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Evaluation of Chitosan-Enriched Medium in Improving Strawberry Micropropagation

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Abstract

The study focused on the possibility of using chitosan in the culture medium for propagating strawberry plants by tissue culture while ensuring reduced culture contamination and increased plantlets growth. The growing tip of the strawberry plant Ruby Gem were cut, superficially sterilized and then planted in the MS medium treated with different concentrations of chitosan (0, 5, 10, 15 mg L^{-1}). The results showed that adding chitosan to the growth medium in the stage of vegetative branches formation was effective in reducing contamination to 10%, increasing the percentage of open buds by 100%, and the average number of branches to 2.75 branches, which increased with increasing the concentration to 10 mg L^{-1}. When it came to branch length, there was no change between the normal medium and the medium that had chitosan added to it. But the amount of stems, leaves, fresh weight, and dry weight were very different between the two media. This was because the amount of chitosan changed these things. The amount of chitosan that was 10 mg L-1 had the most branches, the freshest weight, and the least dry weight. The average was 6.66 branches, 3.96 g of fresh weight, and 0.30 g of dry weight. The group that got 5 mg L-1 had the most leaves, with an average of 5.33 a branch. The best rate was seen in the treatment that had the fewest leaves—2.66 per branch. Put the branch in the medium that had 15 mg L-1 of chitosan added to it. The results showed that supplementing the nutrient medium with different concentrations of chitosan significantly affected the rooting percentage of vegetative branches, the average number of roots and root length with significant differences among the concentrations used. The chitosan at 10 mg L^{-1} resulted in significantly higher rates of rooting percentage (80%), the number of roots (8.10 root $plantlet^{-1}$ and the root length (10.20 cm), respectively.

Keywords:

Strawberry, chitosan, micropropagation.

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Introduction

In the Rosaceae family, there are many different types of plants. The strawberry is one of them. This plant can handle both highs and lows because it comes back every year. You can find it in the wild or in a yard. People all over the world grow the small fruit plant that makes it because it is useful and good for you (Al-Saeedi, 2000). Strawberries are propagated in several ways, including sexual reproduction by seeds or vegetatively by dividing the crown, or by runners. However, vegetatively propagated plants are more vulnerable to infection by pathogens, especially viruses, which are aphids transmitted from infected seedlings to healthy ones. This causes weak plant growth and decreased productivity (Husaini & Xu, 2016). Therefore, attempts were made to propagate economically important strawberry varieties by tissue culture technology using the apical meristem, branch tips or runners to produce new plants free of pathogens and reduce production costs (Rokosa & Mikiciuk, 2017; Campos, 2024).

There are some things that must be in place for tissue culture to work well. What's most important is how clean something is. For this method to work, it needs to be clean (Xie & Fang, 2024). There are different kinds of pollution, like bugs and fungus. They hurt the system from the time it is set up until it grows roots and even grows new plants. Microbes can stop or damage this process at any time, even when it is just starting. Before they are used in tissue culture, plant parts are usually picked up in the field or yard and brought to the lab to be cleaned of germs. On the other hand, a bacterial sickness generally shows up late in the growth process. This makes it more likely for the plants to get hurt and waste time and work (Orlikowska et al., 2017; Murashige & Skoog, 1962).

Chitosan ($C6H_{11}O_4N$)n is a carbohydrate polymer derived from the removal of the acetyl group from the kinetin compound, usually prepared from crustaceans such as shrimp and crabs (Bibi et al., 2021). Being a non-toxic, easily degradable, and naturally derived compound, it is used in a wide range of fields, especially in the agricultural and industrial fields, food preservation, pharmaceutical industries, and many medical uses) (Dias et al., 2013). On the other hand, chitosan shows an anti-fungal, bacterial, and viral effect on soil and plant pathogens, so it is used in sustainable agriculture instead of pesticides. Recently, the role of chitosan in regulating the immune system, secreting enzymes, and activating cells has been observed, and it has also been proven to increase the production of phenolic substances that determine the activity of fungi (Xing et al., 2015). Youssef (2016) found that the best combination (0.5 mg L^{-1} IBA + 1.5 mg L^{-1} TDZ + 25 mg L^{-1} chitosan) in the propagation of strawberry cultivar Festival had the highest rate of number of branches formed from roots, while increasing chitosan in the same combination to 50 mg/L led to the highest rate of number of renewed branches from sepals and petals. The results of the same study indicated that chitosan 25 or 50 mg L^{-1} significantly affected the multiplication of vegetative branches and accelerated the emergence of vegetative branches by 5-7 days with an increase in the number of plantlets. Studies also indicate the efficiency of adding chitosan to the culture medium in positively affecting plant height and increasing the number of vegetative branches of East Java (Mastuti et al., 2021), while it was noted that adding chitosan to the tissue culture medium in banana Musa spp. led to enhanced plant growth and did not allow any fungal or bacterial contamination (Kandha et al., 2021). Based on the above, and the importance of chitosan and its distinctive properties, especially in controlling fungal, bacterial and viral pathogens in soil and plants, the research aimed to introduce chitosan into the strawberry micropropagation program to accelerate plant growth and reduce contamination with pathogens that negatively affect the growth and performance of the resulting plantlets (Doğdu et al., 2021; Pavlenko et al., 2020).

Materials and Methods

The research was done at the University of Kufa from November 2022 to November 2024. It took place in the Plant Tissue Culture Laboratory, the Horticulture Department, and the Horticulture and Landscape Engineering Department in the College of Agriculture. The study included testing the effect of chitosan in strawberry propagation programs using tissue culture technology by conducting 3 separate experiments to test the effect of chitosan in the stages of tissue propagation of strawberries, Ruby Jim variety, the formation stage, the vegetative branch multiplication stage, and the rooting stage.

Preparation of the Culture Medium

It was Mud MS from Murashige and Skoog that was used. A culture platform that is already made. It was made by the Indian company Himedia. 4.43 g of the ready-made culture medium was dissolved according to the manufacturer's recommendations in 600 ml of distilled water free of ions with the addition of growth regulators in different concentrations, prepared in advance as stock solutions (stored at 4°C) with the addition of sucrose 30 g. L^{-1}, then the volume was completed to 1 liter.

The pH of the medium was adjusted to 5.7 ± 0.1 with a 1-norm solution of hydrochloric acid (HCl) or a 1-norm solution of sodium hydroxide (NaOH), and 7 g of agar (type Agar-Agar) was added to it. The components of the medium were mixed and the agar was dissolved using a hot plate magnetic stirrer. The culture medium was then distributed into bottles that had been previously sterilized with a sterilizer at a rate of 40 ml for each vial. The jars had plastic lids on top, and the medium was cleaned in a sterilizer for 20 minutes at 121°C and 1 bar of pressure. After being cleaned, it was put away in a clean place until it was time to use it.

Effect of Chitosan in the Development Stage

The Ruby Jim strawberry type's growing tip was used while the plants were growing. They cut the strawberry's growing tip in half, making each half 2 cm long. First, they cut off the root and any other plants that were growing on it. After running water was used to clean it, 0.5% dangerous botanol was put on it and mixed for 15 minutes with magnets. After that, it was cleaned for 10 minutes with a 3.0% (vol/vol) sodium hypochlorite solution. After that, the plant parts were washed three times, with clean water for five minutes each time. A clean needle was used to move the growth tip to the culture tubes. Then, 10 ml of it was put into MS medium that had 0.5 mg L-1 benzyl adenine and 0.1 mg L-1 IBA in it. There are 15, 10, 5, and 0 mg L-1 of chitosan in a test bottle. There are ten copies of each type. The cultures were kept in a room with 1000 lux of light and a temperature of 25°C for four weeks. There were 16 hours of light and 8 hours of dark every day. As soon as the incubation time was up, the reaction rate (%), the number of branches that formed, and the average length of those branches were all written down. Strawberry shoots grown from plants tips 30 days after planting shown in Figure 1.

Figure 1. Strawberry shoots grown from plants tips 30 days after planting

Effect of Chitosan on the Vegetative Branches Multiplication Stage

An experiment was conducted to show the effect of adding chitosan at concentrations (15, 10, 5, 0 mg L^{-1}) to the multiplication medium (MS medium containing 1 mg L^{-1} benzo adenine in the presence of 0.1 mg L^{-1} IBA) Twenty copies of each treatment were put in the cutting room. They were left there for six weeks with 1000 lux of light, 16 hours of light and 8 hours of darkness. Then, the green group's average number of stems, average branch length, average number of leaves, and average fresh and dry weight (g) were all found.

Effect of Traditional Chitosan on the Rooting Stage of Branches

The branches resulting from the vegetative multiplication stage (from the best treatment) were transferred to MS medium to show the effect of adding four concentrations of traditional chitosan to the MS medium for rooting (15, 10, 5, 0 mg L^{-1}) and the plants were incubated in the growth chamber for 6 weeks where measurements included Rooting percentage, number of Roots and Root length (cm).

Results and Discussion

Efficiency of chitosan in the tissue culture program for strawberry propagation in the seedling establishment stage the results Figure 2 indicate that despite the surface sterilization, the percentage of contamination of the plant parts grown in the nutrient medium in the control treatment was high compared to the percentage of contamination in the medium provided with chitosan in the culture tubes. The chance of pollution going wrong went down as the chitosan level in the food medium rose. Around 10 to 15 mg L-1 was the range where the least contamination was found. Adding chitosan to the food solution changed the reaction rate in a big way as well. All of the buds on the part of the plant that was grown in the MS nutrient medium with 10 and 15 mg L-1 chitosan opened up during ex vivo growth, while only half of the buds on the control treatment opened up. Different amounts of chitosan were used to make the growth medium, and the number of branches also changed in big ways. With 2.75 branches per liter, the MS growth medium made with 10 mg L-1 chitosan had the best rate. In the control group, on the other hand, the rate never went above 1.32 branches. Whether chitosan was present or not did not change how the medium changed the length of the branches.

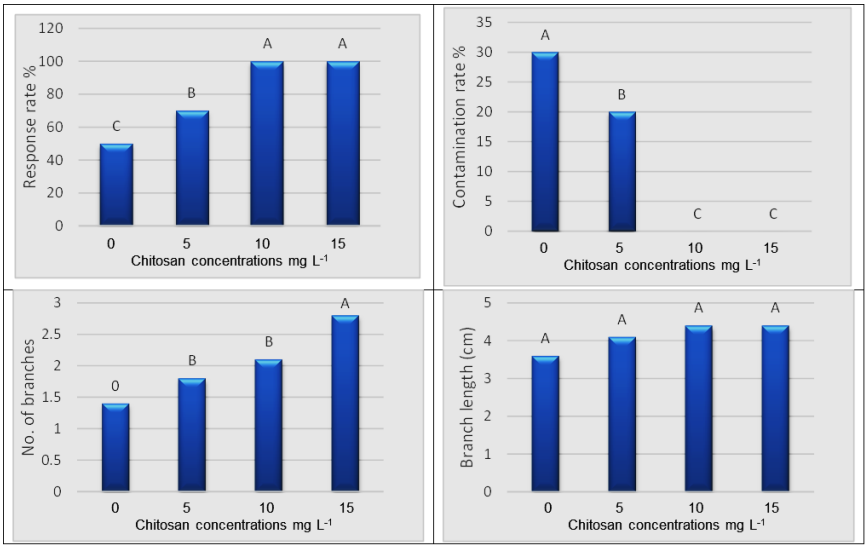

Figure 2. Effect of culture medium treated with chitosan on plantlets contamination %, growth response (%), and the number and length of branches of strawberry plantlets after 4 weeks

Branch Multiplication Stage

As for the effect of chitosan on growth characteristics in the multiplication stage, the results Table 1 indicate that the number of branches, number of leaves, fresh weight and dry weight recorded a significant increase in the medium prepared with chitosan compared to the standard medium, while the control treatment (standard medium) did not differ from the medium supplemented with chitosan in branch length, although the highest average branch length of 4.16 cm was recorded in the nutrient medium prepared with 10 mg L^{-1} chitosan. When it came to fresh weight, this group had the most (6.66), the least (0.30 g) dry weight, and the mostgreen weight. All the other groups and the control group had 3.00 branches, 0.73 g, and 0.14 g, respectively. This was very different. At least 5.33 leaves were on each branch of the medium that had 5 mg L-1 chitosan added to it. At most 2.66 leaves were on each branch of the medium that had 15 mg L-1 chitosan added to it.

Table 1. Effect of culture medium treated with chitosan on strawberry plantlets growth indicators at multiplication stage after 4 weeks

Chitosan mg L^{-1}	**No of branches Branch plantlet^{-1}**	**Branch length cm**	**No of leaves Leaf branch^{-1}**	**Fresh weight (g)**	**Dry weight (g)**
0	3.00b	3.46a	3.66b	0.73d	0.14b
5	2.33b	3.33a	5.33a	1.20c	0.12b
10	6.66 a	4.16a	3.66b	3.96a	0.30a
15	4.00b	3.33a	2.66b	3.36b	0.13b

It's the average of 10 tests that's shown. After the same letter(s) in the same column, Duncan's multiple range test ($P \leq 0.05$) shows that the means do not change.

Rooting Stage

The number of roots that grew changed a lot depending on how much chitosan was used Table 2. There were only 30% roots in the other treatment, but the medium made with 10 mg L-1 of chitosan worked better. In other words, 80% of the plants that were studied did grow roots. The average number of roots changed a lot depending on how much chitosan was used. The same table showed these changes. There were 8.10 sprouted roots per branch in the medium with 10 mg L-1 chitosan, compared to 2.42 sprouted roots per branch in the standard medium. In the treatments with chitosan, the roots also grew at different rates. In the MS medium treatment with 10 mg L-1 chitosan, roots that were 10.20 cm long were longer than roots that were no longer than 7.50 cm long in the control treatment.

Table 2. Effect of culture medium treated with chitosan on rooting indicators of strawberry Ruby Gem plantlets at rooting stage after 4 weeks

Chitosan mg L^{-1}	**Rooting rate (%)**	**No. of roots (root plantlet^{-1})**	**Root length (cm)**
0	30.00b	2.42b	7.50bc
5	40.00b	3.42b	6.50c
10	80.00a	8.10a	10.20a
15	70.00a	7.42a	8.50b

Values are means of 10 replications, where means followed by the same letter(s) within in a column do not differ according to Duncan's multiple range test ($P \leq 0.05$).

Discussion

Tissue culture can work or not work depending on many easy factors. Clean sets are the most important of these. One big reason the micropropagation system fails at different times is because of these things. They can happen when it is starting up, spreading, or even growing. It doesn't matter if they are bacterial or fungal waste. Small living things in the growing medium can stop or hurt the micropropagation at any point in the process. Most of the time, parts of plants that were found in the yard or field are brought to the lab to be germ-free and then grown in tissue culture. Some plants, like bananas, strawberries, and palm trees, are already well on their way to making more plants when bugs start to show up in labs that grow plant tissue. This hurts the crops, which takes time and money (Abdel-Karim, 2017). By adding chitosan to the strawberry plants' food during their growth stage, the response time was sped up and there was less contamination. Chitosan has been used in other studies to lower the activity of germs and the amount of contamination in banana tissue cultures, and the results were similar (Kandha et al., 2022).

Despite the great effectiveness of chitosan as antifungal and antibacterial, the mechanism of action of chitosan against bacteria, whether Gram-negative or positive, is not fully understood. In general, one of these mechanisms involves the nature of the charge between chitosan and bacterial membranes, as negative charges are present in the membranes of Gram-negative bacteria due to the presence of highly electrically charged groups on the lipopolysaccharides and phospholipids that compose them. Researchers suggest that the membrane is the place where chitosan (which has a positive charge) exerts its antimicrobial effects. Chitosan is most active on the surfaces of bacterial cells, where the positive charge of the protein amino group of chitosan interacts with the negatively charged molecules present on the bacterial membrane, which leads to the disruption or restriction of various proteins, which ultimately leads to the death of the bacterial cell (Ke et al., 2021). In addition, soluble chitosan increases the permeability of cell membranes and thus It forces bacteria to empty their contents (Jayakumar et al., 2008). Soluble chitosan also covers bacterial cells and closes the entry and exit points of materials necessary for bacterial growth, thus restricting and killing the cell (Liu et al., 2006).

A positive effect of the chitosan-treated medium on the response rate to bud opening was also observed, which enhances the ability of chitosan to activate and promote meristematic tissues towards cell division and lateral bud growth in the cultivated plant, thus improving overall growth and enhancing vegetative indicators, including branch numbers (Ewhayid et al., 2023). The results agree with (Bayraktar et al., 2016) in Stevia rebaudiana (Acemi et al., 2018) in Ipomoea purpurea, Govindaraju and Indra (Govindaraju & Arulselvi, 2018) in Coleus aromaticus, and (Abogarra et al., 2022) in date palm that treating the culture medium with chitosan gave the best results in both bud opening and bud and leaf numbers.

A clear effect was also recorded in increasing the number of branches in the medium treated with chitosan used to propagate strawberries in the branch multiplication stage. This is in agreement with (Safana et al., 2022a) in Kimquats (Al-Mayahi, 2022) in date palms (Krupa-Malkiewicz et al., 2022) in grapes, and (Ewhayid et al., 2023) in Moringa. The increase in the number of branches due to the addition of chitosan to the nutrient medium is due to the ability of chitosan to stimulate various physiological activities and processes that occur in the plant part, and this is positively reflected in increasing growth and thus improving vegetative indicators, including the number of branches (Charoenwattana & Petprapai, 2013). The addition of chitosan to the nutrient medium may also lead to a temporary increase in endogenous cytokinin levels in plant tissues (Acemi et al., 2018). These slight changes by adding chitosan to the culture medium are the reason for the success of the medium in stimulating branch multiplication, as it was found that adding chitosan to the nutrient medium for strawberry multiplication caused an increase in the number of branches

compared to the control treatment (Youssef, 2016). In addition to the ability of chitosan to activate enzymes and activate various physiological activities due to its ability to cause positive changes in improving growth indicators, including increasing the number of leaves and nodes on the cultivated plant part (Kanchanapoom et al., 2012: Amin, 2013).

Since chitosan is a polysaccharide, it has a positive effect on the process of plant nutrition and strengthening the defense system and may cause an increase in carbon metabolism to double (Ait Barka et al., 2004). In addition, chitosan can chelate nutrients and minerals such as Fe and Cu, which can reduce fungal damage and increase branch growth, with an effect similar to cytokinins important in branch multiplication (Acemi et al., 2018). The results also indicated an increase in the rooting percentage and the number and length of roots in plants grown on the chitosan-enriched medium. This indicates the possibility of using chitosan as a plant growth enhancer by stimulating the biosynthesis signal of some plant hormones such as auxins, as it enhances growth and development through some signaling pathways related to auxin biosynthesis via the tryptophan-independent pathway. This is consistent with previous studies that chitosan stimulates auxin accumulation (Lopez-Moya et al., 2017; Al-Mayahi, 2022). Making cell groups divide is a key part of micropropagation, and auxins help with this. It is also a plant hormone that helps different parts of the plant grow and develop. Chitosan can be thought of as an alternative to or along with the growth factors that are usually made in a lab. Chitosan mostly changes the shape of things in the lab, like how many shoots, roots, and leaves there are (Acemi et al., 2018).

Experimental Design and Statistical Analysis

The tests were given once, and then they were given three more times. The amount of chitosan was the only thing that mattered in their simple experiment design (CRD). There were three copies of each treatment, and each copy had a plant part from the start, growth, and root stages (Compton, 2018). There was an Analysis of Variance in 12th grade that was based on VSN International GenStat 2009. An Apple computer was used to set it up (VSN International, 2009).

Conclusion

The results showed that the culture medium prepared with different concentrations of chitosan used for strawberry propagation by tissue culture showed a positive effect on the vegetative branches formation stage and reduced the contamination rate, as well as increased the percentage of open buds and the average number of branches. The results also indicated that the culture medium treated with chitosan, especially when used at a concentration of 10 mg L^{-1}, significantly enhanced the vegetative growth indicators (number of branches, number of leaves, fresh and dry weight) in addition to its positive effect on the vegetative branches rooting stage by increasing the rooting percentage and the number and length of roots.

Author Contributions

All Authors contributed equally.

Conflict of Interest

The authors declared that no conflict of interest.

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