

Regulatory Role of Plant-Based Biostimulants in Chlorophyll Biosynthesis and Endogenous Hormone Regulation in Plants

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Abstract

Plant-based bio-stimulants offer a sustainable approach to enhancing plant growth by influencing physiological processes such as chlorophyll biosynthesis and hormone regulation. This study investigates the effects of aqueous leaf extracts from *Justicia gendarussa*, *Osmunda regalis*, and *Senna occidentalis* on chlorophyll content and endogenous hormone levels in rice (*Oryza sativa*) plants. Seeds were treated with varying extract concentrations (10%, 20%, and 50%) and analyzed after four weeks. Results showed a significant, dose-dependent increase in chlorophyll a and b content, with *J. gendarussa* at 100% concentration yielding the highest chlorophyll a (7.21 mg/g) and chlorophyll b (3.23 mg/g) levels. Endogenous hormone levels were also elevated, with *J. gendarussa* treatment resulting in auxin (12.53 µg/g), gibberellin (9.63 µg/g), and cytokinin (6.57 µg/g) concentrations. ANOVA confirmed the statistical significance of both plant type and extract concentration on chlorophyll and hormone levels ($p < 0.05$). These findings highlight the potential of these natural extracts, particularly *J. gendarussa*, as eco-friendly bio-stimulants for improving rice plant physiology and promoting sustainable agriculture.

Introduction

Plants are highly sensitive to their environmental conditions, and their growth and development are significantly influenced by various factors, including light, temperature, water availability, and soil nutrients (Balliu et al. 2021; Pant et al. 2021). Among these, the regulation of chlorophyll biosynthesis and the modulation of endogenous hormones are crucial processes that dictate plant health, growth, and overall productivity (Ahsan et al. 2024). Chlorophyll, the green pigment responsible for photosynthesis, is vital for the plant's energy production, while plant hormones, such as auxins, cytokinin, and gibberellins, are key regulators of developmental processes, including cell division, elongation, and differentiation (Hudeček et al. 2023; Sabagh et al. 2022). Recent advancements in plant biotechnology have led to the development of bio-stimulants, substances or microorganisms that enhance plant growth, yield, and stress tolerance without being classified as fertilizers or pesticides (Singh et al. 2022; Ghosh and Pal 2023). Plant-based bio-stimulants, derived from natural sources such as seaweed, plant extracts, and microbial formulations, have gained significant attention due to their ability to stimulate various physiological processes in plants, including chlorophyll biosynthesis and hormonal regulation (Baltazar et al. 2021; Shahrajabian et al. 2021). The impact of plant-based bio-stimulants on chlorophyll development and hormone regulation remains an area of active research. Several studies suggest that these bio-stimulants can enhance chlorophyll content, thereby improving photosynthetic efficiency, and they may also modulate the levels of key plant hormones, further influencing growth and stress responses (Bhupenchandra et al. 2022; Rai et al. 2021). However, the exact molecular mechanisms underlying these effects are still not fully understood, particularly in relation to the interplay between bio-stimulants, chlorophyll biosynthesis, and hormone signaling pathways. This study aims to explore the regulatory role of plant-based bio-stimulants in chlorophyll development and the concentration of endogenous hormones in plants. By examining the effects of different plant extracts on chlorophyll biosynthesis and hormone regulation under varying growth conditions, this research seeks to contribute

to a deeper understanding of how bio-stimulants can be used to optimize plant growth and productivity, particularly under stress conditions such as drought or nutrient deficiency.

Materials and Methods

Sample collection

The plant extracts were prepared from the leaves of three species: *J. gendarussa*, *O. regalis*, and *S. occidentalis*, all collected from various locations within Jhenaidah District, Bangladesh. *J. gendarussa* was sourced from Garagang, near the local market and surrounding agricultural fields. *O. regalis* was gathered from Vatoi Bazar, situated near riverbanks and wetland areas. *S. occidentalis* was collected from Sheikhpura, on the outskirts of the village, in open fields. After collection, the leaves were thoroughly washed, air-dried, and ground into fine powder. The extract was prepared by boiling the powdered leaves in double-distilled water, followed by filtration, and storage at 4°C until further use.

Plant Extract Preparation

Aqueous plant extracts were prepared using a standard extraction procedure. To prepare the extract, 10 grams of dried leaves from each species were boiled in 100 mL of distilled water for 15–20 minutes. The resulting solution was filtered using Whatman No. 1 filter paper to remove any solid residues. The filtrate was stored at 4°C for later use. To create different concentrations of the plant extracts, the following dilution series was prepared: for a 10% concentration, 10 mL of extract was mixed with 90 mL of distilled water; for a 20% concentration, 20 mL of extract was mixed with 80 mL of distilled water; and for a 50% concentration, 50 mL of extract was combined with 50 mL of distilled water.

Seed Preparation

Prior to experimentation, the seeds underwent surface sterilization to eliminate potential contaminants. Initially, the seeds were soaked in 70% ethanol for 1 minute to remove surface impurities. The ethanol was drained, and the seeds were then treated with 3% hydrogen peroxide for 10 minutes to sterilize any remaining microbial contaminants. After this, the seeds were rinsed five times with sterile distilled water to remove any residual chemicals. The sterilized seeds were used immediately to ensure sterility and prevent contamination.

Plant Extract Effect on Chlorophyll Content

To assess the impact of plant extracts on chlorophyll content, fully expanded, mature leaves (5 g) from treated rice plants were harvested after 4 weeks. The leaves were ground in a mortar and pestle with 30 mL of 80% acetone, filtered, and diluted to a final volume of 50 mL with additional 80% acetone. Chlorophyll content was determined by measuring absorbance at 663 nm and 645 nm using a

spectrophotometer for chlorophyll a and b, respectively. The concentrations of chlorophyll a and b were calculated using the following equations based on the absorbance values:

$$\text{Chlorophyll a (mg/L)} = (12.7 \times A_{663}) - (2.69 \times A_{645})$$

$$\text{Chlorophyll b (mg/L)} = (22.9 \times A_{645}) - (4.68 \times A_{663})$$

Where:

A₆₆₃ = Absorbance at 663 nm (for chlorophyll a)

A₆₄₅ = Absorbance at 645 nm (for chlorophyll b)

Plant Extract on Plant Hormonal Concentration

To analyze the effect of plant extracts on plant hormones, 5 g of shoot tissue from the 4-week treated rice plants was ground with 10 mL of 80% acetone, then filtered and diluted to a final volume of 25 mL. The hormonal concentrations were measured by spectrophotometry at A₂₈₀ nm for auxins, A₂₅₄ nm for gibberellins, and A₂₇₀ nm for cytokinin. One-way ANOVA was used to analyze the hormone levels across the treatments. The concentrations were converted to ng/g using the following formula:

$$\text{Concentration (ng/g)} = \frac{\text{Concentration (ng/mL)} \times (\text{Volume (mL of extract)})}{\text{Weight of the shoot sample (g)}}$$

Results

Measurement of Chlorophyll Content on Plant Extract Treatment

The effect of different plant extracts and their concentrations on chlorophyll content was evaluated using the quantified levels of chlorophyll a and b, as presented in Table 1. The findings revealed a notable increase in chlorophyll concentrations with increasing doses of *J. gendarussa*, *O. regalis*, and *S. occidentalis* extracts, indicating a dose-dependent stimulatory effect on chlorophyll biosynthesis. Among all treatments, *J. gendarussa* extract at 100% concentration recorded the highest chlorophyll accumulation, with 15.49 mg/L of chlorophyll a and 20.14 mg/L of chlorophyll b. Similarly, *O. regalis* at 100% concentration showed elevated chlorophyll levels, registering 14.01 mg/L (chlorophyll a) and 19.38 mg/L (chlorophyll b). The extract from *S. occidentalis* also enhanced chlorophyll content, albeit to a slightly lower extent than the other two, with 12.75 mg/L of chlorophyll a and 17.65 mg/L of chlorophyll b at full concentration. In contrast, the control group exhibited the lowest chlorophyll content (1.84 mg/L for chlorophyll a and 1.95 mg/L for chlorophyll b). ANOVA analysis confirmed significant effects of the plant extract type ($p < 0.01$) and concentration ($p < 0.001$) on chlorophyll synthesis, with an F-value of 50.70 for the plant factor and 183.07 for concentration (Table S9). The interaction between plant extract

and concentration also had a significant impact ($p < 0.01$), with an F-value of 20.95. However, the residual variation was relatively low, confirming that the model's explanatory power was high ($p > 0.05$). This analysis supports the conclusion that both the type of plant extract and its concentration significantly influenced chlorophyll content, and the interaction between these factors was also statistically significant (Fig. 1).

Table 1
Overall data of chlorophyll content.

Plant Extract	Concentration (%)	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)
J. gendarussa	20	2.84	3.77
J. gendarussa	50	8.09	9.73
J. gendarussa	100	15.49	20.14
O. regalis	20	3.44	4.87
O. regalis	50	9.24	12.37
O. regalis	100	14.01	19.38
S. occidentalis	20	3.06	3.91
S. occidentalis	50	7.45	11.06
S. occidentalis	100	12.75	17.65
Control	100	1.84	1.95

Exploration of Plant Extract on Plant Hormonal Concentration

The hormonal concentrations, including auxins, gibberellins, and cytokinin, were measured for various plant extracts at different concentrations, as summarized in Table 2. The results indicated that both the plant type and concentration significantly influenced the concentrations of auxins, gibberellins, and cytokinin, though the interaction between these two factors was less pronounced. For auxins, *J. gendarussa* at 100% concentration showed the highest levels (46.5 ng/g), followed by *O. regalis* (52.1 ng/g) at the same concentration (Fig. 2). On the other hand, *S. occidentalis* demonstrated the lowest auxin concentration at all concentrations, with 26.3 ng/g at 100% concentration. The control group exhibited 14.8 ng/g of auxins. ANOVA results for auxins indicated that both the plant extract type ($p < 0.0255$) and concentration ($p < 0.0119$) had statistically significant effects, with the interaction between plant extract and concentration not being significant ($p < 0.4374$). Similarly, for gibberellins, *J. gendarussa* at 100% concentration showed the highest value (36.7 ng/g), followed by *O. regalis* at 100% concentration (41.3 ng/g). The lowest gibberellin levels were observed in *S. occidentalis*, particularly at 20% concentration (9.2 ng/g). The control had 12.4 ng/g of gibberellins. The ANOVA results for gibberellins indicated significant effects of both plant type ($p < 0.0355$) and concentration ($p < 0.0205$),

with no significant interaction between the two factors ($p < 0.5712$). As for cytokinin, *J. gendarussa* at 100% concentration showed the highest value (15.8 ng/g), with *O. regalis* and *S. occidentalis* also showing increasing cytokinin concentrations at higher concentrations of their extracts. The control group exhibited a cytokinin concentration of 6.9 ng/g. ANOVA analysis revealed that both the plant type ($p < 0.00934$) and concentration ($p < 0.01609$) significantly affected cytokinin concentrations, while the interaction between plant extract and concentration was not significant ($p < 0.66029$).

Table 2
Overall data of plant hormonal concentration indicating the effect of plant extract.

Plant Extract	Concentration (%)	Auxins (ng/g)	Gibberellins (ng/g)	Cytokinin (ng/g)
J. gendarussa	20%	17.2	13.5	8.6
J. gendarussa	50%	36.8	30.2	13.4
J. gendarussa	100%	46.5	36.7	15.8
O. regalis	20%	31.5	26.2	16.4
O. regalis	50%	41.7	36.5	18.9
O. regalis	100%	52.1	41.3	20.7
S. occidentalis	20%	11.4	9.2	5.6
S. occidentalis	50%	21.5	16.1	9.8
S. occidentalis	100%	26.3	21.7	11.6
Control	100%	14.8	12.4	6.9

Discussion

Plant Extracts Effects on Chlorophyll Content

The significant increase in chlorophyll a and b levels observed in this study suggests that the applied plant extracts positively influenced photosynthetic pigment synthesis, likely by enhancing nutrient uptake and reducing oxidative stress. Notably, the presence of phenolic compounds in *J. gendarussa* may have contributed to this increase by upregulating genes involved in tetrapyrrole metabolism, a key pathway in chlorophyll biosynthesis. These findings are in agreement with earlier research, such as the study by Vashishth et al. (2023), which demonstrated that various plant extracts applied to *Callistemon viminalis* cuttings improved chlorophyll levels. The bioactive compounds present in these extracts are believed to either stimulate chlorophyll biosynthesis or protect existing chlorophyll molecules from degradation due to oxidative stress. Among the three plant species tested, *J. gendarussa* consistently showed the highest chlorophyll content, followed by *O. regalis* and *S. occidentalis*, with all showing dose-dependent effects. This suggests that higher concentrations of plant-based bio-stimulants may more effectively enhance photosynthetic efficiency and overall plant vigor. Further research, including transcriptomic and

proteomic analyses, is recommended to identify the specific genes and chlorophyll-binding proteins involved in this enhancement and to better understand the underlying molecular mechanisms.

Plant Extracts Effects on Hormonal Concentration

The application of plant extracts also led to notable increases in the concentrations of key endogenous hormones, including auxins, gibberellins, and cytokinins. This hormonal modulation likely plays a role in promoting plant growth and enhancing stress resilience. Among the treatments, *J. gendarussa* and *O. regalis* showed the strongest stimulatory effects on auxin and gibberellin levels, while *S. occidentalis* had relatively moderate effects. The elevated auxin concentrations may promote root and shoot elongation, while increased gibberellins could enhance stem elongation and seed germination. Cytokinins, which influence cell division and delay leaf senescence, were also significantly elevated, particularly in treatments with higher extract concentrations. These hormonal shifts suggest that bioactive compounds in the extracts may interact with hormonal biosynthesis or signaling pathways, amplifying growth responses. Although the interaction between plant type and extract concentration was not statistically significant for hormones, both factors independently showed strong effects, emphasizing their importance in optimizing plant growth regulators. Further studies should investigate the molecular pathways triggered by these plant-based bio-stimulants, including potential cross-talk among hormone signaling networks. Insights into these mechanisms will be essential for developing sustainable bio-stimulant formulations for use in stress-prone agricultural environments.

Conclusions

This study demonstrates that plant-based bio-stimulants derived from *Justicia gendarussa*, *Osmunda regalis*, and *Senna occidentalis* significantly enhance chlorophyll content and regulate endogenous hormone levels in rice plants. The effect was concentration-dependent, with 100% extracts showing the highest improvement in both chlorophyll a and b levels, as well as increased concentrations of auxins, gibberellins, and cytokinin. *J. gendarussa* was particularly effective in promoting chlorophyll biosynthesis and hormonal modulation. These findings suggest that such natural plant extracts hold great potential as eco-friendly alternatives to chemical enhancers, contributing to sustainable agriculture by improving plant vigor and stress tolerance. Further molecular studies are needed to identify the precise pathways involved and to validate their use across different crop species.

Declarations

Ethics declarations

This article does not include any studies by any of the authors that used human or animal participants. All authors are conscious and accept responsibility for the manuscript. No part of the manuscript content has been published or accepted for publication elsewhere.

Conflict of interest

The authors declare no competing interests.

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Author Contribution

A.A.M. comprehended and planned the study, carried out the analysis, wrote the manuscript, and prepared the graphs and illustrations; S.K. contributed to the critical revision of the manuscript and wrote the manuscript; A.A.M. supervised the whole work, and all authors approved the final manuscript.

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Data availability

The corresponding author Abdullah Al Mamun is responsible for all data and materials.

Code availability

There was no code available.

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Figures

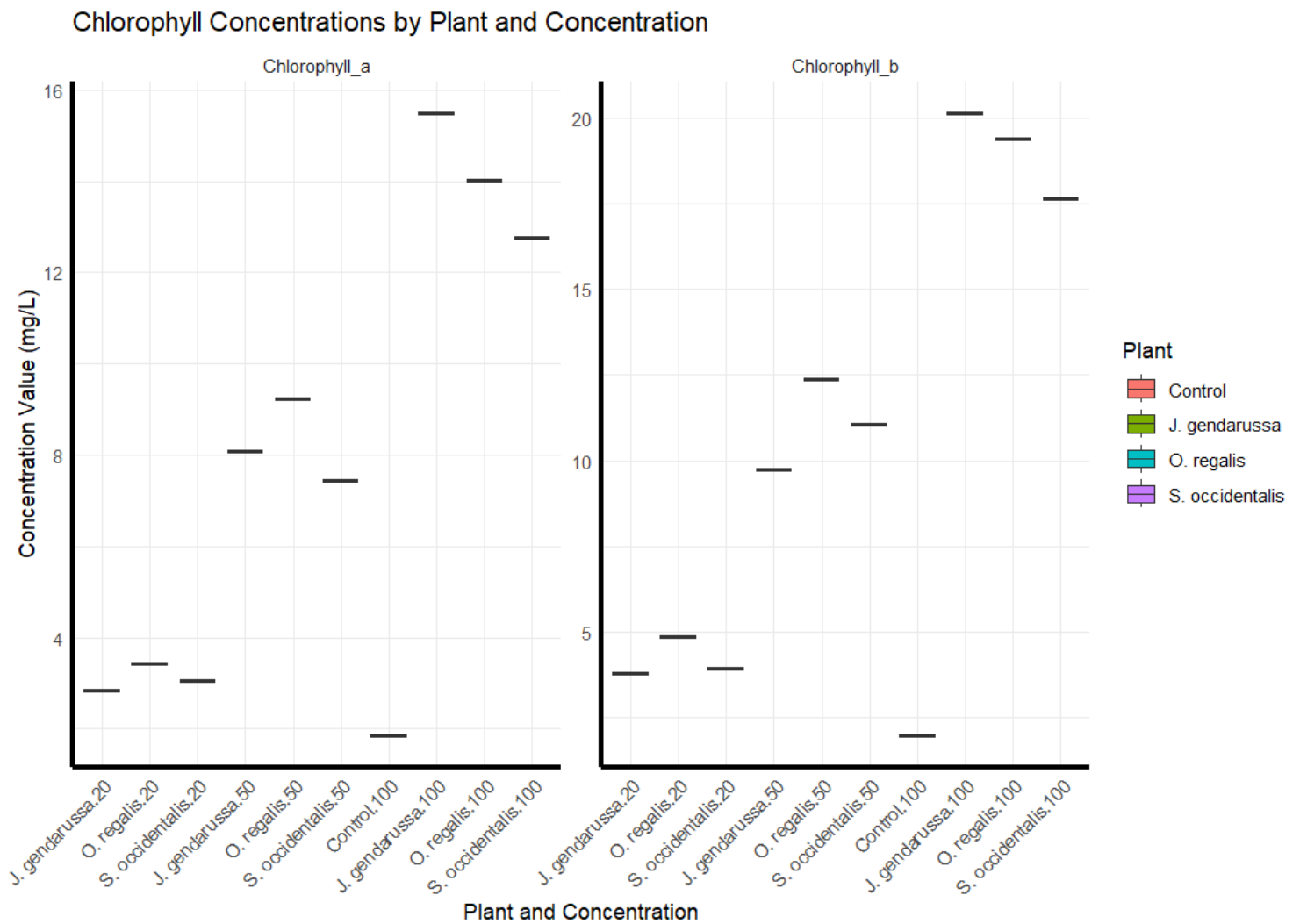


Figure 1

Chlorophyll Concentrations in Different Plant Species at Varying Concentration Levels. Boxplot showing the concentration of Chlorophyll a and Chlorophyll b in different plant species (*J. gendarussa*, *O. regalis*, *S. occidentalis*, and Control) at concentrations of 20%, 50%, and 100%. The data illustrate the significant differences in chlorophyll content across plants and concentrations. Statistical significance was determined using ANOVA, with p-values indicating differences between treatment groups.

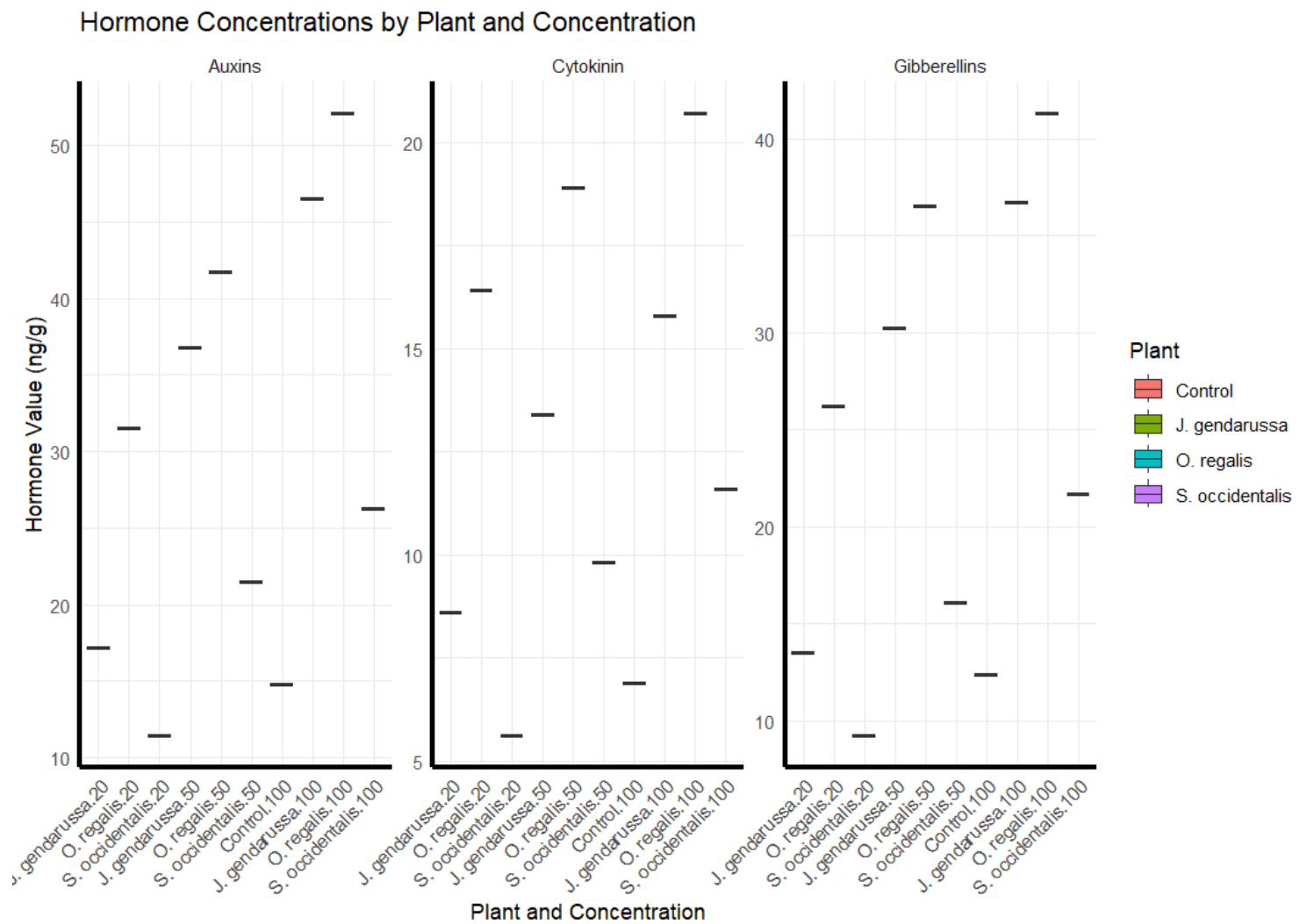


Figure 2

Hormone Concentrations in Different Plant Species at Varying Concentration Levels. Boxplot showing the concentration of Auxins, Gibberellins, and Cytokinin in different plant species (*J. gendarussa*, *O. regalis*, *S. occidentalis*, and Control) at concentrations of 20%, 50%, and 100%.