

Bio-Physicochemical Studies on Water Calyx Fluid in the African Tulip Tree, *Spathodea campanulata* P. Beauv.

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ABSTRACT

Background: Flowers of *Spathodea campanulata* P. Beauv. development is unique as inner whorls develop immersed in a fluid called water calyces. **Purpose:** The present study is an investigation into the biochemical composition and physicochemical properties of water calyx fluid from *Spathodea campanulata* P. Beauv. **Methods:** Fluid composition of small, medium and large stages of *S. campanulata* flower buds were analyzed qualitatively and quantitatively for biomolecules viz., reducing sugars, total free amino acids, proteins, phenols and Indole-3-Acetic Acid (IAA). Physicochemical analyses were done using particle size analyzer. **Results:** Reducing sugars, total free amino acids, proteins and IAA were present in appreciable amounts whereas total phenol content was low and the content of biomolecules were significant between groups and within the groups. The water calyx fluid is highly stable and absolute necessity for the floral development. **Conclusions:** The concentrations of all the analyzed biomolecules were found to be decreasing with bud maturity. The contents of water calyx fluid is obligatory for the development of floral whorls.

Keywords: Water calyx fluid, Biochemical composition, Zeta potential.

INTRODUCTION

The phenomenon of flowering in plants is one of the most explored areas in the field of reproductive biology which is governed by both endogenous and exogenous metabolites. Floral whorls in the African tulip tree, *Spathodea campanulata* P. Beauv. develop inside the closed gamosepalous calyx submerged in the watery fluid often referred to as water calyces or water jacket. The presence of water calyx in *S. campanulata* was ascribed to be beneficial in the floral development and the liquid is reported to be alkaline in nature with traces of hydrochlorides, carbonates, nitrates, ammonia; sulphates of potassium, sodium and calcium. The secretion is often called as “fluid produced from calyx hydathodes instead of nectar”.¹⁻² The cup-like water calyces serve merely as defense tool in deterring floral herbivores and in protecting the sex organs during unfavorable consequences of strong transpiration.²⁻⁴ The fluid has been demonstrated to possess antimicrobial and multi-potential biomolecules necessary for the development.⁵ Primary metabolites are involved in plethora of cellular reactions resulting in the overall growth of the plant body.⁶ Sugars in plant act as a signaling molecule and are required for the development of reproductive structures.⁷ Amino acids are the fundamental basic component of proteins in plants besides its cellular signaling.⁶ Amino acid metabolism plays a key role in protein synthesis, flower initiation, development and floral differentiation as these are the only endogenous nitrogenous compounds available within the plants.⁸ Phenolics play a vital role in pigment production and plant protection from biotic and abiotic stress.⁹ Plant growth hormone

auxin is involved in cell fate and many organ and flower development.^{10,11} Significant information is provided on the nitrogen metabolism, enzymes and phenolic acid metabolism in *S. campanulata* P. Beauv.¹²⁻¹⁴ However, the existing knowledge on the biochemical composition of aforementioned molecules in the fluid and its physicochemical properties are fragmented except mineral content and antimicrobial activity. Hence, the present work was undertaken to provide a systematic account of bio-physicochemical composition in the water calyx fluid of *S. campanulata* and its role during the course of flower development.

MATERIAL AND METHODS

Flower collection and water calyx sample preparation

Flower buds of *S. campanulata* were collected from different locations in the city of Bengaluru where the tree is planted as an avenue tree owing to its ornamental value. The work was carried out with freshly collected materials in two different flowering seasons. The healthy buds were selected and grouped into small, medium and large sizes based on the size cum weight of intact flower buds. The calyx fluid was extracted from the respective flower buds with the help of sterile syringes. For physicochemical study, calyx fluid from all the bud sizes were collected freshly, mixed and analyzed.

Qualitative test for biomolecules¹⁵

Test for reducing sugars

Benedict's reagent method

To 1 ml of sample, 1 ml of Benedict's reagent was

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added and heated in a boiling water bath for 10 min. The formation of red/yellow precipitate was observed as positive reaction.

Test for amino acids

Ninhydrin method

To 1 ml of sample, 1 ml of 0.1% freshly prepared ninhydrin solution was added. The contents were mixed and boiled for 2 min, allowed to cool. The appearance of violet/purple complex was observed as positive reaction.

Test for proteins

Biuret reaction

To 2 ml of sample, 2 ml of 10% sodium hydroxide was added followed by 2 drops of 0.1 % copper sulphate solution and mixed thoroughly. The formation of violet colour was observed as positive reaction.

Test for ammonia^{16,17}

It was carried out using Nessler's method, using a test kit for ammonia estimation. 1 ml of the fluid from the water calyces of different bud sizes were taken in test tubes, mixed with 1 ml of Nessler's reagent and the colour reaction and precipitation was observed.

Quantitative estimation of biomolecules¹⁵

Estimation of reducing sugars

Reducing sugars were estimated by Nelson-Somogyi method. To 1 ml of fresh fluid, 1 ml of fresh alkaline copper tartarate reagent was added. The mixture was heated for 20 min in a boiling water bath, cooled and 1 ml of arseno molybdate reagent was added. The solution was mixed and diluted to 25 ml with distilled water. The resulting blue colour was read at 620 nm using Genesys visible spectrophotometer. 1 ml of distilled water with all other reagent except sample was used as blank. Reducing sugars in the samples were calculated from glucose standards.

Estimation of total free amino acids

Total free amino acids were estimated by Ninhydrin method. To 1 ml of fresh sample, 1 ml of Ninhydrin reagent was added. The tube was heated in a boiling water bath for 20 min, cooled to room temperature. 5 ml of diluents solution (equal volume of water and n-propanol) was added and mixed. The volume was raised to 25 ml with distilled water and the intensity of purple colour was read using Genesys visible spectrophotometer at 570 nm. 1 ml of distilled water with all other reagent except sample was used as blank. Total amino acids in the fluid was calculated from glycine standards.

Estimation of proteins

Proteins were estimated by Lowry's method. To 1 ml of sample 5 ml of alkaline copper solution was added mixed well and allowed to stand for 10 min at room temperature. 0.5 ml of folin-ciocalteu reagent was added, mixed and incubated at room temperature in dark for 30 min. The volume was raised to 25 ml with distilled water. The intensity of the blue coloured complex was read using Genesys visible spectrophotometer at 660 nm. 1 ml of distilled water with all other reagent except sample was used as blank. Proteins in the fluid was calculated from bovine serum albumin (BSA) standards.

Estimation of total phenols

Total phenols were estimated by Folin-Ciocalteu reagent. To 1 ml of Folin-Ciocalteu reagent 1 ml of fresh sample was added, followed by 2 ml of 20% sodium carbonate. The mixture was heated in boiling water bath for one min. The volume was raised to 25 ml with distilled water and the intensity of blue colour was read using Genesys visible

spectrophotometer at 725 nm. 1 ml of distilled water with all other reagent except sample was used as blank. Total phenols in the fluid was calculated from catechol standards.

Estimation of Indole Acetic Acid¹⁸

IAA was estimated by Salkowski's reagent method. 1 ml of fluid was mixed with 2 ml of Salkowski's reagent (0.5 M ferric chloride in 35 % perchloric acid). The tubes were mixed well and incubated in dark for 30 min. The absorbance was recorded at 530 nm using Genesys visible spectrophotometer. 1 ml of distilled water with all other reagent except sample was used as blank. IAA concentration in the fluid were obtained using standard curve of synthetic IAA from Himedia.

Physicochemical analysis

The collected fluid was analyzed for pH, mobility, zeta potential, polarity, conductivity and viscosity using particle size analyzer (Make Microtrac, Model Nanotracer wave, USA). Density of the fluid was calculated by specific gravity method.¹⁹ pH of the fluid was determined by using (Make Elico, Model LI-614 pH analyser).

Statistical analysis

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

RESULTS

The mean concentration of biomolecules *viz.*, reducing sugars, total free amino acids, proteins, total phenols and IAA were found to be significant at ($p < 0.05$) across the maturity of the buds. The inference of physicochemical study reveals that the water calyx fluid is highly stable

Biochemical estimations

For reducing sugars (Figure 1), mean was significant with ($F_{2,6} = 273.160, p = 0.00$). A Tukey post hoc test revealed that the concentration of the reducing sugars statistically significantly lowered with the maturity of large buds ($268.73 \mu\text{g/ml} \pm 31.53, p = 0.00$) and medium buds ($599.63 \mu\text{g/ml} \pm 18.25, p = 0.02$) compared to the concentration of small buds ($727.75 \mu\text{g/ml} \pm 22.80, p = 0.00$).

Changes in total free amino acids (Figure 2) was statistically significantly between groups ($F_{2,6} = 46.38, p = 0.00$) and within groups concentration decreased statistically significantly with maturity of large buds ($3.31 \mu\text{g/ml} \pm 1.76, p = 0.00$) and medium buds ($6.73 \mu\text{g/ml} \pm 1.68, p = 0.02$) compared to concentration of small buds ($14.21 \mu\text{g/ml} \pm 0.24, p = 0.02$).

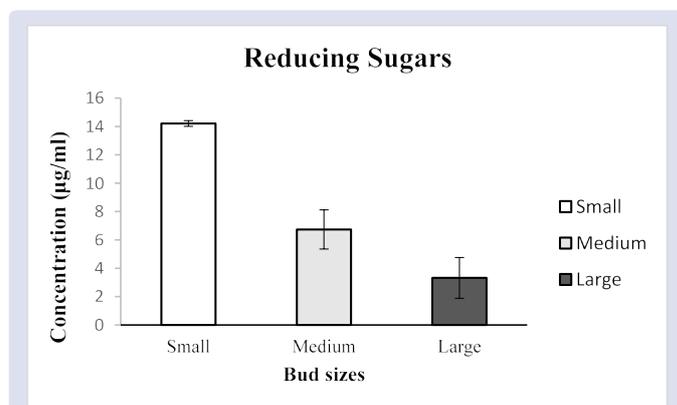


Figure 1: Concentration of reducing sugars in 3 different stages of floral development.

Concentration of proteins (Figure 3) showed statistically significant between groups ($F_{2,6} = 134.26$, $p = 0.00$) and multiple comparison revealed that within the groups concentrations of proteins decreased statistically significantly with maturity of large buds ($23.74 \mu\text{g/ml} \pm 5.51$, $p = 0.00$) and medium buds ($58.84 \mu\text{g/ml} \pm 10.94$, $p = 0.00$) when compared with small buds ($123.15 \mu\text{g/ml} \pm 4.48$, $p = 0.00$).

Composition of total phenols (Figure 4) was statistically significant between groups ($F_{2,6} = 24.72$, $p = 0.001$) and from Tukey post hoc test concentration of total phenols within groups was statistically significantly between large buds ($0.85 \mu\text{g/ml} \pm 0.79$, $p = 0.01$) and small buds ($13.26 \mu\text{g/ml} \pm 3.22$, $p = 0.01$). There was no statistically significant difference between the medium buds and small buds ($p = 0.197$); medium and large buds ($p = 0.07$).

Endogenous plant hormone IAA (Figure 5) was statistically significant between groups ($F_{2,6} = 31.10$, $p = 0.001$). Within groups the concentration of IAA decreased statistically significant with large buds ($16.35 \mu\text{g/ml} \pm 4.07$, $p = 0.01$) and small buds ($264.06 \mu\text{g/ml} \pm 57.40$, $p = 0.01$); medium buds ($77.43 \mu\text{g/ml} \pm 38.81$, $p = 0.03$) and small buds ($264.06 \mu\text{g/ml} \pm 57.40$, $p = 0.01$). There was no statistically significant difference between the medium buds and large buds ($p = 0.228$).

Physicochemical analysis

The pH of the calyx fluid was found to be alkaline with a pH of 8.5.

The mobility and conductivity of the fluid was -15.63 u/s/V/cm and $11.672 \mu\text{S/cm}$ respectively.

The viscosity of the fluid was $0.9 \text{ m}^2/\text{s}$ with a density of 998.01 Kg/m^3 almost to that of water density 1000 Kg/m^3 .

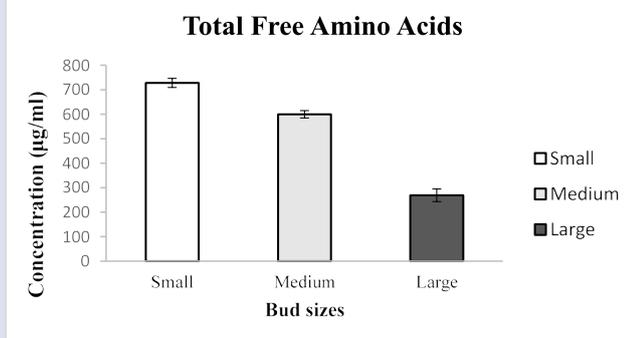


Figure 2: Concentration of amino acids in 3 different stages of floral development.

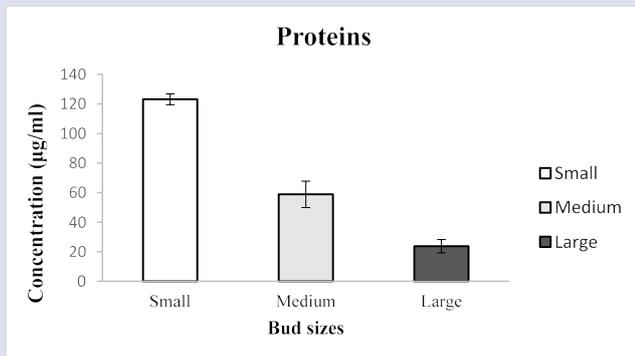


Figure 3: Concentration of proteins in 3 different stages of floral development.

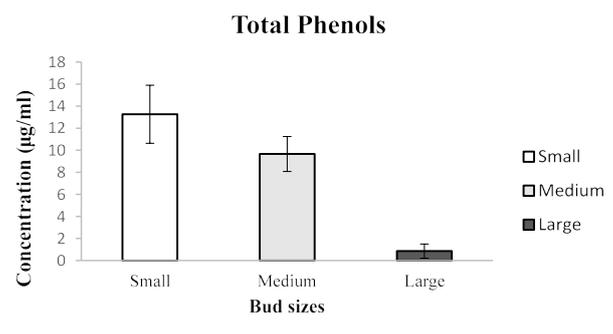


Figure 4: Concentration of total phenols in 3 different stages of floral development.

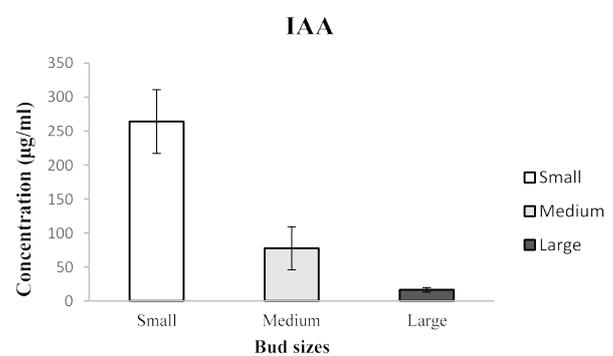


Figure 5: Concentration of IAA in 3 different stages of floral development.

The dielectric constant of the fluid was 79 at 24.53°C with negative polarity of charge -3.402 fC . The dielectric constant of the fluid is almost equal to that of the dielectric constant of water i.e., 78.3 at 25°C .

The zeta potential of the fluid was -200.0 mv at a pH 8.5.

DISCUSSION

The preliminary qualitative analyzes of biomolecules *viz.*, reducing sugars, total free amino acids, proteins, total phenols and ammonia showed positive reaction irrespective of bud maturity. The quantitative estimations of aforementioned biomolecules except ammonia were screened and were found to be varying in concentrations irrespective of the bud maturity. A statistically significant difference between groups was determined by one-way ANOVA ($p < 0.05$) in the mean concentrations of all the screened biomolecules quantitatively.

Carbohydrate metabolism is linked with the development of flowers and maturity of the flowering and other storage organs.^{20,21} Reducing sugars are usually found to be in abundance during the flower development as per the study.²¹⁻²³ The higher content of reducing sugars in small buds is due to sucrose catabolism into glucose and fructose which generally is utilized by the plants during respiration for the production of energy so that it could be used in the floral development and blooming by forming intermediate metabolites.²⁴

The reported concentration of amino acids corresponds to the 15 reported amino acids.¹² The amino acids are believed to be declining as the flower develops.²³ The amount of amino acid recorded would have pumped through vascular tissues from the root-source to the sink-flower. There are evidences amino acids could also synthesized through *de novo* pathways.²⁵ The fluid acts as amino acids pool which are used as building blocks during the flower development for the synthesis of

enzymes and structural proteins. These amino acids could also be the reason behind the ammonical nature of the calyx fluid. *In vitro* studies have shown floral development enhanced with exogenous application.²⁶

A significant decline in soluble protein content during flower development reported correlates with the present study.²³ Initial increase in the concentration of proteins implies that increased protein synthesis during bud development and thereafter declining trend in protein concentration is apparent in flower opening and cell expansion.²⁷

Our results on phenols are partly in conformity with earlier reports but decrease in phenolics concentration occurred as the bud maturity increased.²³ Phenols are secondary compounds having antioxidant properties basically protecting the cells from oxidative damage.²⁸ High phenol content in the calyx fluid during the early bud development stages would be beneficial for the differentiating floral tissues as it could be protecting the growing regions of the floral tissues in unopened flower buds. The variation in the phenol concentration merely does not depend on petal expansion but also on environmental and physiological cues as relevant in the present study.²⁹

Many studies are conducted on the impact of IAA on flower development. One such example is of *Arabidopsis thaliana* suggesting endogenous auxin are important factor in the differentiation and formation of floral meristem and similarly high amount of IAA was also evaluated in *Raphanus sativus* during the flowering period.^{30,31} The increase in endogenous IAA content during floral bud initiation and differentiation is a regular phenomenon.^{32,33} IAA production is at peak during flowering with its continual growth and decreases as fruitlets are formed which is congruent with the study where the concentration is found declining with the maturity of floral buds. Hence, it is very evident from the previous studies that optimum level of endogenous IAA is required for the initiation and differentiation of floral whorls which is why the calyx fluid of smaller buds have shown maximum amount of IAA when compared to the fluid of larger buds. The present findings in this regard are in conformity with the previous studies.

The pH of the calyx fluid was found to be alkaline with a pH of 8.5 is contradictory to earlier reports claiming it to be acidic with pH of 6.4.⁵ High alkalinity of the calyx fluid is due to the high concentration of amino acids under deamination reaction to form ammonia which is clear from the present study.⁶ The mobility and conductivity of the fluid was -15.63 u/s/V/cm and 11.672 μ S/cm respectively which is because of the presence of dissolved biomolecules since conductivity is usually a measure of both organic and inorganic molecules as.³⁴ The presence of 7 different minerals in the fluid could have also contributed to the conductivity.⁵ The viscosity of the fluid was 0.9 m²/s with a density of 998.01 Kg/m³ almost to that of water density 1000 Kg/m³. Water is the only solvent that is used by the plant cell for maintaining its hydrostatic pressure within cells for the movement of water and solutes in xylem and phloem and also for plant support.³⁵ Thus, calyx fluid also conducts properties as of water in the developing flower buds for maintaining the turgidity of the floral whorls. The dielectric constant of the fluid was 79 at 24.53°C with negative polarity of charge -3.402 fC. The dielectric constant of the fluid is almost equal to that of the dielectric constant of water i.e., 78.3 at 25°C.³⁶ Hence the fluid is a good solvent for charged particles with polarity of negative with a charge of -3.402 fC.³⁵ The stability of the calyx fluid was assessed through zeta potential. The zeta potential of the fluid was -200.0 mv at a pH 8.5. Since the zeta potential value is more than -25 mv, it creates an ideal optimum environment with great stability of biomolecules which in turn could be utilized by the developing floral buds.³⁷ Zeta potential is not only dependent on pH but also by the amount of ions in the dispersed medium which is why the conductivity of the calyx fluid was found to be greater and alkaline in nature.³⁸

The water calyx fluid may be playing a major role in the development of floral parts as it was present and analyzed to contain many biochemical

appreciable quantity in all three different stages of flower development. Presence of reducing sugars, total free amino acids, proteins, total phenols and IAA were biochemically established. A significant decrease in the concentrations of all the screened biomolecules was evident from small to large sized buds owing to its utilization by the developing flower buds as immediate source for various physiological activities. The studied biomolecules confer various physiological and pharmaceutical advantages as in many plants.³⁹ The present findings therefore prove that osmolytes of the fluid are obligatory for the development of the floral buds and fruits. Identification of all biomolecules in the water calyx fluid will throw light on its role in floral development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

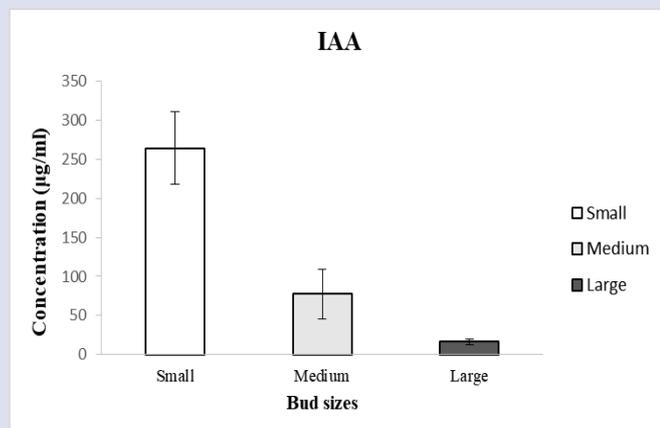
IAA: Indole-3-Acetic Acid.

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GRAPHICAL ABSTRACT



SUMMARY

Metabolites in water calyx fluid of *S. campanulata* P. Beauv. are favorable osmolytes (reducing sugars, total amino acids, protein, total phenols and IAA) and obligatory for flower development. The evaluated biomolecules are significant in quantity in various stages of floral buds and contribute to floral ontogeny. Physicochemical parameters revealed the fluid is highly stable. Hence, the water calyx fluid provides optimum physiological condition for the developing floral buds.

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