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Research paper

Effect of chitosan on morpho-physiological traits and regeneration of *Iris pseudacorus* plantlets under *in vitro* conditions

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Abstract
Iris pseudacorus is a highly valuable ornamental and medicinal plant. Chitin is one of the most abundant polysaccharides in nature and is widely used in agriculture for seed germination to stimulate plant growth. Chitosan can be used as an antibacterial component and can increase plant resistance to diseases. This study was conducted based on a completely randomized design. Treatments consisted of different concentrations of chitosan (0, 5, 10, 20, 40, 80, and 120 ppm) with five replications. Morphological and physiological traits including leaf number, leaf weight, plantlets height, leaf area, percentage of regeneration, chlorophyll a, b and total, total phenol, and flavonoids were evaluated. Results of the analysis of variance showed that chitosan significantly affected leaf number, regeneration percentage, phenol content, and leaf fresh weight. Flavonoids, chlorophyll a and b, total chlorophyll were also significantly affected. Results also showed that the highest (31.60 mg/g gallic acid) and lowest (15.51 mg/g gallic acid) total phenol content was obtained from 120 ppm chitosan and control samples, respectively. The highest flavonoid content (5.78 mM/g) was obtained by 120 ppm chitosan and the lowest value (3.20 mM/g) was recorded in the control treatment. In general, our investigation showed that chitosan had a positive effect on all measured traits. In most of the measured traits, the best chitosan concentration was 120 ppm.

Keywords: chitosan; *Iris pseudacorus*; regeneration; secondary metabolites; tissue culture

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Introduction

Iris pseudacorus which is called the yellow iris and yellow flag, is a perennial herbaceous plant native to Europe, western Asia, and northwestern Africa. *Iris pseudacorus* grows near wetlands and tolerates waterlogging, low pH, and non-ferrous soils and is used as a wastewater treatment (Villasentor *et al.* 2007).

Plant tissue culture is a technique that makes it possible to produce and propagate a whole plant from different plant tissues. In addition, plant tissue culture can be a suitable means for the conservation of native or endangered species and genotypes as valuable resources of germplasm (Arikat *et al.* 2004).

In many plants, the use of bio-stimulators is one of the ways to reduce the harmful effects of stress and increase their yield and quality (Gornik *et al.* 2008). Several substances with elicitor properties have been identified, including chitosan, which stimulate the response to stress and defense mechanisms (Kowalski *et al.* 2006). Chitosan is an organic polymer made from the hard shells of aquatic animals such as crabs and shrimp, which has recently been considered for its antibacterial properties (Kumar *et al.* 2006). Also, the positive

effects of chitosan were reported on some traits in *Lilium regale* such as fresh weight, root number, regeneration percentage and total chlorophyll (Pourbeyrami Hir *et al.* 2021). The results of another experiment on the regeneration of *L. ledebourii* and *L. dandie* showed that chitosan significantly ($p \leq 0.01$) affected most of the morphological traits and secondary metabolites (Khalafi *et al*. 2021).

Today, the use of chitosan as a non-toxic, degradable, and environmentally friendly substance to reduce and improve the effects of various stresses, including drought (Dzung *et al.* 2011) and salinity stress (Ma *et al.* 2012) has been considered and many researchers have demonstrated increased yield and germination in plants with the use of chitosan (Guan *et al.* 2009; Mahdavi *et al.* 2014; Amiri *et al.* 2015). Khan *et al*. (2016) used different concentrations of humic acid (0, 1, 2, and 3 g/l) and chitosan (0, 40, 60, 80 mg/l) as a leaf spray and reported that these compounds significantly affected the growth and yield of the pea plant.

The effect of chitosan on the growth and reproduction of *Panisea uniflora* (Lindl.) plantlets showed that the culture medium containing 92 mg/l chitosan had the greatest effect on the morphological indicators (Ritti *et al.* 2016). Chitosan increased the fresh weight of roots and stems, soluble sugars, proline, phenols, and flavonoids of *Ocimum basilicum L.* plants under water-deficit stress and normal conditions (Malekpoor *et al.* 2017). The appropriate effect of different levels of chitosan on improving yield and growth traits of tomatoes and peppers (Monirul *et al.* 2018) and *Satureja isophylla L.* has also been reported (Salehi *et al.* 2016). The present study aimed to investigate the effect of chitosan on *in vitro* regeneration of *Iris pseudacorus.*

Materials and Methods

Plant materials

The present study was conducted in the Faculty of Agriculture and Natural Resources of Mohaghegh Ardabili University, Iran. The physiological dormancy of seeds was broken by placing the seeds in 20 molar NaOH solution for 24 hours (Sun *et al.* 2006). The seeds were then sterilized with 70% ethanol for 1 minute and 3% sodium hypochlorite for 15 minutes.

Media preparation and treatments

The sterilized seeds were cultured in a 1.2 MS medium. One month after seed germination, leaves and roots were removed from the plantlets, and the hypocotyl was cultured in a MS medium supplemented with 30 mg/L sucrose and 7 mg/L agar. The samples were treated with different concentrations of chitosan [0 (control), 5, 10, 20, 40, 80, and 120 ppm]. This experiment was performed as a completely randomized design with five replications. The cultured media were placed in a growth chamber at 24± 2 °C equipped with 2000-lx fluorescent lamps (16 h light and 8 h dark). After transferring the cultured explants to the growth chamber, daily controls were carried out to investigate changes in the growth and regeneration of explants and remove the infected cultures. After two months, the regenerated explants and plantlets were examined and the necessary data were recorded.

Measured traits

Characteristics measured in this study included plantlet fresh weight, plantlet height, number of leaves, leaf area (Maleki *et al*. 2018), regeneration percentage, total phenols (Slinkard and Singleton (1977), total flavonoids (Krizk *et al.* 1993), and chlorophyll a, b, and total chlorophyll content (Arnon 1949).

Statistical analysis

After analysis of variance, the treatment means were compared using Duncan's multiple-range test and the graphs were drawn using Excel software. The data were analyzed by SPSS software.

Results and Discussion

Plantlet fresh weight

Significant differences were observed in the morphologies characteristics of the *Iris pseudacorus* plantlets en treated with different chitosan concentrations (Table 1). All chitosan concentrations had significant and positive effects on the fresh weight of the plantlets. However, there were no significant differences among 5, 10, 20, 40, and 80 ppm chitosan concentrations. The highest fresh weight (3.4 g) was achieved in the media treated with 120 ppm of chitosan concentration (Figure 1).

Table 1. Analysis of variance of the chitosan effect on morphological characteristics of *Iris pseudacorus*

Source of variation	df	Mean Square				
		Fresh weight	Plant height	Leaf number	Leaf area	Regeneration percentage
Chitosan	6	0.572**	3.157*	57.448*	1.861**	9.26*
Error	28	0.073	0.682	15.749	0.171	0.029
CV (%)		17.02	14.36	25.25	30.23	13.15

*, **: Significant at 5% and 1% probability levels, respectively.

Plantlet height

Plantlet height was affected significantly when treated with different concentrations of chitosan (Table 1). The height of plantlets increased as the chitosan concentration increased. However, at lower concentrations (5, 10, 20, 40, 60, and 80 ppm) the effect was not significant. Chitosan at the rate of 120 ppm showed positive and significant effects on the *in vitro* plantlets' height compared to the control and lower concentrations (5, 10, and 20 ppm) (Figure 2).

Plantlet leaf number

The number of plantlet leaves was significantly affected by different concentrations of chitosan (Table 1). An increase in the chitosan concentration increased leaf number, however, concentrations higher than 20 ppm showed larger effects and were significantly different from the control and the treatment with 5 ppm of chitosan. Plants growing on media containing 120 ppm of chitosan produced about two times more leaves than the media containing no chitosan (Figure 3).

Leaf area

The effect of chitosan on leaf area index of the *Iris pseudacorus* plantlets was also significant at the 1% probability level (Table 1). There was an

increasing trend in leaf area with increasing chitosan concentration. Plants treated with 120 ppm chitosan had the largest leaves. On the other hand, the lower concentrations of chitosan did not affect leaf size significantly (Figure 4).

Regeneration percentage

As shown in Table 1, chitosan affected the regeneration of *Iris pseudacorus* plantlets significantly. However, lower concentrations had no significant effect on the regeneration percentage. On the other hand, 80 and 100 ppm of chitosan caused about 100% regeneration, which was more than 20% more than the control and lower concentrations (Figure 5).

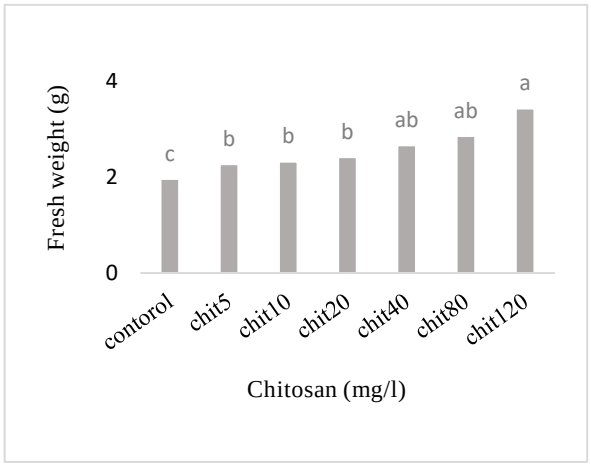

Figure 1. The effect of chitosan on the fresh weight of *Iris pseudacorus*.

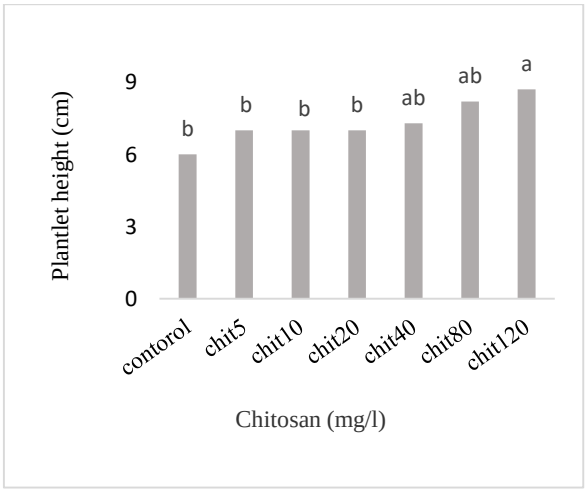

Figure 2. The effect of chitosan on the plantlet height of *Iris pseudacorus.*

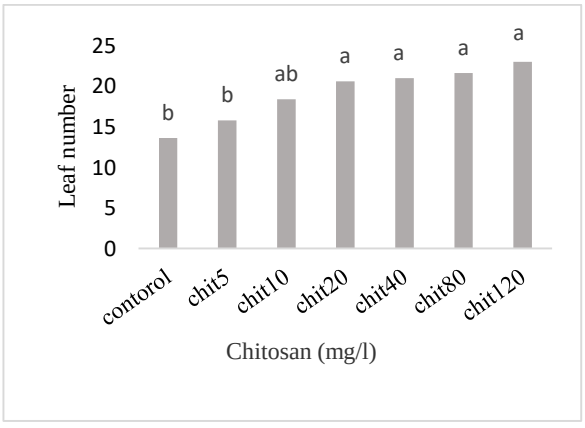

Figure 3. The effect of chitosan on the number of plantlet leaves in *Iris pseudacorus.*

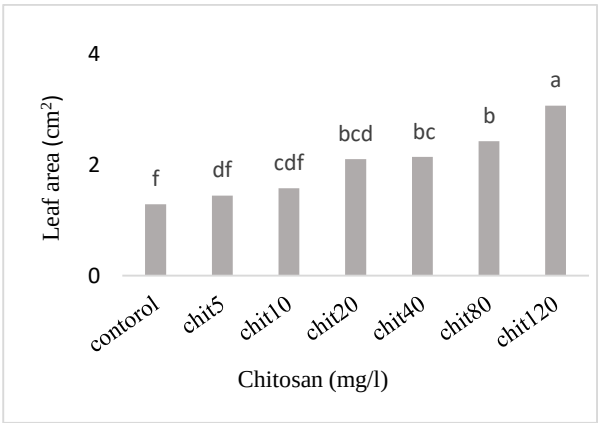

Figure 4. The effect of chitosan on the plantlet leaf area in *Iris pseudacorus.*

Chlorophyll content

The results showed that chitosan significantly affected chlorophyll a, chlorophyll b, and total chlorophyll content (Table 2). The amount of chlorophyll a, b, and total chlorophyll increased with the increase of chitosan. The highest chlorophyll content was obtained from the media containing 120 ppm of chitosan. There was no significant difference between the control and 5 ppm chitosan concerning chlorophyll content. (Figures 6, 7, and 8).

Table 2. Analysis of variance of the chitosan effect on some physiological traits of *Iris pseudacorus.*

Source of variation	df	Mean Square				
		Total flavonoids	Total phenols	Total chlorophyll	Chlorophyll b	Chlorophyll a
Chitosan	6	3.562**	101.694*	44.79**	5.71**	19.31**
Error	28	0.534	17.790	5.35	0.452	1.50
CV		23.3	27.94	37.19	38.60	37.57

*, **; Significant at 5% and 1% probability levels, respectively.

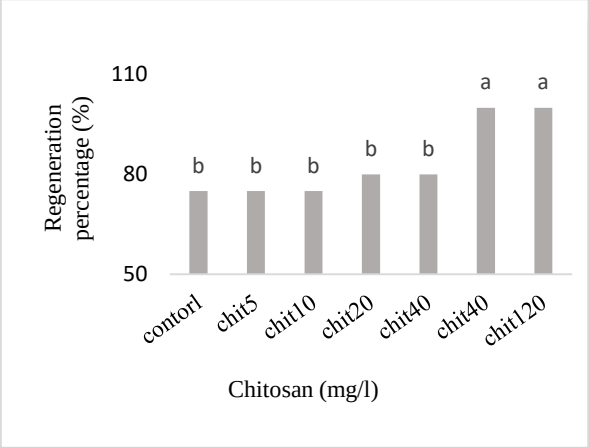

Figure 5. The effect of chitosan on regeneration percentage of explants of *Iris pseudacorus.*

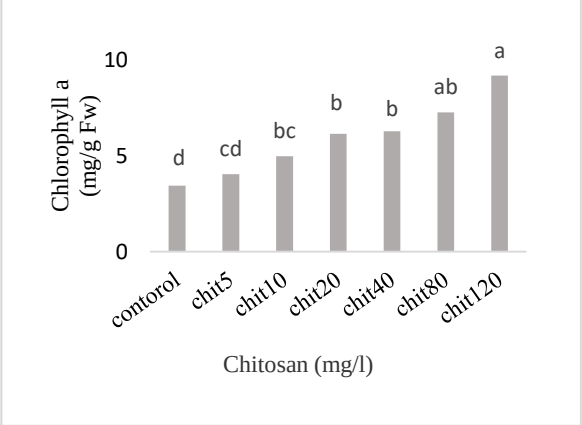

Figure 6. The effect of chitosan on the chlorophyll a content of *Iris pseudacorus* plantlets.

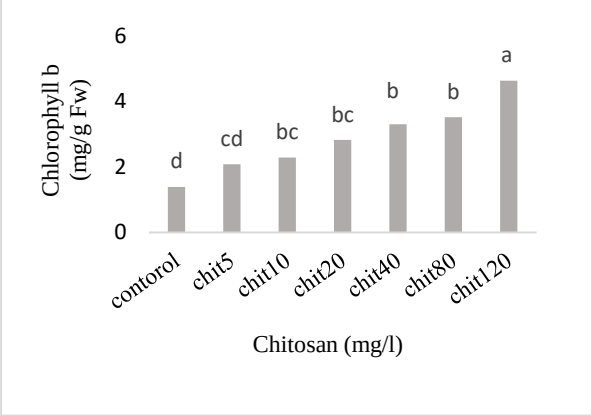

Figure 7. The effect of chitosan on chlorophyll b content of *Iris pseudacorus* plantlets.

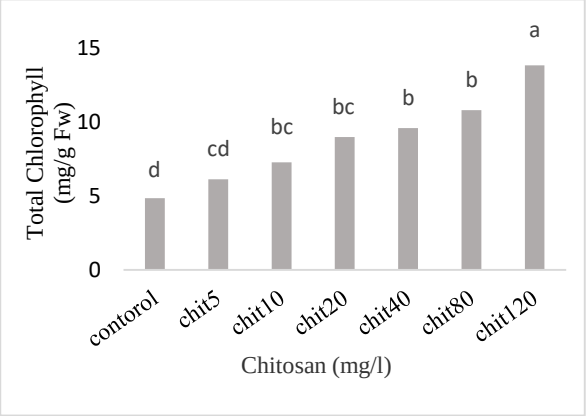

Figure 8. The effect of chitosan on the total chlorophyll content of *Iris pseudacorus* plantlets.

Total phenols

Total phenol content was influenced by the chitosan treatments (Table 2). There was an increasing trend in total phenol content with increasing chitosan concentration. Chitosan at the rate of 120 ppm had the highest total phenol content with the amount of 31.60 mg gallic acid per gram. The plantlets from the media containing concentrations higher than 20 ppm of chitosan had higher phenol content compared to the control (Figure 9).

Total flavonoids

Analysis of variance showed that chitosan significantly ($p \leq 0.01$) affected the total flavonoid content of plantlets of *Iris pseudacorus*. Similar to the total phenol content, the total flavonoid content increased with increasing the chitosan concentration. The concentration of 120 ppm chitosan produced the highest flavonoid content. The plantlets from the media containing higher than 20 ppm of chitosan, had higher flavonoids compared to the control (Figure 10).

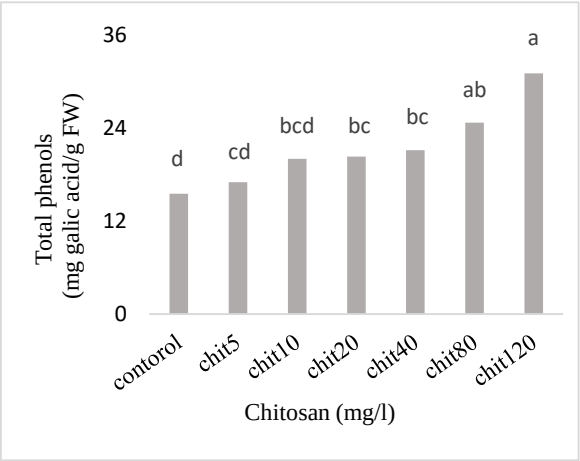

Figure 9. The effect of chitosan on the total phenol content of *Iris pseudacorus* plantlets.

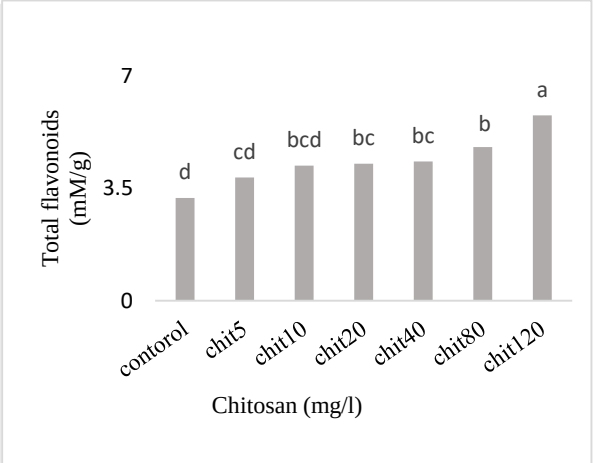

Figure 10. The effect of chitosan on the total flavonoid content of *Iris pseudacorus* plantlets.

Discussion

Plantlet fresh weight and height

In the present study, the highest fresh weight and height of the plantlets were observed at 120 ppm of chitosan. The stimulating effect of chitosan on plantlets growth is due to the increased absorption of water and essential elements and reduced accumulation of free oxygen radicals by increasing the activity of antioxidant enzymes. The reason for the increase in plant's weight and height following the use of chitosan is its effect on stimulating physiological processes, increasing CO2 stabilization, and improving vegetative growth, (Amiri *et al.* 2015). According to Mahdavi *et al.* (2014), chitosan increased the fresh weight of plantlets by stimulating the growth of stems and roots and thus increasing the absorption of water and nutrients and better transfer of these substances in the plant organs. The mechanism of action of chitosan on increasing plant growth may be related to the presence of nitrogen in the amino acids structure. Chitosan may regulate plant growth

through signal pathways that lead to auxin biosynthesis (Malekpoor *et al.* 2017). Nourafkan (2019) investigated the effect of chitosan on the fresh weight of *Lemon verbena* and found that with foliar application of chitosan, the fresh weight of the leaves increased and reached the highest level at 15 mg/l. Consumption of chitosan in Chinese cabbage increases the fresh weight of the plant (Ouyang and Langlai 2003). Forouzandeh *et al.* (2019) found that the highest height of the fennel (*Foeniculum vulgare*) plant with an average of 23.6 cm was observed in the treatment of 1.5 mM salicylic acid and 200 mg/l chitosan and the lowest height of the plant was observed in the control. Guan *et al.* (2009) also reported that chitosan has a positive effect on the growth and yield of maize, which is consistent with the results of the present study.

Leaf number and leaf area

In the present study, the highest number of leaves and the highest leaf area were observed in the treatment of 120 ppm chitosan, which was significantly different from the control treatment. Chitosan also affected and increased the leaf chlorophyll content. Chitosan consumption may increase chlorophyll production and leaf area by affecting the genes responsible for chlorophyll production (Yin *et al.* 2012). Studies have shown the positive effect of chitosan on the number of leaves of *Lemon verbena* (Nourafkan 2019) and *Salvia leriifolia* (Jami *et al.* 2018) and it has been found that with increasing the amount of chitosan, the number of leaves of plantlets increases. In another experiment, foliar application of chitosan (0.2 g/l) increased the number of *Satureja* leaves, which is consistent with the results of the present study. Khan *et al.* (2018) also showed that plant growth and yield were significantly affected by the combination of chitosan and humic acid. In another study, the effect of chitosan on the growth and yield of some plants such as soybean, rice, potato, lettuce, and radish was investigated. The results showed that chitosan has a strong effect on plant growth. The use of 0.5% chitosan was suitable for growing soybeans and rice, but its concentration of 0.1% had a greater effect on lettuce and potatoes. The lettuce leaf area increased by 50 to 60% compared to the control group after three times spraying with 0.1% chitosan and radish leaf area increased by 100% with 0.5% chitosan concentration (Chibu and Shibayama 2003).

Regeneration percentage

It has been indicated that chitosan promotes growth and development by increasing the activity of key enzymes in nitrogen metabolism (nitrate reductase, glutamine, and protease synthetase) and improving nitrogen transport (Mondal *et al.* 2016). In the present study, the highest regeneration was observed in 120 ppm of chitosan (Figures 5 and 11). The mechanism of action of chitosan is not fully understood but according to some researchers, it seems chitosan can stimulate the roots, growth, and induction of certain enzymes such as chitinase, pectinase, and gluconase (Hien 2004). Coskun *et al.* (2015) investigated the effect of chitosan on the hypocotyl, stem tip, and leaves

isolated from the *Melissa officinalis L.* plantlets. They reported that all concentrations of chitosan improved plant regeneration and the highest regeneration was observed in the chitosan concentration of 30 mg/l in the single-node sections. Pourbeyrami Hir *et al.* (2021) also observed that the higher concentration of chitosan lead to an increase in the regeneration percentage in *Lilium regael*.

Chlorophyll content

It seems that chitosan as an elicitor activates and increases the expression of genes in the biosynthesis pathway of the chlorophyll and as a result increases the amount of chlorophyll (Limpanavech *et al*., 2008; Yin *et al*. 2012; Malekpoor *et al.* 2017). Khaje *et al.* (2014) investigated the effect of chitosan elicitor on *Dracocephalum*. They reported that the chitosan increased the amount of chlorophyll pigments. Dzung *et al.* (2002) reported that chitosan increased the chlorophyll content of soybeans, peanuts, and coffee. These results are in agreement with the present study regarding the increase in plant chlorophyll content with increasing chitosan concentration.

Salachna and Zawadzinska (2014) reported that the use of chitosan increased the amount of chlorophyll content in the coffee leaf. Due to the existence of nitrogen in chitosan and the structural role of this element in the chlorophyll tetrapyrrole rings, this increase is explainable. Yadollahi Dahcheshme *et al.* (2012) stated that using chitosan increased the chlorophyll a and b content in the sunflower such that fiver grams per liter chitosan increased the chlorophyll a and b content by 17.11% compared to the control. Limpanavech *et al.* (2008) investigated the effect of chitosan on the orchid plant *Dendrobium nobile*. They observed that the amount of chloroplasts in the treated young plants leaves with concentrations of 10 and 50 ppm chitosan was significantly higher than the control group. Naderi *et al.* (2015) indicated that chitosan can act as a bio-elicitor and increase the chlorophyll content in *Ocimum basilicum* plant and also can increase the activity of antioxidant enzymes and secondary metabolites.

Total phenol and flavonoid content

Phenolic compounds are potent inhibitors of oxidative stress and participate in the accumulation or removal of hydrogen peroxide in cooperation with peroxidases (Michalak *et al.* 2006). In the present research, the chitosan increased total phenols and flavonoids at the 120 ppm concentration. Although bio-elicitors such as chitosan have been widely used in the production of secondary metabolites, their mechanism of action on the production of secondary metabolites in plants is not well understood. Generally, elicitors detect cells by stimulating cellular signals and molecular interactions between plant receptors at the cell membrane or cytoplasmic level. As a result, the signal received by the plant cells stimulates the expression of related genes on the pathway and causes the synthesis of secondary metabolites (Zhao *et al.* 2003). Different factors such as the source of the elicitor, its specificity, the concentration of the elicitor, the growth stage of the plant, the time of adding the elicitor, and the length of time the plant is exposed to the elicitor, increase the production of secondary metabolites

Figure 11. The effect of chitosan concentrations on in *vitro* regeneration and proliferation of *Iris pseudacorus.*

(Vasconsuelo *et al.* 2007).

Chitosan has increased the phenolic compounds in the tomato plant (Liu *et al.* 2007). Palida *et al.* (2014) reported that the foliar application of chitosan in the tea plant and white flax significantly increased the phenol content of the leaves compared to the control group. Kim *et al.* (2007) indicated that chitosan as a bio-elicitor probably has the potential for removing free radicals. Khalafi *et al.* (2021) investigated the effect of chitosan on the production of the secondary metabolites of two species of lily flower and revealed that chitosan can positively affect the production of secondary metabolites and in most cases the highest concentration of this component enhanced the productivity of the measured traits.

According to Naderi *et al.* (2015), chitosan improves the biosynthesis of secondary metabolites, increases carbohydrates in the roots, and increases growth in the basil plant. Emami Bistagani *et al.* (2015) investigated the effect of chitosan on thyme. They found that with increasing chitosan concentration, the total phenol content in thyme increased. Forouzandeh *et al.* (2019) also observed that with increasing salicylic acid and chitosan, the total phenol and flavonoid content of The fennel increased. Chitosan also increased the production of flavonoids in *Salvia leriifolia* (Jami *et al.* 2018). These results again are consistent with the results of the present study.

Conclusion

In the present study, the morphological characteristics of *Iris pseudacorus* was positively affected by high concentrations of chitosan (80 and 120 ppm) and this compound increased the plantlets' height, fresh weight, leaf area, and number of leaves. Moreover, application of chitosan increased the biochemical characteristics, and the highest amount of total phenols and flavonoids was obtained at 120 ppm of Chitosan. Also, chitosan increased the chlorophyll a, b, and total chlorophyll, and the highest amount of these traits was observed at the concentration of 120 ppm chitosan. In general, the highest concentration of chitosan (120 ppm) most efficiently improved the morphological, physiological, and biochemical characteristics of *Iris seudacorus* and can be used in the mass cultivation of this plant.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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اثر کیتوزان بر صفات مرفوفیزیولوژیکی و باززایی گیاهچه‌های *Iris pseudacorus* در شرایط درون شیشه‌ای

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چکیده

زنبق زرد یک گیاه زینتی و دارویی بسیار ارزشمند است. کیتین یکی از فراوان ترین پلی ساکاریدها در طبیعت است و به طور گسترده در کشاورزی برای جوانه‌زنی بذر به منظور تحریک رشد گیاه استفاده می‌شود. کیتوزان میتواند به عنوان یک ماده ضد باکتری استفاده شود و مقاومت گیاه را در برابر بیماری‌ها افزایش دهد. این مطالعه بر اساس طرح کاملا تصادفی انجام شد. تیمارها شامل غلظت‌های مختلف کیتوزان (۰، ۵، ۱۰، ۲۰، ۴۰، ۸۰ و ۱۲۰ پی پی ام) با پنج تکرار بود. صفات مورفولوژیکی و بیوشیمیایی شامل تعداد برگ، وزن برگ، ارتفاع بوته، سطح برگ، درصد باززایی، کلروفیل a، b و کل، فنل کل و فلاونوئیدها مورد ارزیابی قرار گرفتند. نتایج تجزیه واریانس نشان داد که کیتوزان بر تعداد برگ، درصد باززایی، محتوای فنل و وزن تر برگ تأثیر معنی‌داری داشت. فلاونوئیدها، کلروفیل a و b، و کلروفیل کل نیز به طور قابل توجهی تحت تأثیر قرار گرفتند. همچنین نتایج نشان داد که بیشترین (۳۱/۶۰ میلی‌گرم در گرم اسید گالیک) و کمترین (۱۵/۵۱ میلی‌گرم در گرم اسید گالیک) فنل کل به ترتیب از ۱۲۰ پی‌پی‌ام کیتوزان و نمونه شاهد به‌دست آمد. بیشترین مقدار فلاونوئید (۵/۷۸ mM/g) توسط ۱۲۰ پی پی ام کیتوزان و کمترین مقدار (۳/۲۰ mM/g) در تیمار شاهد به دست آمد. به طور کلی، این بررسی نشان داد که کیتوزان بر تمامی صفات اندازه گیری شده اثر مثبت داشت. در اکثر صفات اندازه گیری شده بهترین غلظت کیتوزان ۱۲۰ پی پی ام بود.

واژه‌های کلیدی: باززایی؛ کشت بافت؛ کیتوزان؛ متابولیت‌های ثانویه؛ *Iris pseudacorus*