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Original Article

Biochemical Changes During Flowering of *Spathodea campanulata* P. Beauv.

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ABSTRACT

Backround: *Spathodea campanulata* P. Beauv., exhibits a phenomenal structure often referred to as water calyces from within which the entire floral whorls presumed to be developing. **Purpose:** The present study was an attempt on the exceptional development of corolla under the influence of the bathing fluid of water calyces in *Spathodea campanulata*. **Method:** Three different developmental stages of flower buds were used for anatomical, physiological (PWC, RWC and ΨW) and biochemical studies (reducing sugars, amino acids, proteins, phenols, IAA and amylase activity) using standard methods. **Results:** Biochemical composition was found to be directly influencing the unique pattern of floral ontogeny. The exponential expansion of the corolla was found to be corresponding with significant increase in IAA concentration. **Conclusions:** The bathing fluid with its contents *viz.,* sugars, amino acids, proteins, IAA and phenols seems to play a pivotal role in the development, nourishing and protecting the inner whorls of the flower buds. Anatomical studies also support the idea that the biochemical changes, especially the quantity of sugars and IAA could be the reason for the exponential expansion of the petals.

Key words: Biochemical composition, Flower development, Water calyces.

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INTRODUCTION

S. campanulata P. Beauv. belonging to Bignoniaceae family produces buds with inner floral whorls developing in an unique way with enclosed calyx called water calyces. Water calyx refers to gamosepalous condition having watery fluid enclosing the other inner floral whorls viz., corolla, androecium and gynoecium. The water calyx fluid is believed to be obligatory for the floral development and it is reported that the fluid accumulates because of water secreting glands present on the inner walls of the calyx.[1,2] Success of flowering plants relies in the formation of flowers. The phenomenon of flowering and overall plant development is an integral part of plants metabolome as machinery behind cell wall formation, remobilization of flux between source and sink, pollination and in defense processes.[3] Several reports are available on the changes of various biochemicals occurring during flowering. Carbohydrates and Nitrogen ratio is crucial for cellular functions, plant growth, flowering and completion of life cycle. Carbon nutrients include especially sucrose and glucose while nitrogen nutrients include inorganic nitrates, ammonia and organic compounds constituting all amino acids. It is reported that, sugars are the energy currencies reflecting the plant energy status whose effect is diverse in plant growth and development and also as osmoregulant.[4-6] Amino acids are the key elements in building the cells resulting in various forms of proteins.[7,8] Amino acids are also the precursors for Phytohormones involved in many developmental processes ranging from seed germination to flowering.[9] One such natural phytohormone is auxin playing a classical role in initiation of floral primordia and specific floral organs and their pattern formation within the floral buds.[10,11] Several reports on various secondary metabolites and their antioxidant properties on *S. campanulata* is reported.[12-17] But the primary biochemical composition during flowering is uncertain in *S. campanulata*. Hence, the present study was focused on biochemical changes during the flower development of *S. campanulata* with special reference to flowering within water calyces.

MATERIAL AND METHODS

Sample collection

Flower buds of *S. campanulata* were collected from different locations in Bengaluru. The healthy buds were selected and grouped into small, medium and large based on the size of intact flower buds.

Surface area and fresh and dry weight determination[18]

Surface area of the unopened and opened petals were determined by using graph sheet and the fresh and dry weight of the same was also determined in triplicates.

Water status of corolla[18]

Petal Water Content (PWC): The fresh weight (FW) of corolla from large size buds and opened buds were weighed. The dry weight (DW) of the same was weighed after drying completely in oven at 60°C for 6 days. PWC % was calculated as total petal weight using the formula: [(FW – DW)/FW×100].

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Relative water content[19]

Discs of corolla with 1 cm^3 area were punctured using cork borer. Fresh weight of 10 such corolla discs were weighed and placed in petri dishes containing distilled water for 4 hours at room temperature. After incubation, discs were blotted gently and turgid weight was measured. The discs were kept for drying in hot air over at 60°C overnight for dry weight. After 6 days, dry weights of discs were measured and relative water content was calculated accordingly.

Water potential (ΨW) by gravimetric method[20]

Different concentrations of sucrose solutions were prepared (0.2, 0.4 up to 1.0 M) using 1 M stock sucrose solution. Using 1 cm^3 cork borer, discs of corolla were punctured and weighed for initial weight and incubated for 1 hour at room temperature separately in different concentrations of sucrose solution. Final weight of the same were measured and water potential was calculated using formula $22.4*M*T/T_0$.

Amylase activity[21]

Extraction of enzyme

5 grams of freshly collected flowers of unopened and opened flower corolla were extracted for enzyme analysis by grinding in a pre-cooled mortar and pestle using phosphate buffer (pH 6.5, 0.2 M), centrifuged at 5000 rpm in a refrigerated centrifuge for 20 min. The supernatant was used as enzyme source.

Estimation

1 ml of enzyme + 1 ml of 0.2% starch + 1 ml of phosphate buffer were incubated for 15 minutes and 30 minutes intervals. 25 µl of Iodine was added after incubation time to the reaction mixture. The blue color developed was measured at 600 nm using Genesys 10 UV-Vis spectrophotometer. The quantity of residual starch was plotted against standard 0.2% starch solution. Zero time control was maintained. The enzyme activity was represented as amount of starch degraded per ml of enzyme per minute.

Anatomical studies

Free hand sections of the calyx and corolla were taken. Both unstained and stained sections were observed under high power magnification and photographed. Details of calyx glands and cell size of corolla were obtained. Photomicrography was performed for free hand sections of calyx and corolla using Magnus MLX Plus microscope.

Extraction for quantitative estimations

5 g of fresh weight of tissues *viz*., whole buds, bud devoid of calyx fluid, calyx and inner whorls of buds without calyx were extracted in 80% boiling ethanol in a water bath and ground using a mortar and pestle. The extract was centrifuged at 4000 rpm for 10 minutes, supernatant was collected and the tissues were re-extracted in 80% ethanol until the tissues became colorless. The ethanolic extracts were pooled and were made up to 100 ml and stored at 4°C for quantitative estimations.

Estimation of reducing sugars[21]

Reducing sugars were estimated by Nelson-Somogyi method using Genesys visible spectrophotometer at 620 nm. Reducing sugars in the samples were calculated from glucose standards and expressed as microgram per gram fresh weight of the tissue.

Estimation of total free amino acids[21]

Total free amino acids were estimated by Ninhydrin method using Genesys visible spectrophotometer at 570 nm. Total amino acids in the fluid were calculated from glycine standards and expressed as microgram per gram fresh weight of the tissue.

Estimation of proteins[21]

Proteins were estimated by Lowry's method using Genesys visible spectrophotometer at 660 nm. Proteins in the fluid was calculated from bovine serum albumin (BSA) standards and expressed as microgram per gram fresh weight of the tissue.

Estimation of total phenols[21]

Total phenols were estimated by Folin-Ciocalteu reagent method using Genesys visible spectrophotometer at 725 nm. Total phenols in the fluid was calculated from catechol standards and expressed as microgram per gram fresh weight of the tissue.

Estimation of indole acetic acid[22]

IAA was estimated by Salkowski's method. The absorbance was recorded at 530 nm using Genesys visible spectrophotometer. IAA concentration in the fluid were obtained using standard curve of synthetic IAA from Himedia and expressed as microgram per gram fresh weight of the tissue.

Statistical analysis

IBM SPSS Statistics, version 20, was used for One-way ANOVA to compare means ($p < 0.05$) and Tukey HSD Post Hoc test was conducted for multiple comparisons.

RESULTS AND DISCUSSION

The present study provides information on the influence of biochemical components in the flower development of *Spathodea campanulata* from bud to open flower stage.

The flower buds reach their maximum size (large) around 7 days with calyx completely closed, enclosing all the inner whorl. At the just open stage around day 10 the calyx breaks open and a part of corolla emerges out. By overnight the entire corolla expands pushing the calyx towards one side and the corolla reaches maximum size and it is retained throughout its life.

Flower area, fresh mass, ΨW and water content of corolla increased as the flower development progressed towards opening, similar to the earlier studies on *Narcissus*.[23,24] The area of the unopened corolla was 39 cm^2 and opened corolla was 94 cm^2 respectively, more than twice of the unopened showing the exponential expansion of corolla. The dry weight of the opened corolla is less than the unopened corolla confirming that the biochemical components present in unopened corolla is used up for exponential expansion of opened corolla.

The difference in PWC of the unopened corolla to opened corolla increased by nearly 8% indicating the intake of water into the system to facilitate the development of turgidity of petal cell and to help in its enlargement and consequent formation of intercellular spaces leading to exponential expansion of petal (Figure 1). RWC of unopened corolla (75%) to opened corolla (55%) decreased, indicating that the fluid of the water calyx play a role in RWC status of the petal tissue. Once out of the bud the petals are exposed to dry environment resulting in lower RWC of opened petals (Figure 1). Increase in the water content in corolla tissues is attributed to the increase in the cell turgidity, which is important for flower opening.[18,25] RWC is a measure of water status of tissues in relation to its turgid condition. From Figure 1, it is evident that the corolla has the potentiality to imbibe 30% of watery fluid which is produced by water calyces. Based on earlier studies, the amount of biomolecules in the fluid is seem to be linearly decreasing as the flower is developing within closed buds. Henceforth, the osmolytes present in

the fluid could be reabsorbed by the developing floral whorls especially by the corolla aiding in the rapid expansion of petal cells and overall floral development.[14,26]

Amylase activity was found to be significant in unopened and opened corolla with ($F_{3,11}$ = 19.20, p = 0.01). It was very high in unopened and opened corolla. The activity decreased with time in unopened whereas in opened corolla it increased tremendously with increase in time indicating that the osmolyte required for the turgidity and substrate required for respiration by the exponentially expanding petal cells is being supplied by the activity of amylase by converting the starch into sugars. The biochemical status, higher starch content of unopened petal and higher sugar content of opened petal also reflects role played by them in influencing RWC status. Water potential status of unopened corolla (- 12.76 bars) and opened corolla (-15.64 bars) indicates the potential for greater intake of water by the open petal compared to unopened petal, the same inference is also reflected biochemically by more starch and less sugar in unopened and less starch and more sugar in opened petals and the greater activity of amylase in unopened petals (Figure 2).[27]

The anatomical studies revealed the changes in cell shape of corolla from spherical, compactly packed cells without intercellular spaces to elongated branched cells with large air spaces, similar to the earlier studies on Rose and Carnation (Figures 3 and 4).[28] IAA in plant tissues is known for hypertrophy and hyperplasia. This kind of anatomical metamorphosis of normal cells into elongated branched turgid cells with large intercellular spaces and with thinner cell wall may be due to the effect of higher IAA concentration in the tissues and the calyx fluid.[29] The anatomy of calyx showed the presence of multicellular

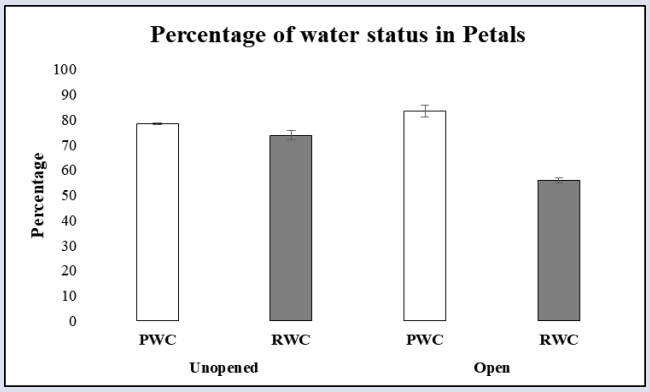

Figure 1: Graph showing percentage of water status in unopened and open flowers. PWC: Petal water content; RWC: Relative water content.

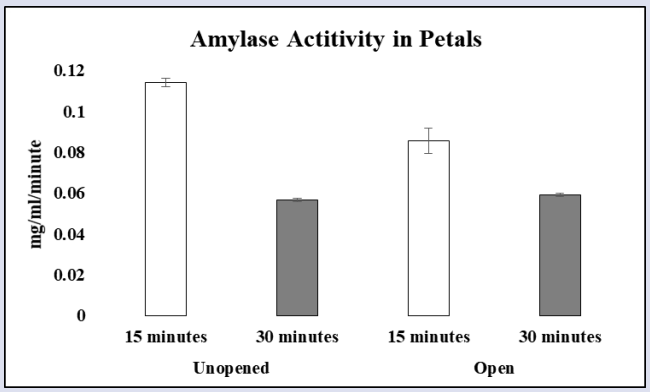

Figure 2: Graph showing amylase activity per minute in unopened and open flowers.

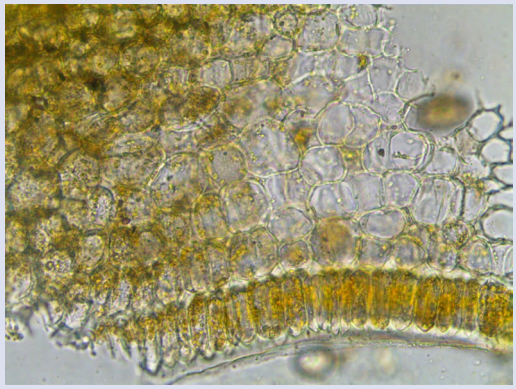

Figure 3: T.S. of corolla showing compactly arranged cells in small sized bud.

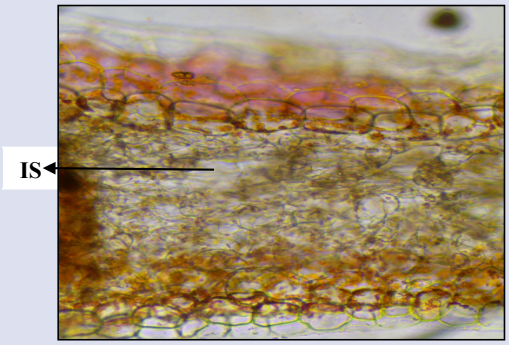

Figure 4: T.S. of corolla showing large intercellular spaces in large sized bud.IS: Intercellular space.

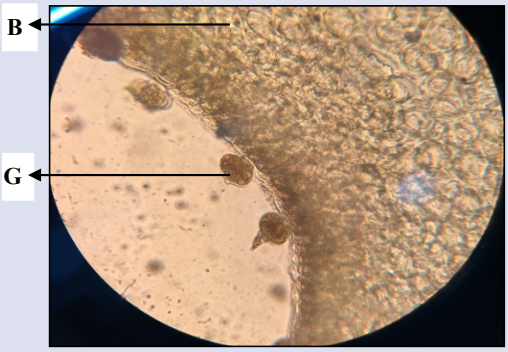

Figure 5: T.S. of calyx showing stalked peltate glands on inner walls. B: bacteria; G: peltate gland.

stalked peltate glands along their inner wall of the calyx harboring motile endophytic microorganisms (Figure 5).

Concentration of reducing sugars was found to be significant in whole buds ($F_{2,6}$ = 16.02, p = 0.004) and inner whorl ($F_{2,6}$ = 34.71, p = 0.001). The reducing sugar content remained uniform in calyx with minor decrease towards maturation. Whereas, the reducing sugar concentration in corolla showed 45% increase at the medium stage and remained constant (Figure 6). The increase may be necessitated by cellular development and for the turgidity of the cells to help in opening of corolla. The higher concentration of sugar in medium stage is the reason for the higher water content and may be the reason for the turgidity required for breaking opening of calyx.[25] The decrease in sugar

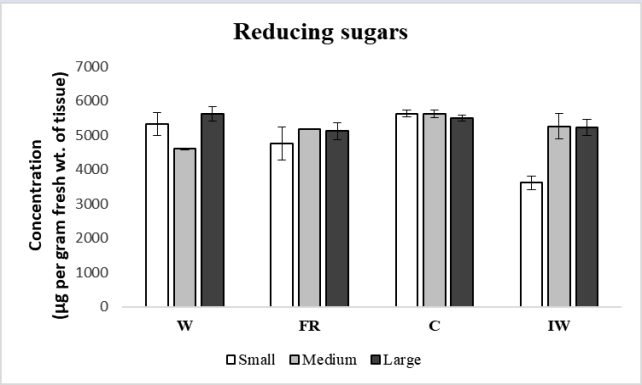

Figure 6: Graph showing concentration of reducing sugars in three stages of flower bud tissues.

content of calyx may be due to senescence as reported in *Hemrocallis, Helleborus, Consolida* and *Rosa*.[30]

Concentrations of amino acids were significant between groups in all the stages of bud. The concentration of free amino acid in the calyx varied in different stages with increase of about 350% in the middle stage followed by a decrease in large stage (Figure 7). In *S. campanulata* the abundant free amino acid concentration in the calyx of middle stage is unique and different from rest of the floral development studies, which may be due to the endophytic microorganism present in the glands contributing to amino acid content by nitrogen fixation.[31,32] The free amino acid concentration in corolla exhibited high quantity than calyx, indicating the demand for the same in the actively developing corolla, which is about to open, similar to *Hemrocallis and Consolida.*[33-36] The decrease in free amino acid in large stage could be due to senescence and transition of amino acid pool to the other developing floral organs.[37]

Protein content was found to be significant in whole bud (p = 0.49), calyx (p = 0.23) and inner whorl (p = 0.006) whereas it was not significant in fluid removed buds (p = 0.495). Inner whorl had least content of protein followed by calyx, fluid removed and whole bud. The quantity of protein was reflective of the quantity of amino acid in different whorls at different stages. There was an increase at the medium stage in both calyx and corolla indicating the need for proteins as enzymes helping in cellular changes (Figure 8). The decrease may be due to translocation during senescence.[37]

Total phenolics in Whole buds, fluid removed buds and calyx were not significant (p = 0.486, p = 0.194, p = 0.079) respectively. However the phenol concentration was significant in Inner whorl ($F_{2,6}$ = 5.71, p = 0.04 and among groups significant difference was found in medium and large, p = 0.05.). Maximum in middle sized inner whorl with a mean concentration 3317.67 µg/g fresh weight of tissue and least in large sized inner whorl with a mean concentration of 2952.77 µg/g fresh weight of tissue (Figure 9). Generally, the phenolic composition decreases from bud towards senescence stage and this characteristic trend is also observed in the present study.[18,24] The maximum amount of phenol present in calyx reflects its role as defense compound in the protective whorl enclosing the nutrient rich bathing fluid and the inner tissues. The calyx contained more of phenols than corolla as it is developing under the protection of calyx.[38]

IAA content was found to be significantly increasing in all stages of whole buds ($F_{2,6}$ = 797.15, p = 0.000), FR buds ($F_{2,6}$ = 265.78, p = 0.000) and inner whorl ($F_{2,6}$ = 76.17, p = 0.000). The concentration of IAA in whole bud is greater when compared to the fluid removed buds (Figure 10). The difference in IAA concentration of whole buds could be due to the presence of calyx fluid containing IAA.[14] No statistical significance was seen in the IAA concentration with calyx tissue (p = 0.673). The increase in IAA concentration from small to middle stage, both in intact and fluid removed treatments was drastic (161% and 121% respectively) which clearly indicates that the exponential expansion of corolla is due to the very high concentration of IAA, part of which maybe a contribution from the calyx fluid (i.e., approximately 40%). IAA concentration was found to be increasing with the development of flower buds in all bud categories (Figure 10). This pattern of increase in IAA concentration is in confirmation with other studies too.[39] The overall concentration of IAA in calyx and corolla seems to reflect its role in the progressive development of calyx and especially the sudden development and exponential expansion of corolla (25% in the

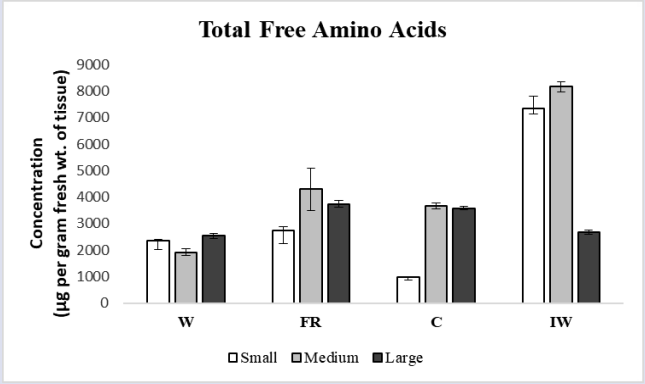

Figure 7: Graph showing concentration of total free amino acids in three stages of flower bud tissues.

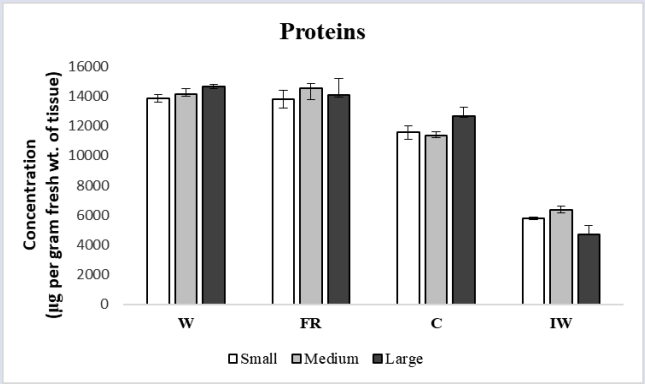

Figure 8: Graph showing concentration of proteins in three stages of flower bud tissues.

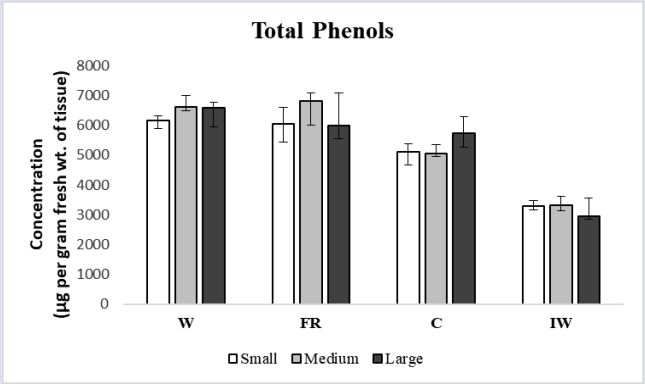

Figure 9: Graph showing concentration of total phenols in three stages of flower bud tissues.

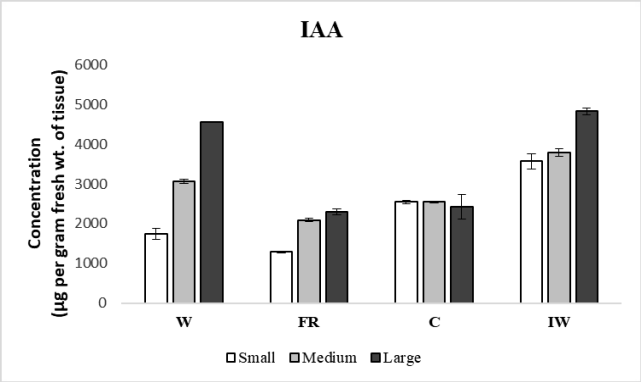

Figure 10: Graph showing concentration of IAA in three stages of flower bud tissues.

middle sized bud). IAA in plant tissues is known for hypertrophy and hyperplasia.[29] The anatomical studies of young petals showed that the spherical compactly arranged parenchyma cells metamorphosed into elongated branched turgid cells with large intercellular spaces with thinner cell wall may be due to the effect of IAA concentration in the tissues and the calyx fluid.

CONCLUSION

The obligatory requirement of closed calyx and the presence of bathing fluid in the floral development in *S. campanulata* are unique. The present study reveals that the biochemical changes happening during the development is also unique that the exponential increase in the plant hormone IAA in the fluid and the tissues in bud maturity suggest that the calyx fluid has a vital role in the exponential development of corolla. The content of reducing sugars increased by 45% at the blooming stage indicating its role as an osmolyte and the energy source for the petal expansion. The present study reveals that water calyx plays a role in the floral development of *S. campanulata* by influencing the changes in biochemical components and enzyme activity during different stages of development.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

PWC: Petal water content; RWC: Relative water content; ΨW: Water potential; IAA: Indole-3- Acetic Acid.

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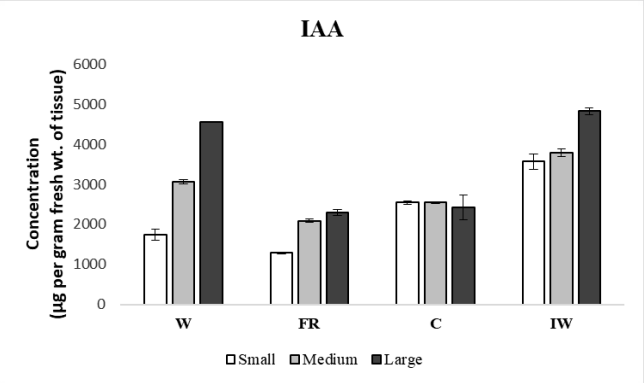
GRAPHICAL ABSTRACT



SUMMARY

The physiological, biochemical, enzymological and anatomical studies on *Spathodea campanulata* P. Beauv. flowers, were carried out to understand the unique obligatory requirement of closed calyx and the presence of bathing fluid. The present study reveals that water calyx plays a role in the floral development of *S. campanulata* by influencing the changes in biochemical components and enzyme activity during different stages of development. The biochemical changes happening during the development is also unique that the exponential increase in the plant hormone IAA in the fluid and the tissues in bud maturity suggest that the calyx fluid has a vital role in the exponential development of corolla.

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