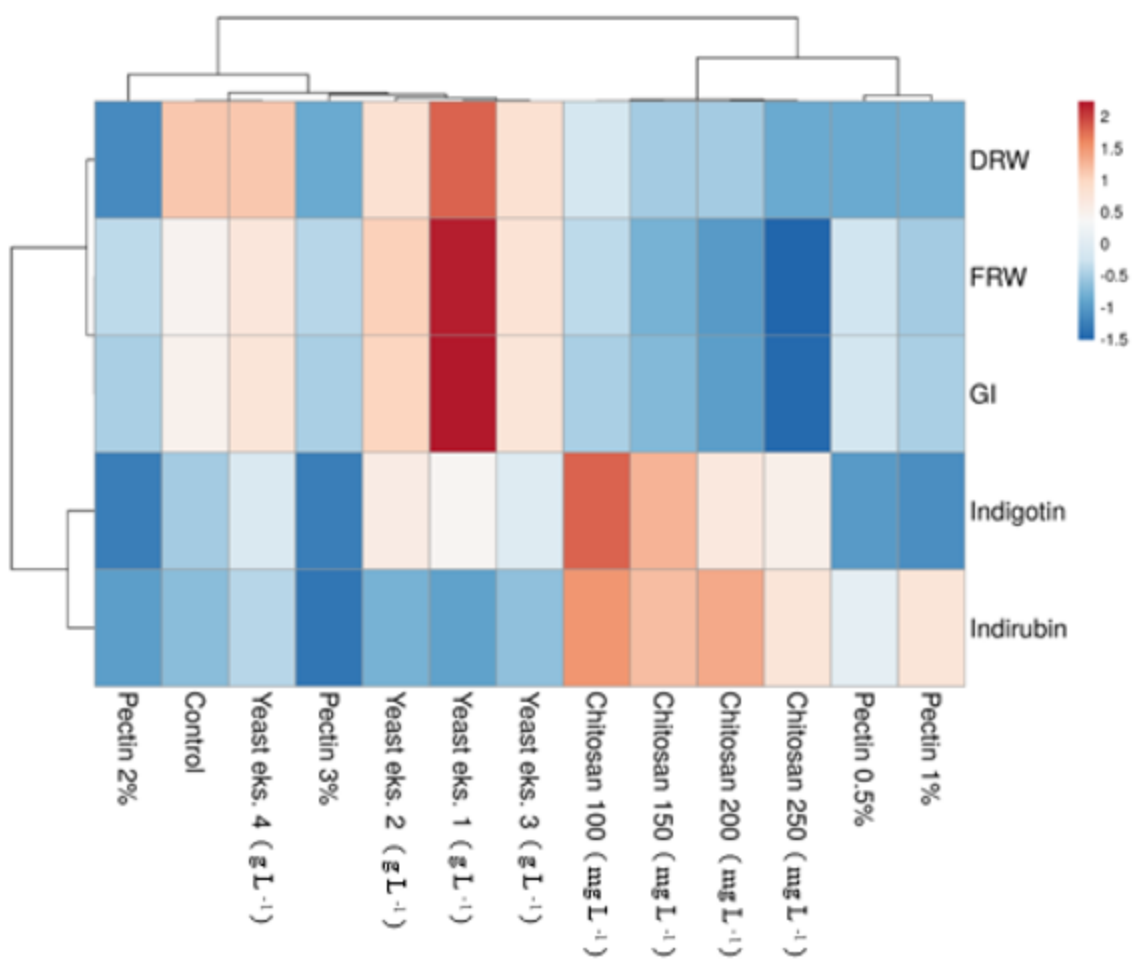


Figure 7

Heat map representing changes induced by applying biotic elicitors to root cultures in root growth and accumulation of indigotin and indirubin