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Investigating auxin import and export proteins in *Chlorella vulgaris*

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SUMMARY

***Chlorella vulgaris* is a unicellular freshwater green alga that has promising applications in biofuel and food production. Current research on *C. vulgaris* focuses on cell wall degradation methods to extract nutrients, but research into other areas of *C. vulgaris* can improve current methods of resource production. One clear candidate for further study is auxin, a plant hormone that stimulates cell division and macromolecule accumulation in *C. vulgaris*; auxin secretion has also been implicated in cell communication in other species of algae. Unfortunately, *C. vulgaris*' response to auxin is not well studied, and it is unknown if *C. vulgaris* even secretes auxin. To shed light on these unknowns, we hypothesized that *C. vulgaris* possesses membrane-bound auxin transport proteins. We tested this hypothesis by treating *C. vulgaris* with cellulase to create protoplasts, cells without cell walls, and observing whether they swelled in response to auxin. Provisional evidence for swelling helped set up further experiments assaying *C. vulgaris* cultures for auxin secretion. Protoplasts were treated with supernatant extracted from centrifuged cell culture and then observed for swelling. The results of the experiment showed significant increases in protoplast size, indicating auxin presence in the supernatant, and suggest that *C. vulgaris* is capable of secreting auxin. In addition, the rapid swelling of protoplasts suggests the presence of auxin import proteins and alternate auxin signaling pathways. These results contribute to a better understanding of auxin transport and response in *C. vulgaris*, which have implications for more efficient growth and resource extraction of *C. vulgaris*.**

INTRODUCTION

Chlorella vulgaris of the phylum Chlorophyta is a unicellular freshwater green alga, with applications in human nutrition and biofuel (1). Standing between easy access to *C. vulgaris'* resources and humans, however, is a robust cell wall (2). For this reason, much of the recent research concerning *C. vulgaris* has been focused on methods for degrading the cell wall to form protoplasts, or cells without cell walls (1–5). Once the cell wall is degraded, the nutrients inside the cell can be converted to forms that have numerous uses such as food coloring, nutritional additives, and fuel, due to *C. vulgaris*'s high protein and lipid content (1). Unfortunately, commercial production of *C. vulgaris* is an expensive process (1). Though the cell wall is often studied, knowledge about C. vulgaris's growth behavior and interaction with phytohormones is lacking. Studying other characteristics of *C. vulgaris* may shed light on new ways to improve resource production and lower the cost of growing *C. vulgaris*.

One candidate for further study is auxin, a class of small molecules that are synthesized by land plants and play an influential role in the growth and cell differentiation processes; auxin plays a similar role in algae by stimulating cell division (5). The most common form of auxin in nature is indole-3-acetic acid (IAA) (6). In *C. vulgaris*, the application of 0.1 μM IAA caused a 53% increase in cell count and an 81% increase in protein content over 48 hours, a strong indicator that IAA can boost resource availability in *C. vulgaris* (7). The authors of the study noted that auxin's ability to promote algae growth could have commercial benefits (7). IAA can also increase macromolecule accumulation, as demonstrated by a transcriptomic study of IAA-treated *C. vulgaris* (8). The authors of the study found that IAA treatment upregulated an array of genes that play roles in lipid accumulation and photoprotective pigment production, suggesting that IAA also enhances C. vulgaris viability (8). In addition to changing gene regulation, which is considered a slow response, IAA also stimulates rapid responses in plants. In maize protoplasts, the auxin binding protein (ABP1) IAA receptor, located inside the cell on the endoplasmic reticulum, can detect a concentration of 1 μM exogenous IAA (9). This detection leads to a rapid increase in protoplast size, peaking at 4% after one hour (10, 11). The sensitive response to IAA mediated by ABP1 will be key in our experiments probing the existence of *C. vulgaris* membrane IAA exporters.

The mechanisms that *C. vulgaris* has in place to transport auxin are unclear. Before the 21st century, researchers believed that *C. vulgaris* had no auxin export proteins and did not secrete IAA in measurable amounts, but this belief seems to be contradicted by a more recent genomic study showing that *C. vulgaris* possessed DNA sequences resembling those that code for known auxin export proteins in Arabidopsis thaliana, a model land plant (12–14). The authors of the genomic study were careful to note that the function of the *C. vulgaris* sequences could not be inferred solely from similarity to A. thaliana, so this evidence is not definitive (14). Additional evidence suggesting that *C. vulgaris* may have a mechanism for auxin export can be found in other species of algae. One such species is *Chlorella sorokiniana*, which is related to *C. vulgaris* and is known to secrete dilute, nanomolar concentrations of IAA in water culture (15, 16). Given their relatedness, it is plausible that *C. vulgaris* is also capable of similar IAA secretion. The existence of IAA import proteins

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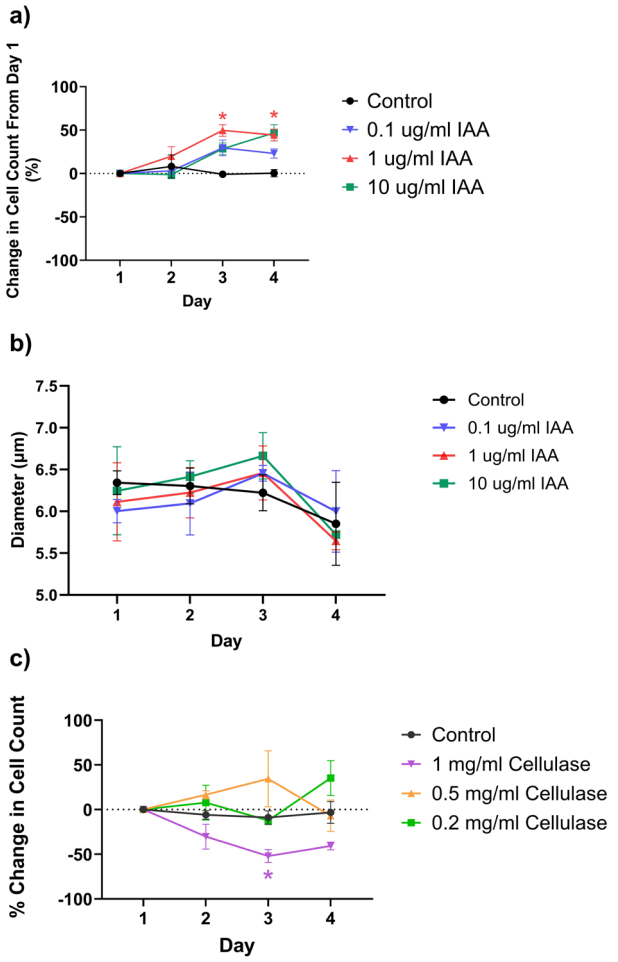

Figure 1. The effect of different concentrations of IAA and cellulase on *C. vulgaris*. A) Line graph showing changes in cell count over time (mean ± SEM). *C. vulgaris* cells were treated with 0 (black), 0.1 (blue), 1 (red) or 10 µg/mL (green) IAA. Cells were counted and their sizes measured using a hemocytometer (n = 3, 2-way ANOVA). B) Change in the average size of cells over three days after being treated with IAA (2-way ANOVA). C) Changes in cell count over three days, after cellulase treatment (n = 3, 2-way ANOVA). Cells were treated with cellulase at concentrations of 0 (black), 0.2 (green), 0.5 (yellow), and 1 mg/mL (magenta). *p < 0.05

in *C. vulgaris* similarly hinges on indirect genetic evidence. IAA import proteins have been found in *C. sorokiniana*, but that does not mean that *C. vulgaris* necessarily possesses IAA import proteins. Moreover, while *C. vulgaris* is known to respond to IAA, which implies the ability of IAA to cross the cell membrane, this observation does not shed light on if the transport of IAA is passive or active. Viewed as a whole, the knowledge concerning *C. vulgaris* auxin transport has direction but lacks precise details. Knowledge about the specifics of auxin transport will improve our understanding of *C. vulgaris*, and may lead to more cost-effective methods of growth. As such, the goal of this study is to search for auxin import and export proteins in *C. vulgaris*.

Based on published research, we hypothesized that *C. vulgaris* possesses auxin import and export proteins. Using cellulase enzyme, we degraded the cell wall to form protoplasts, allowing us to additionally probe the question of if these transport proteins are in the cell membrane or cell wall. Treating *C. vulgaris* protoplasts with exogenous IAA and observing for rapid swelling allowed us to test the presence of auxin import proteins. We also assayed supernatant from cells and protoplasts for auxin secretion, which may act as an indicator of auxin export proteins. The results of our experiments indicated that *C. vulgaris* protoplasts responded to auxin by swelling. In addition, our tests on *C. vulgaris* protoplast and cell culture supernatant suggested that there was auxin present in the supernatant. These results are evidence in support of our hypothesis that *C. vulgaris* possesses auxin import and export proteins. These findings may open doors to new and more cost-effective methods for *C. vulgaris* growth.

RESULTS

To verify the expected behavior for IAA and cellulase and to find the optimal concentrations for experiments in this study, *C. vulgaris* was treated with several different concentrations of IAA and cellulase. *C. vulgaris* treated with IAA proliferated (**Figure 1a**), but IAA treatment did not affect cell size (**Figure 1b**). The 1 µg/mL IAA group exhibited significant growth compared to the control on day 3 ($p = 0.0212$, ANOVA) and day 4 ($p = 0.0265$, ANOVA, **Figure 1a**). Cells experienced a decrease in size on day 4 regardless of treatment type or concentration. *C. vulgaris* cells in the control group, as well as those treated with 0.2 or 0.5 mg/mL cellulase showed no significant difference in cell count, but 1 mg/mL cellulase inhibited growth on the third day ($p = 0.0476$, ANOVA, **Figure 1c**). Furthermore, concentrations of 0.5 mg/mL cellulase left cell debris that lowered image quality and hampered accurate cell counting. All subsequent experiments used 0.2 mg/mL cellulase to create protoplasts.

To test if *C. vulgaris* protoplasts swelled in response to IAA, we treated protoplasts with 1 µg/mL exogenous IAA and measured changes in protoplast size after one hour. After one hour, treated protoplasts swelled by 3.8% and the control, which was treated with Guillard Ryther broth, shrank by -4.8% (**Figure 2**). The difference between groups was not statistically significant ($p = 0.10$, t-test). We observed the two

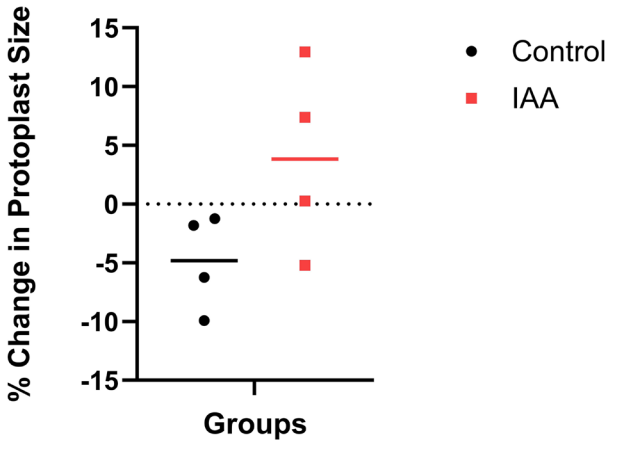

Figure 2. The effect of treating protoplasts with exogenous IAA. Changes in protoplast size one hour after being treated with 1 µg/mL exogenous IAA (red) or broth (black). Individual points are displayed; the mean is depicted as a horizonal bar (n = 4, unpaired t-test, p = 0.10).

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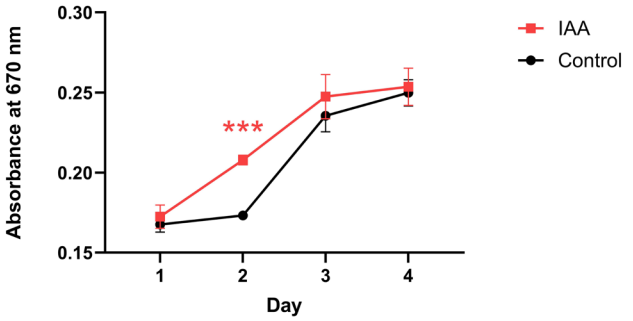

Figure 3. The effect of IAA on division of *C. vulgaris* protoplasts. Changes in absorbance at 670 nm of protoplast cultures treated with either 1 µg/mL IAA (red) or broth (black) over three days. Each point represents the mean ± SEM (n = 4, 2-way ANOVA). *p < 0.05, **p < 0.001, ***p < 0.0001

groups of protoplasts for three days after initial IAA treatment to see if there were any differences in growth compared to the control group. The spectrophotometer showed no significant differences in cell culture density compared to the control, indicating that the IAA-treated protoplasts were not dividing more rapidly (**Figure 3**). The results characterized protoplast response to IAA over a longer period of time and gave us an idea of what we should expect in the experiment testing for IAA secretion.

In order to test for membrane-bound IAA export proteins in *C. vulgaris*, the culture that *C. vulgaris* grows in must be analyzed for the presence of IAA, which would indicate secretion by cells. However, if *C. vulgaris* even secretes IAA, it may do it on the nanomolar scale like *C. sorokiniana* (16), which would be too dilute for us to detect. To stimulate more concentrated secretions in *C. vulgaris*, two groups of C. vulgaris cells and protoplasts were treated with 10 µg/mL IAA. This exogenous application was meant to increase uptake of IAA, which could theoretically cause more IAA to be secreted as well. We chose the concentration of 10 µg/mL exogenous IAA instead of 1 µg/mL IAA because there was concern that only a fraction of the exogenous IAA would be secreted. The rationale was that even if only 10% of the 10 µg/mL exogenous IAA were secreted, it would still be concentrated enough to cause a noticeable response in *C. vulgaris*. On the other hand, 1 µg/mL exogenous IAA may be diluted to the range of 0.1 µg/mL IAA, which we knew from our preliminary experiment to be not concentrated enough to elicit a significant response. Furthermore, we knew that 10 µg/mL IAA would not stunt growth and that it was within the optimal range of IAA concentrations for *C. vulgaris*.

After one day of growth, both groups treated with exogenous IAA were centrifuged to remove exogenous IAA, and fresh Guillard Ryther broth was added. After another day of growth, supernatant from the two groups was transferred to a different group of protoplasts. Protoplasts treated with exogenous IAA, supernatant from cells, or supernatant from protoplasts all experienced significant swelling after 1 hour ($p = 0.0409$, 0.0146, and 0.0344, respectively; Student's t test, **Figure 4a**). Compared to the control group, the average change in protoplast size was 5.58% greater for the cell supernatant group, 6.81% greater for the protoplast supernatant group, and 5.70% greater for the exogenous IAA group. We found no significant difference in protoplast

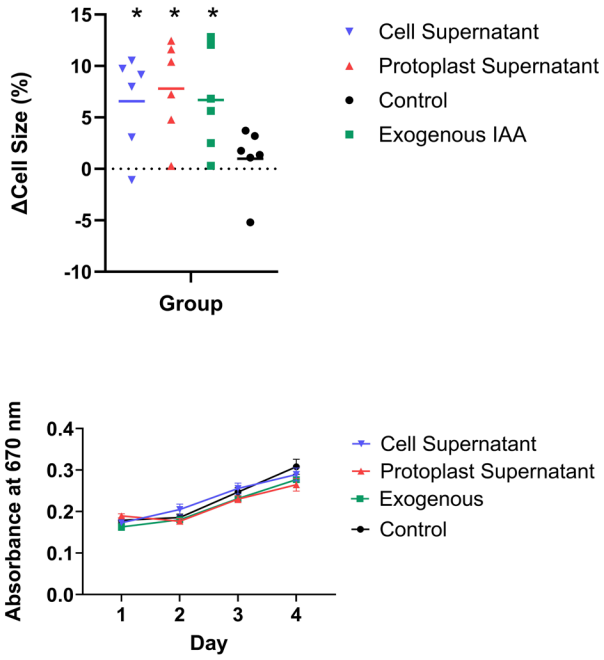

Figure 4. Testing for IAA secretion by treating protoplasts with supernatant from cell culture. A) The average and individual percent changes in protoplast size one hour after treatment for the three treatment groups and control group. Protoplasts were treated with broth (black), 1 µg/mL exogenous IAA (green), or supernatant from intact cells (blue) or protoplasts (red). Each bar represents the mean (n = 6, one-way ANOVA, Students t-test). B) The change in absorbance at 670 nm for the same four experimental groups over three days, after treatment. Each point represents the mean ± SEM (2-way ANOVA). *p < 0.05.

swelling across the four groups ($p = 0.0612$, ANOVA, **Figure 4a**). Following the same experiment revealed no significant differences in cell culture density across the four groups after three days ($p = 0.3489$, ANOVA, **Figure 4b**).

DISCUSSION

The results of the preliminary experiments found that 1 and 10 µg/mL IAA promoted cell division the most, corresponding with prior research (7, 12, 17). The sudden cell size decrease for all four experimental groups on day 4 was unexpected as prior research suggests that IAA does not impact cell size in C. vulgaris with intact cell walls (17). It is possible that this decrease reflects cell division that created smaller cells.

We did not see significant differences in culture density over a three day time course of IAA treated protoplasts, a surprising observation since IAA clearly promotes cell division in cells with intact cell walls and since treatment of cells with cellulase would not remove the intracellular IAA pathways that lead to cell division. A possible explanation for this lack of protoplast division in response to IAA could lie in the cell wall. The cell wall of plant cells is a more acidic environment than the neutral cytosol, which causes the IAA in the cell wall matrix to be protonated and lipophilic and allowing it to pass through the cell membrane (18). Degradation of the cell wall may have removed this acidic environment, which made it more difficult for large amounts of IAA to diffuse into the cell. While protoplasts did not divide, they did swell in response to

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IAA. This observation led us to a protein called auxin binding protein 1 (ABP1), an auxin receptor that is located inside the cell on the endoplasmic reticulum.

In maize, ABP1 is responsible for protoplast swelling of around 4% after treatment with IAA (11). In our own experiments treating *C. vulgaris* with IAA, protoplasts swelled 3.85% compared to the untreated control. The similar increases in size for both maize and *C. vulgaris* protoplasts suggest that there is an ABP1-like receptor in *C. vulgaris*. Two studies comparing *C. vulgaris* gene sequences with A. thaliana gene sequences using BLAST came to different conclusions. One study found no ABP1 orthologs, while the other succeeded in identifying orthologs (14, 8). Further research is needed to confirm ABP1's existence in *C. vulgaris*. We recommend using a gene knockout method to knock out suspected ABP1 sequences in *C. vulgaris*, then repeating the IAA treatment and measuring protoplast size. If swelling is not present, then there will be strong evidence that the sequences do code for an ABP1-like protein.

Our experiments on *C. vulgaris* protoplast swelling indicate that IAA enters protoplasts through methods other than passive diffusion. As mentioned previously, degrading cell walls removes the acidic wall environment and prevents IAA from diffusing passively into the cell (18). However, our experiments suggest that a putative ABP1 located inside the cell and on the endoplasmic reticulum responded to IAA. These results suggest that *C. vulgaris* actively transported IAA into the cell using membrane bound IAA import proteins. To reconcile our results showing protoplast swelling but not protoplast division in response to IAA, we propose that *C. vulgaris* auxin import proteins transport IAA into the cell at levels sufficient for the putative ABP1 to induce protoplast swelling but insufficient for cell division. For this to happen, it would mean that the intracellular pathways stimulating cell division are less sensitive to IAA than the putative ABP1 receptor. Few papers go in depth into the sensitivity of these receptors or pathways, so we recommend future research testing the affinity of ABP1 for IAA to see if our speculation is correct. Another explanation could be that, unlike in maize, the putative ABP1 proteins in *C. vulgaris* exist on the cell membrane. This would negate the need for an active IAA import protein. However, given that ABP1 is located on the endoplasmic reticulum in land plants, we deem this explanation unlikely (9).

Our hypothesis of auxin export proteins in *C. vulgaris* was supported by observing protoplast swelling after treatment with supernatant from both cells and protoplasts. Protoplast swelling indicates IAA presence in the supernatant, suggesting that IAA was secreted by *C. vulgaris* cells. Furthermore, we know that IAA did not simply "leak" out of the cells because IAA is ionized in the cytosol and cannot passively diffuse through the cell membrane (18). This finding suggests that any IAA in the supernatant was actively secreted using auxin export proteins. Lastly, our results showing that supernatant from both cells with intact walls and protoplasts caused swelling. As such, removing the cell wall did not impact IAA secretion, suggesting that IAA export proteins are localized to the plasma membrane.

Following the same experiment over three days showed no significant differences in cell growth across all treatment groups, supporting our findings when treating protoplasts with exogenous IAA. Protoplast swelling seen in the experimental groups (**Figure 4a**) corroborates what we saw in our experiment treating protoplasts with exogenous IAA (**Figure 2**). The swelling of protoplasts in response to both supernatant and IAA once again reinforcing our belief that there is an IAA import protein.

Our study did not focus on other *C. vulgaris* secretions, but in retrospect, we should have considered this topic very carefully. *C. vulgaris* is known to secrete vitamins, and it is almost a certainty that it secretes influential signaling molecules that may have been partly responsible for the swelling seen in our experiments (19, **Figure 4a**). This possibility highlights an error in our experimental design. It was important for there to be a fifth group of protoplasts treated with supernatant from *C. vulgaris* culture grown without exogenous IAA, which would have exposed protoplasts to "natural" levels of C. vulgaris secretion not augmented by exogenous auxin. Further experiments using five treatment groups instead of four can show the possibility of endogenous IAA secretion as seen in *C. sorokiniana*, and it would have also controlled for secretions besides just IAA. Out of all suggestions for future research, we believe that including this fifth group is most immediate. Additionally, we recommend using a gene knockout method to disable suspected coding sequences of auxin transport proteins in *C. vulgaris*. Repeating our experiments with these knockouts can more definitively establish the presence of auxin import and export proteins.

The results of our study found that IAA induced rapid swelling in *C. vulgaris* protoplasts and that supernatant from cells and protoplasts caused swelling in another group of protoplasts. These results suggest the presence of auxin export and import proteins, as well as an ABP1-like protein responsible for protoplast swelling in *C. vulgaris*. These results contributed to a better understanding of *C. vulgaris* auxin response and transport and may play a future role in developing better ways to grow *C. vulgaris*.

MATERIALS AND METHODS

Algae

C. vulgaris was purchased from Algae Research Supply and grown in modified Guillard Ryther broth (Algae Research Supply) under algae light (Algae Research Supply) for 18 hours per day. For all experiments, *C. vulgaris* stock was diluted with broth in a 1:10 ratio and pH was buffered with a bicarbonate salt mix (Algae Research Supply). The pH of the algae cultures and replicates were not monitored throughout experimentation. Algae solutions were shaken once per day by hand to maintain uniform culture density.

Optimal Auxin Concentrations for *C. vulgaris* Growth

To determine optimal auxin concentrations for growth, IAA (Chemimpex, Cat# 39290) was dissolved in 91% isopropanol and applied at 0.1, 1.0, and 10 µg/mL concentrations (n = 3; three independent replicates observed at the same time of day). The control was an equal volume of 91% isopropanol. Cell size and cell count were observed using a light microscope and hemocytometer (Amazon) every day for three days using 10 µL of algae culture under 100x magnification. Each observation only removed 1% of the total replicate volume and likely did not change the culture density. For each count, the same six squares on the hemocytometer grid were selected for cell counting. Cells were automatically

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counted using the ImageJ watershed function, with particle area restricted to 15–60 μm^2 to prevent counting debris or artifacts. These magnification, hemocytometer, and ImageJ settings were the same for all future experiments.

Protoplast Formation

Cellulase (Amazon) was dissolved in Guillard Ryther growth medium and applied in concentrations of 0.2, 0.5, and 1 mg/ml, with the control being an equal volume of growth medium (n = 3; three independent replicates observed at the same time of day). Cells were grown for three days, with observations for cell count and size made every day, including the day of application, for a total of four observations. Each observation used 10 μL of algae culture.

Protoplast IAA Time Course

Protoplasts were produced by treating *C. vulgaris* with 0.2 mg/mL cellulase for one day, then treated with 1 μg/mL IAA (n = 6; six replicates observed at the same time). Cell culture density was measured every day for three days using a SpectroVis+ spectrophotometer (Vernier) at 670 nm. Each reading was done using 2 mL cell culture in plastic cuvettes (Amazon).

Protoplast Swelling

Protoplasts were produced by treating *C. vulgaris* with 0.2 mg/mL cellulase. Then, after 24 hours, the experimental group was treated with 1 μg/mL IAA (n = 4; four replicates observed at the same time) and compared to a control of untreated protoplasts. Cell size was measured one hour after treatment using a microscope at 100x magnification and a hemocytometer.

Testing for Auxin Secretion Using Protoplast Swelling as a Measuring Point

Protoplasts and intact cells were treated with IAA at a concentration of 10 μg/mL for one day then centrifuged at 3000 rpm for three minutes (n = 6; six replicates observed at the same time). The supernatant containing exogenous IAA was discarded and 1 mL of Guillard Ryther broth free of IAA was added, resuspending the pellet. After another day, IAA treated cells and protoplasts were again centrifuged at the same settings and the supernatant was added to pellets of different protoplasts (n = 6). Another two groups of different protoplasts were treated at the same time with 1 μg/mL exogenous IAA and broth for the control (n = 6). Protoplast sizes were measured an hour after treatment using a microscope and hemocytometer. The same experiment was followed for three more days where cell culture density was measured using a spectrophotometer at 670 nm.

Statistical Tests

The statistical tests used in this study were the t-test and 1-way and 2-way ANOVA. The independence and data type conditions were met. There were no glaring issues with homogeneous variance or normality in our data, but the small number of replicates used meant that we proceeded with our tests with caution.

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