PRELIMINARY REPORT ON THE FLOWER COMPOSITION OF NANDI FLAME

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Abstract

Spathodea is a monotypic genus in the flowering plant family Bignoniaceae. The single species, *Spathodea campanulata*, is commonly known as the Fountain Tree, African Tulip Tree Pichkari or Nandi Flame., The flower bud is ampule-shaped, contains watery liquid and called Water calyx. Water calyces may be present for the protection of floral buds from floral herbivores. Water calyces were first described in *Spathodea campanulata* over 100 years ago by Treub in 1889.Water calyces hold and often secrete liquid, causing buds to develop under an aqueous or mucilaginous mixture. In 1890, in the Annales of the Botanic Garden of Buitenzorg, in a paper on the flower buds of *Spathodea campanulata*, Dr. Treub mentions the fact that various bacteria occur normally in the liquid secreted inside the closed calyx and partially gave the chemical composition of the fluid and reported that it is ammoniacal.

In the present work, chemical composition of the fluid from water calyces of *Spathodea campanulata* with respect to Starch, Reducing sugars, Amino acids, Ammonia, Phenols, pH was analysed by routine methods and occurrence of bacteria by microscopic observations, isolation and culturing on Nitrogen free media was carried out. Results reveal that the pH is 9 and fluid contains trace amount of reducing sugars, phenols, amino acids and large amount of ammonia. The presence of few motile, many capsulated, gram-negative bacilli was recorded microscopically and isolation trials resulted in a thin pellicle forming bacteria on nitrogen free semi solid (NFb) medium at pH 9, which produced yellow colonies on nitrogen free malate agar medium supplemented with yeast extract turning the media alkaline. The role of the fluid present in the calyx and the bacteria is discussed as an indication to a primitive symbiosis.

Key words

Spathodea companulata, Bignoniaceae, water calyx, alkalophilic, microaerophilic, capsulated, Gram-negative bacterium, NFb media, Biochemical tests, peltate glands, mucilaginous colony, nitrogen fixation, associative symbiosis.

1. Introduction

The African tulip tree, *Spathodea campanulata.* Beauv. is a monotypic genus of Bignoniaceae which exhibits a phenomenal structure often referred to as water calyces from which the entire flower is presumed to be developed. Water calyx in *S. companulata* was first discovered by Trueb, and in his paper 'flower buds of *S. campanulata*' has inferred that this specialized structure contains colonies of microorganisms which are beneficial for the floral development [1]. In 1897 Mr. S. H. Korrders, reported the occurrence of microorganisms particularly bacteria in the water calyces of *S. campanulata* and fungi in other plants containing water calyces. He also ascribes that the fluid is said to be alkaline if it contains bacteria and acidic if fungi is found in the water calyces [1]. The mutual benefit between the microbes present in the water calyces and the floral buds is not clear. The fluid of *S.Campanulata* calyx is suggested to be to be protecting the young floral buds from the herbivores [2, 3]. The present work is an attempt to study the biochemical and microbial composition of the fluid inside the calyx of *Spathodea campanulata,* to establish associative symbiosis.

Figure 1. Inflorescence of *Spathodea campanulata*

2. Material and methods:

2.1. Collection of Inflorescence

The flower buds of *S. campanulata* were collected from Richmond, Langford Road, Bangalore. The work was carried out in 2 seasons.

2.2. Studies conducted with the flower buds

The inner surface of the calyx was examined under Stereozoom. The fluid from water calyces of *S. campanulata* was collected from flower buds (younger to older buds) and was used for biochemical estimations. The fluid was tested and estimated for the presence of starch, reducing sugars, amino acids and ammonia. The pH of the fluid present in the bud was studied in an increasing order of bud maturity by using pH paper/meter. The fluid was used for bacterial isolation and culturing.

2.3. Biochemical tests

Test for Starch: 1ml of the fluid from the calyx of different sizes was mixed with 0.5ml of Iodine reagent in a separate test tube and the colour reaction was observed [4].

2.3.1. **Test for reducing sugars:** 1ml of the fluid from the water calyx of different sizes was mixed with 1ml of Benedict's reagent and heated in a boiling water bath for 10 minutes. The colour reaction was observed and compared to the colour chart provided with reagent [4].

2.3.2. **Estimation of reducing sugars:** The reducing sugar present in the fluid was estimated using Nelson-Somagyi method and quantified using a standard curve prepared with glucose. 1 ml of the fluid from the water calyx of different sizes were taken in 25ml marked test tube, mixed with 1ml of alkaline copper reagent, boiled in a water bath for 10 min and cooled. 1 ml of arsenomolybdate reagent was added and the blue colour was estimated at 640nm against a blank and the concentration was calculated using a standard graph prepared with glucose [4].

2.3.3. **Estimation of free amino acids:** Ninhydrin reagent was used for the estimation of free amino acids in the fluid of the water calyx. 1ml of the fluid from the water of different sizes were taken separate test tubes, mixed with 1ml Ninhydrin reagent, boiled in water bath for 5 min with the mouth of the tubes covered. 5ml

of diluent solution (methyl cellosolve) was added to each tube and made up to 25 ml with distilled water. The intensity of the purple colour was measured against 535 nm in a spectrophotometer and the concentrations were calculated using a standard graph. [4]

2.3.4. **Estimation of ammonia:** It was carried out using Nessler's method, using a test kit for ammonia estimation from fish tank water. 1 ml of the fluid from the water calyx of different sizes was taken in test tubes, mixed with 1ml of Nessler's reagent, the colour reaction and precipitation were observed. [5, 6]

2.4. Microscopic studies of the fluid

The microscopic studies of the fluid were carried out using light and phase contrast microscopes. Staining techniques like Gram staining and capsule staining and hanging drop technique for motility were conducted following routine microbial methods. [6]

2.5. Isolation and culturing of the bacteria from the fluid

The organism from fluid was isolated on NFb semi solid medium, 0.1ml of fluid was injected into 1ml of medium (5 samples). Purification of pellicles was carried out on NFb semi solid medium to get pure cultures. culturing was done on NFb+Yeast Extract agar medium. [7, 8]

3. Results

3.1. Examination of the inner wall of the water calyx and pH of calyx fluid

Inner side of the young and old water calyces showed the presence of thick mucilaginous bacterial colonies (Fig 2 & Fig 3).

Figure 2. Mucilagenous bacterial colony on the inner side of the calyx in young bud

Figure 3. Mucilagenous bacterial colony on the inner side of the calyx in older bud

The pH of calyx fluid was found to be alkaline ranging from pH 8.9 to pH 9.1 from small buds, medium buds to large buds (Table 1)

Table 1: pH test

Floral buds	pH paper	pH meter
1	9	8.9
2	9	8.9
3	9	9
4	9	9.1
5	9	9

3.2. Biochemical tests

The biochemical tests for starch, reducing sugars, amino acids and ammonia qualitatively showed that the fluid was rich in reducing sugar, amino acid and ammonia (Fig 4) but the fluid is absent for starch. (Table 2)

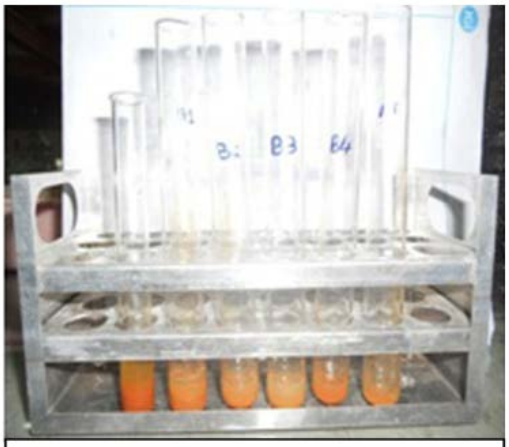

Figure 4. Positive test for presence of ammonia

Table 2: Biochemical estimations

Molecules screened	Method	Result	ug/ml avg of 5 samples
Starch	Iodine test	Absent	-
Reducing sugars	Benedict's	Absent	-
	Nelson's – Somogyi test	Present	43
Amino acids	Ninhydrin test	Present	86
Ammonia	Nessler's Reagent test	Present	-

3.3. Microscopic studies of the fluid

The microscopic studies of the fluid carried out using light and phase contrast microscopes revealed the presence of Bacteria in the calyx fluid, which increased in number from smaller bud to larger bud (Fig 10 & Fig 11). Hanging drop technique showed the bacteria were motile in nature (Fig 5). Gram staining showed the calyx fluid bacteria were gram negative (Fig 6) and capsule staining showed the presence of thick capsule around the bacterial cell (Fig 7) (Table 3).

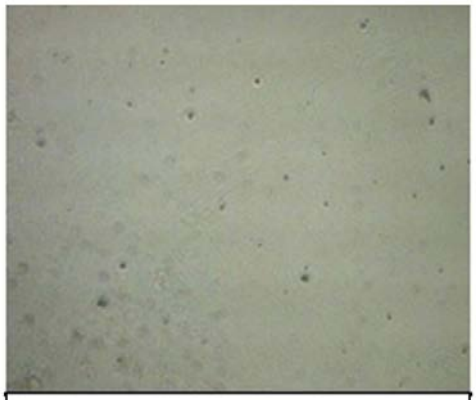

Figure 5. Microscopic view of motile capsulated bacteria

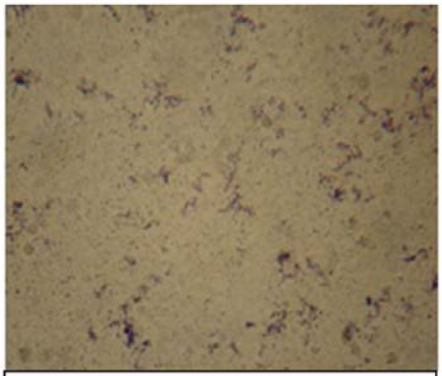

Figure 6. Microscopic view of Gram-negative bacteria upon Gram staining

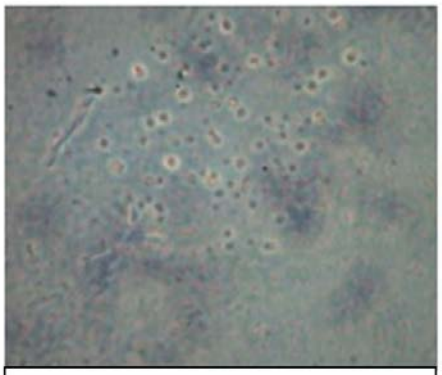

Figure 7. Microscopic view of capsulated bacteria upon capsule staining

3.4. Isolation and culturing of the bacteria from the fluid

Bacteria were isolate in NFb semi solid medium The older buds showed white pellicle formation on 7th day and younger buds on 10th day after inoculation.

Purification of pellicles was carried out on NFb semi solid medium to get pure cultures. Pure culture was inoculated on NFb+YE agar medium and yellow, shining and glossy mucilaginous colonies were obtained after incubation period (Fig 8 & Fig 9) (Table 3)

Table 3: Microscopic observations of bacteria using light and Phase contrast microscope, isolation and culturing.

Technique	Observations
Gram staining	Gram negative
Capsule staining	Capsulated
Motility by hanging drop	Motile
Nitrogen free semi solid medium	White pellicle formation
Nitrogen free agar medium with Yeast Extract	Yellow colonies

Figure 8. Isolation of bacteria in NFb semi solid media

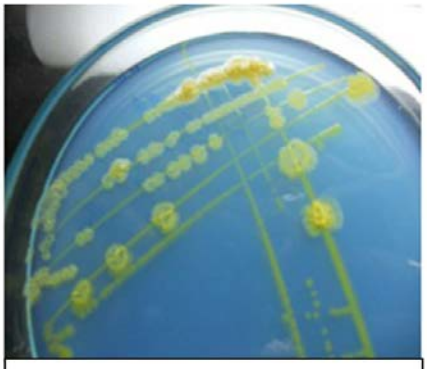

Figure 9. Growth of bacterial colonies on NFb+YE agar

Microscopic studies also revealed that the number of bacteria increased with age/size of the water calyces and also presence of peltate glands were observed on the inner side of the calyx, which contains motile bacteria (Fig 8)

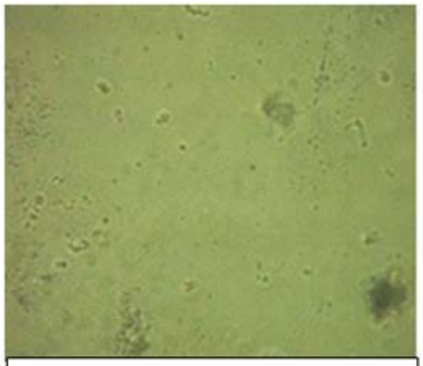

Figure 10. Microscopic view of bacteria from younger

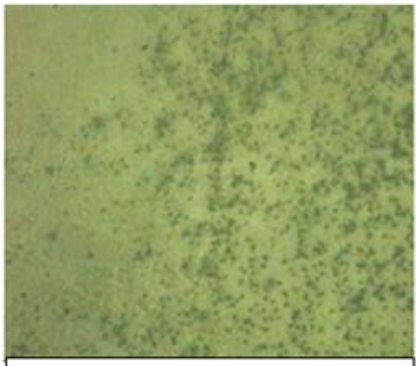

Figure 11. Microscopic view of bacteria from older bud

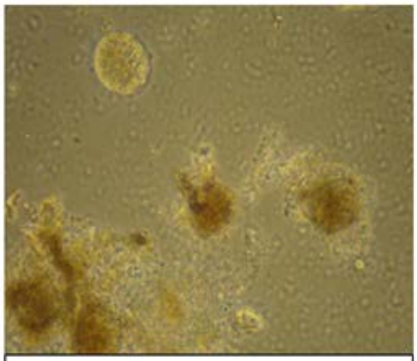

Figure 12. Peltate glands found on the inner side of

4. Discussion:

The water calyx in *S. campanulata* is an interesting feature which could be to be protecting the developing floral organs. In late 19th century, Trueb had reported the presence of microbes and partially gave the chemical composition of the fluid ascribing it to be ammoniacal and putrid. But he failed to report both, the role of bacteria and the fluid during the floral development. Although the earlier work of Jintana *et al*., (2010) has reported that the flower contains acetic acid bacterium and according to Kallider *et al*., (2011) the pH of fluid is acidic i.e., 6.4 [9,10]. The pH was found to be alkaline around pH 9 from the study which disproves the earlier work by Kallider *et al.*, (2011) [9,10].

The present work reveals the presence of large quantity of ammonia by Nessler's reagent test. The fluid was positive for Ninhydrin reagent suggesting that amino acid may be present but Ninhydrin reacts positively for ammonia also giving characteristic pink colour. Hence the

result prove that large quantity of ammonia occurs in the fluid in increasing quantity from younger to mature buds and pH was always found to be around 9 in all stages of maturity indicating that the fluid is of alkaline which is seen to be contradicting with the earlier mentioned reports [9,11]

Microscope studies proved the presence of motile capsulated gram-negative bacilli. The number of organisms seems to be increasing with age of the buds. Big sized colony with large number capsulated bacteria was obtained from older buds and small colony from younger buds indicating the increase in population of bacteria with age and size of water calyx. Isolation of the bacteria from the fluid using NFb semisolid medium at neutral pH failed or inconclusive, but at pH 8.5 in the semisolid medium pellicle formation in five days was observed consistently in all the isolation trails. The isolate was growing on NFB agar medium supplemented with yeast extract, producing yellow-coloured colonies. These results indicate that bacteria seem to be, microaerophilic since it occurs in the viscous liquid, alkalophilic and nitrogen fixing since isolation could be done in alkaline nitrogen free medium. The ammonia accumulated in the fluid of the calyx may be excretion by the bacteria [12]. *Stenotrophomonas maltophila* is reported secrete ammonia into the medium [13]. Plant growth promoting rhizobacteria are known to be associated with roots of many plants, offering benefits of nitrogen fixation, phosphate solubilisation, antagonistic action and plant hormone Indole-3-acetic acid (IAA) production [14,15]. A similar type of plant growth promoting benefit may be provided by the bacteria in water calyx to the plant leading to an associative symbiosis, unique among flowering plants and microbes.

5. Conclusion:

The study reveals the presence of bacteria within the cells of calyx and in the calyx fluid. The qualitative estimation of biochemical compounds from the calyx fluid showed that the calyx fluid was rich in Sugars and amino acids. The fluid was alkaline in condition, possible due to presence of ammonia, which may be produced as a result of nitrogen assimilation. The isolation and culturing of bacteria on NFB semi solid media and NFB solid media showed prominent pellicle formation and mucilaginous colonies respectively, proving the bacteria associated may be a potential nitrogen fixing bacteria and may provide plant growth promoting benefit in water calyx of *Spathodea campanulate*. Therefore, this study is a preliminary report on occurrence of alkalophilic, microaerophillic, nitrogen fixing bacteria from the water calyx of *Spathodea campanulata P.Beauv.*

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