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BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN *AZOLLA-ANABAENA AZOLLAE* SYMBIOTIC SYSTEM UNDER GRADIENT CONCENTRATIONS OF NH_4NO_3

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ABSTRACT

The effect of NH_4NO_3 on *Azolla pinnata* association was evaluated using morphological and biochemical analysis by growing the plants in gradient concentrations of NH_4NO_3. The association showed a doubling time of 4.25 to 4.5days in control conditions. The supplementation of NH_4NO_3 reduced the doubling time and 5mM NH_4NO_3 gave better growth rate. Root length analysis showed that the root length increased with decreasing supply of nitrogen. *Azolla* underwent morphological modifications such as root length and root number increase in order to increase nitrogen uptake under nitrogen limitation. The increased concentration of NH_4NO_3 supply increased the GS activity with a maximum activity in 5mM NH_4NO_3. The prolonged supply of NH_4NO_3 reduced the GS specific activity as evident from the reduced GS specific activity on 14th day, which was further increased on 21st day suggesting the possibility of a regulatory mechanism for the uptake of ammonium. Chlorophyll content, total sugar and soluble sugar are directly influenced by nitrogen supply.CN analysis detected that the reduction in total nitrogen increased the C/N ratio at higher concentrations of NH_4NO_3 which is due to the inhibition of ammonium uptake at higher concentrations of NH_4NO_3.

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INTRODUCTION

Azolla pinnata is a symbiotic system which fixes atmospheric nitrogen with the help of the endophyte *Anabaena* to meet the nitrogen requirements of the association (Mayashree, *et al.*, 2017). *Azolla* is capable of growing in nitrogen free media (Ray *et al*., 1979) as *Anabaena* supplies ammonia to the fern, and the fern in turn provides the cyanobacterium with photosynthetic assimilates (van Hove and Lejeune 2002) thus helps to maintain the C-N balance in the association. The ammonium supplied by the cyanobacterium is assimilated by the fern with the help of Glutamine synthetase (GS), which catalyzes the incorporation of ammonium into glutamate and generate glutamine (Tobin and Yamaya, 2001), which provides nitrogen groups, either directly or via glutamate for the biosynthesis of all nitrogenous compounds in the plant (Lea *et al*., 1990). The GS activity is dependent on the ammonium concentration, thus the measure of GS activity suggest the level of ammonium intake and rate of ammonium assimilation in the symbiotic system. Nitrogen is a limiting factor for plant growth and development; and its deficiency cause changes in plant growth and development. Ammonium nitrate (NH_4NO_3) serves as a nitrogen source for *Azolla* in Hoaglands nutrient medium. The concentration of NH_4NO_3 determines the growth characteristics of the association. In the present study the effect of gradient concentrations of NH_4NO_3 on the morphological and biochemical characteristics were analyzed to understand the significance of NH_4NO_3 concentration on the association. Morphological parameters such as doubling time, relative growth rate and root length of *Azolla pinnata* grown in gradient concentrations of NH_4NO_3 were analysed on the 7th, 14th and 21st day. Morphological analysis gives a qualitative validation of *Azolla* growth in gradient concentrations of NH_4NO_3 when compared to ambient atmospheric conditions and selecting the ideal concentration of NH_4NO_3 for better growth. Average root length of 50 roots was measured to find the response of *Azolla* towards nitrogen limitation. NH_4NO_3 supplied above an optimum concentration can also affect the growth as it retards

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the root length and relative growth rate. Biochemical analysis of the association under different concentrations of NH_4NO_3 helps to understand the assimilation potential of *A.pinnata* with respect to gradient concentrations of NH_4NO_3. The growth analysis in relation to GS specific activity, chlorophyll content, total sugar content, soluble sugar content, total C%, total N% and C/N ratio on the 7th, 14th and 21st day of growth is measured. All these parameters were compared with that of *Azolla* under control condition and in nitrogen free media.

MATERIALS AND METHODS

Plant material

A. pinnata fronds collected from the Botanical garden, University of Calicut were thoroughly washed several times in tap water to remove adhering green algae and other microorganisms, followed by surface sterilization with 0.12% (v/v) sodium hypochlorite solution containing few drops of Tween 20 for 10 mins. The fronds were then washed with sterile distilled water, blotted dry and transferred to Hoagland's nutrient solution.

Culture medium and Gradient concentrations of NH_4NO_3

A. pinnata, fronds were grown in Modified Hoaglands solution with or without combined nitrogen. Gradient concentrations of NH_4NO_3 at 1mM, 2.5mM, 5mM and 10mM concentrations were added to N free Hoaglands medium. The cultures were kept in 16-hr light, with a light intensity of 1500$\mu Em^{-2}s^{-1}$ and a temperature of 25±2ºC. Illumination was provided by cool white fluorescent, incandescent lights. *Azolla* under ambient conditions and in N free Hoaglands medium served as the controls.

Doubling time of *A. pinnata*

Growth rate of *Azolla* cultures in different experimental conditions was measured by calculating the doubling time using the formula derived by Aziz and Watanabe (1983).

Doubling time = t/r
Where, t = duration of growth
r = [log (Wt/Wo)]/0.301
Wt= Weight of *Azolla* after't' days.
W_0= Weight of initial inoculums

Root number and length

The root number and root length were measured from each experiment by counting individual roots from randomly selected fronds. The root length was measured by placing the representative fronds on a graph paper, and an average of at least 50 roots were measured.

GS extraction and Transferase assay

100mg of *A. pinnata* fronds from all the experiments were powdered in liquid nitrogen, emulsified in an extraction buffer containing 100mM Tris HCL (pH 7.2), 3mg DTT, 0.2g PVP, and 0.2ml PMSF using a mortar and pestle kept on ice. The homogenate was then centrifuged at 10,000 rpm for 10mins at 4ºC. The supernatant was collected and used for GS transferase assay and protein quantification. GS transferase assay was carried out according to the protocol explained by Shapiro and Stadtman (1970). The optical density was measured at 540nm against blank to which the stop solution was added prior to addition of enzyme extract and incubated at 30ºC for 15min. Total protein was quantified according to Lowry *et al*. (1951) using gradient concentrations of bovine serum albumin (BSA,1mg/ml) as standard. The concentration of γ-glutamyl hydroxamate and protein was calculated from their corresponding standard graphs and the specific activity of GS was calculated.

Quantification of Chlorophyll, total sugar and soluble sugar

Total chlorophyll content of the *Azolla* fronds under different growth conditions was quantified according to Arnon's protocol (1949). Chlorophyll a, chlorophyll b and total chlorophyll was measured at corresponding optical density and calculated according to the equations by Mac Kinney (1941).Total sugar and soluble sugar from the *Azolla* cultures were quantified using Anthrone method (Hedge and Hofreiter 1962) from standard graph developed using glucose as standard.

Total Carbon

Total carbon of *A.pinnata* grown under different experimental conditions was measured using CHNS analyser (Model: Elementar Vario EL III). Dried samples were ground to a fine powder using mortar and pestle and subsequently measured with sulfanilic acid as standard.

RESULTS

Doubling time (Dt)

Azolla pinnata grown in 5mM NH_4NO_3 showed the shortest doubling time of 3.96±0.07 days. Plants under nitrogen starvation (without any nitrogen source) showed a higher doubling time 7.13±0.29 days. *Azolla* grown under 10mM NH_4NO_3 showed a doubling time with an average of 4.98±0.17 days while plants under natural conditions showed an average doubling time of 4.25±0.20 days (Fig.1). *Azolla* in 5mM NH_4NO_3 with the least doubling time showed better growth than those under *in vivo* conditions.

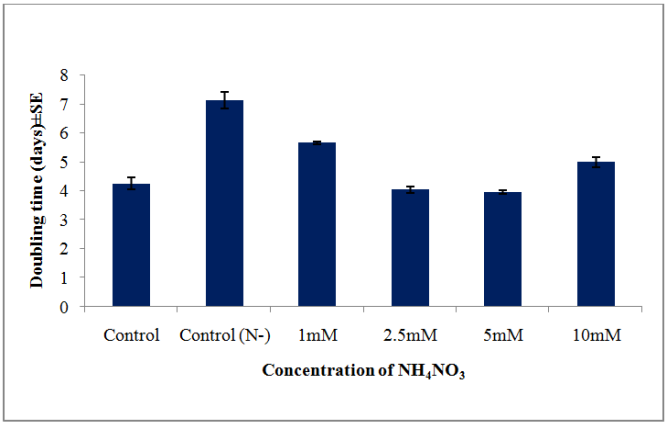

Fig. 1. Doubling time (days) of the *A. pinnata* association grown in gradient concentrations of NH_4NO_3

Relative growth rate (RGR)

The relative growth rate increased from the 7th to 14th day and decreased on the 21st day, it was the highest in *Azolla* treated with 5mM NH_4NO_3 on the 14th day. Relative growth rate was the least on the 21st day for *Azolla* treated with 10mM NH_4NO_3. Plants under nitrogen starvation showed a lower relative growth rate than plants under control conditions and those supplied with NH_4NO_3 (Fig.2). Excessive ammonium supply can also hamper the growth of *Azolla;* by reducing the relative growth rate.NH_4NO_3 supplied above 5mM affected the growth of *Azolla* leading to a decrease in RGR.

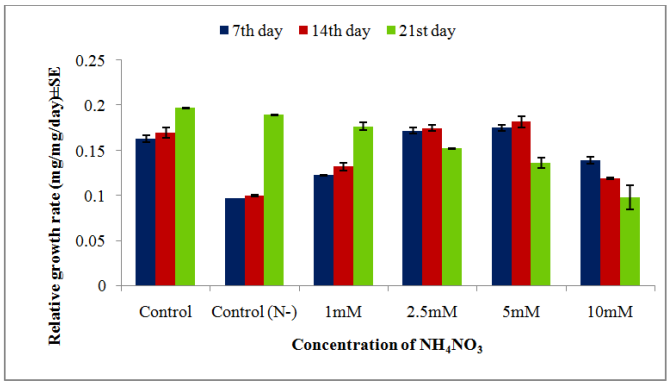

Fig. 2. Relative growth rate (RGR mg/mg/day) of *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on 7th, 14th and 21st day

Root Length analysis

The root length increased as the supply of nitrogen decreased. *A. pinnata* cultures under nitrogen starving condition (Control [N^-]) showed the longest roots with an average of 1543.95±1.6mm on the 21st day, followed by 1365±1.7mm on the 7th and 1302.74±1.6mm on the 14th day. Plants grown under normal atmospheric conditions showed an average root length of 461.33±0.1mm on the 7th day and with slight changes on 14th and 21st day. With the increase in concentration of NH_4NO_3 the root length decreased (Fig.3). The smallest root length was observed in cultures treated with 10mM NH_4NO_3 and the longest in *Azolla* treated with 1mM NH_4NO_3. Supply of NH_4NO_3 above 5mM affected the root length; the roots started shedding and became too short in length.

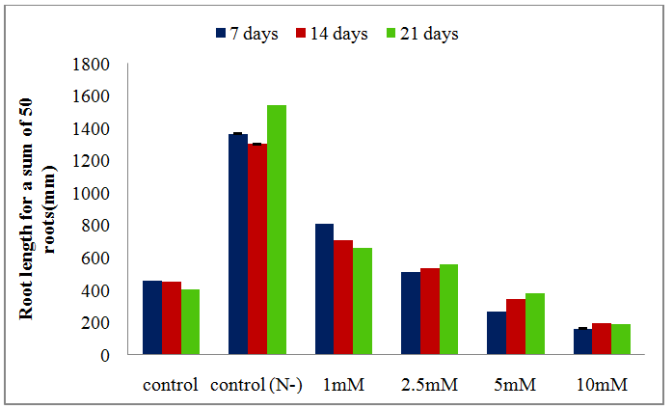

Fig. 3. Root length of the *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on 7th, 14th, and 21st day

GS Specific activity

GS specific activity was highest in *A. pinnata* grown in 5mM NH_4NO_3 on the 7th day followed by 10mM, control, N-deficient control, 2.5mM NH_4NO_3 and 1mM NH_4NO_3. After 14th day of culture the GS specific activity decreased in all the concentrations except in 1mM NH_4NO_3 and 2.5mM NH_4NO_3. Generally the GS specific activity decreased from the 7th to 14th day. On the 21st day the plants showed a gradual increase in GS specific activity compared to 14th day with highest in 5mM NH_4NO_3 treated plants. Control plants and N- deficient plants showed a better GS activity 278.11±0.44 and 223.58±0.89 on the 7th day which reduced drastically on the 14th day and then increased on the 21st day (Fig.4). The initial increase in GS specific activity was due to the increased uptake of ammonium and once the internal concentration increased the uptake decreased so does the GS specific activity.

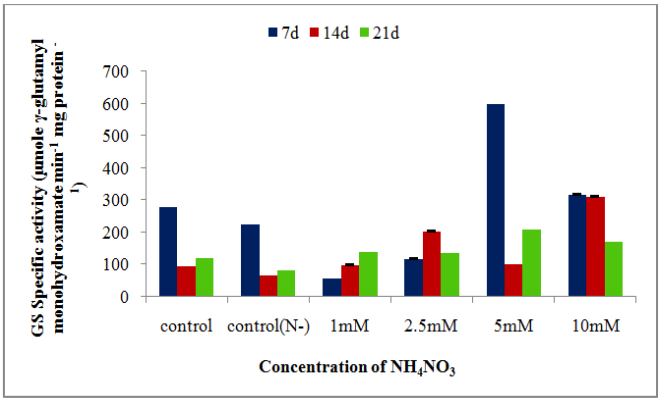

Fig. 4. GS specific activity (µmole γ-glutamyl hydroxamate min^{-1}mg $protein^{-1}$) in *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on 7th, 14th, and 21st day

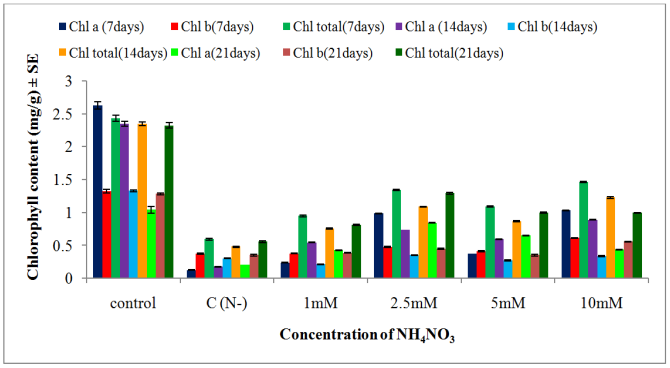

Fig. 5. Chl a, chl b and total chl content of *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on 7th, 14th, and 21st day

Quantification of chlorophyll

NH_4 concentration in the medium influenced the chlorophyll content in *A. pinnata.* The total chlorophyll content of *A. pinnata* grown in the control was higher on the 7th, 14th and 21st day. *Azolla* grown without nitrogen (N-) supply showed the least chlorophyll content. Of the different concentrations of NH_4NO_3 used the total chlorophyll content was highest in 10mM with 1.47±0.01mg/g on the 7th day which slightly increased on the 14th day but dropped drastically on the 21st day. The next highest chlorophyll content was observed in 2.5mM NH_4NO_3 on the 7th day which decreased a little on the 14th day then increased on the 21st day. Chlorophyll a was highest in 2.5mM NH_4NO_3, with 0.87±0.002mg/g but chlorophyll b was highest in 10mM NH_4NO_3 with

0.61±0.005mg/g. Even though 10mM NH_4NO_3 showed highest chlorophyll content initially, the chlorophyll content decreased drastically towards the end. *A. pinnata* grown in 2.5mM NH_4NO_3 the chlorophyll content was less compared to 10mM NH_4NO_3, it increased after a drop on the 14th day. During the 21st day a slight increase in chl a, chl b and total chl was observed in all the treatments except in the higher concentration 10mM NH_4NO_3 (Fig.5). The drastic drop in chlorophyll content on the 21st day in 10mM NH_4NO_3 indicates the difficulty of survival for *A. pinnata* under extremely higher concentrations of ammonia and the chlorophyll content is dependent on the internal N levels and exposure period.

Total sugar quantification

The highest quantity of total sugar was measured in the control plants grown on the 7th day, which gradually increased on the 14th day and later decreased on the 21st day. Whereas in control (N-) without N supply, there was a hike in total sugar content on the 14th day and then decreased. The gradient concentrations of NH_4NO_3 showed a similar pattern of increase on the 14th day and then decreased on the 21st day except 10mM NH_4NO_3 which increased on the 21st day (Fig.6). The increase in sugar content on the 14th day shows its relation with N assimilation. The decrease in N assimilation on 14th day can be the reason for increased sugar content on 14th day.

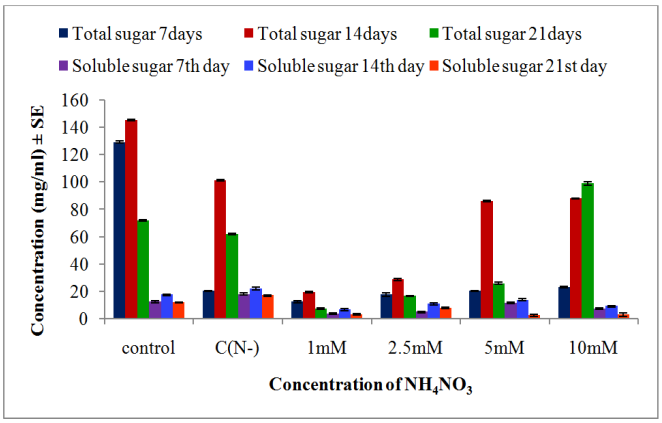

Fig.6. Total sugar and soluble sugar content of the *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on the 7th, 14th, and 21st day

Soluble sugar estimation

Among the *A. pinnata* grown as control and those under gradient concentrations of NH_4NO_3, highest quantities of soluble sugar was observed in control without nitrogen (N-) supply on the 7th, 14th and 21st day compared to all other samples. It showed a gradual increase from day 7 to 14 and then dropped on the 21st day. In the cultures kept in gradient concentrations of NH_4NO_3, *A. pinnata* grown in 5mM NH_4NO_3 showed highest soluble sugar on the 7th and 14th day later decreased drastically on the 21st day. All the growth conditions showed a similar pattern of increasing soluble sugar level on the 14th day from that of the 7th day and a decrease on the 21st day (Fig.6). This can be due to the decrease in N assimilation on the 14th day. The control (N-) plants showing increased levels of soluble sugar indicates its association with nitrogen availability. As there is a lack of nitrogen in control plants, lesser quantity of sugars will be taken for carbon assimilation.

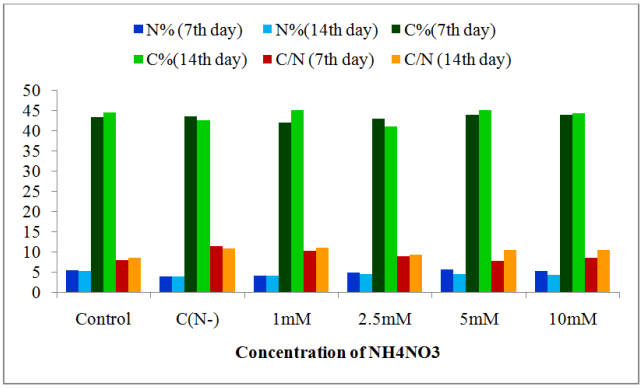

Fig.7. C/N ratio and total CN content in *A. pinnata* association grown in gradient concentrations of NH_4NO_3 on the 7th and 14th day

CN analysis

CN analysis of the association under varying concentrations of NH_4NO_3, confirms its role in determining the total carbon and nitrogen contents. C/N ratio is an indicator of nitrogen limitation of plants, and adequate supply of C and N nutrients are critical for plant growth development and response to a wide array of stresses. Maintaining an appropriate balance of C and N nutrients is critical from the metabolic point of view. The C/N ratio increased with decreasing levels of nitrogen intake. Increased supply of N reduced the C/N ratio to some extent only; concentration above 2.5mM NH_4NO_3 increased the C/N ratio. The control plants showed nitrogen content of 5.48% and C content of 43.39% on the 7th day which gave a C/N ratio of 7.93. On 14th day nitrogen content of the control (+N) plants decreased to 5.27%, carbon content increased to 44.67% increasing the C/N ratio to 8.46. Control plants without nitrogen supply showed 3.82% N and of 43.67% C increasing the C/N ratio to 11.42 than control +N (Fig.7). It is clear from the observation that increased C/N ratio indicates a demand for nitrogen.

DISCUSSION

Ammonium (NH_4^+) and nitrate (NO_3^-) are the major N sources for the plant growth and development. Ammonium is the preferred N source over nitrate by plants. But, when ammonium salts are supplied to plants as the only source of nitrogen in a growth medium the pH will fall to a strongly acidic state which will affect the plant growth. Similarly when nitrate salt alone is supplied as N source the pH will eventually shift to alkalinity (Arnon, 1937). The deleterious effect of ammonia alone as a sole source of N has been reported in many plants (Errebi and Wilcox, 2008). The addition of nitrate however, alleviated the inhibitory effects of NH_4 on growth (Ota and Yamamoto, 1989). In several crops, combinations of NH_4 and NO_3 usually result in greater vegetative growth than when either N form is used (Edwards and Horton, 1982; Schrader *et al*., 1972). The effect of NH_4NO_3 on growth rate, morphological changes, chlorophyll content, total sugars, soluble sugars and C-N ratio was analyzed in *A. pinnata* and proved to be influenced by the varying concentrations of NH_4NO_3. It is already proven that, if plant N nutrition is based on either NH_4^+ or NO_3^-; uptake, this has a range of secondary effects on the overall physiology of the plant effecting its

uptake, assimilation and transport, differences in plant and root morphology (Marschner, 1995).The ammonium assimilation potential of the association was studied by analysing GS specific activity and quantifying the nutritional status including chlorophyll content, total sugar and C-N ratio.

Effect of NH_4NO_3 on morphological characteristics

Azolla-Anabaena association lives in symbiosis with each other sharing the carbon and nitrogen sources. *Azolla* provides the required carbon while endophyte supplies the necessary nitrogen in the form of NH_3 thereby maintaining a balance. The morphological characters analyzed in this study such as doubling time, relative growth rate and root length exhibited significant responses towards varying concentration of NH_4NO_3.Under ambient conditions the association exhibited an average doubling time ranging between 4 to 4.5days. The doubling time increased with age of the culture as overcrowding cause scarcity of nutrients. When $NH_4NO_3$was provided the doubling time reduced indicating increased growth and biomass in short time. The association supplied with 5mM NH_4NO_3showed the lowest doubling time and maximum growth rate further increase in concentration increased the doubling time and reduced the growth rate. Even though association is dependent on the nitrogen fixed by the endophyte it can also assimilate combined nitrogen from the medium (Meeks *et al*., 1987).Cary and Weerts (1992) showed that *A. pinnata* and *A. filiculoides* can obtain externally supplied nitrogen, even though both species benefit from a symbiotic association. The external supply of nitrogen in the form of combined nitrogen, however, depresses nitrogen fixation by the symbiont. As a result a balance is maintained between the nitrogen inputs from the atmosphere and the medium in order to maintain a relative growth rate and doubling time beneficial for the association. The combined nitrogen source in the form of NH_4NO_3 at low concentration up to 5mM can make up the loss of nitrogen input from the endophyte as a result of reduction in nitrogen fixing activity. A study in *A. japonica* proved that a concentration of ammonium up to 2.5mM and nitrate up to 1.25mM in the medium had no apparent effect on its growth, as the growth was comparable to that of fronds grown in N alone. But, further increase in the concentrations of these combined nitrogen sources decreased the growth rate (Kitoh and Shiomi, 1991).The inhibitory effects of combined nitrogen on the nitrogen-fixing activity in *Azolla* species has been confirmed by many investigators (Yatazawa *et al*. 1980; Peters and Mayne 1974). However, only few studies were conducted on the effect of combined nitrogen sources in the medium on growth. Yatazawa *et al*. (1980) reported that ammonium at a concentration as low as 1mM caused a considerable decrease in the growth of *A. pinnata*. The present results showed that ammonium nitrate in the medium exert an unfavourable effect on the growth of the association when their concentrations increased above 5mM. Jampeetong *et al*., (2016) proved that *A. pinnata* grown in below 5mM NH_4^+ had higher relative growth; however, increasing NH_4^+ concentration above 5mM significantly decreased the growth characteristics. Generally, plant growth decreases under N supply exceeding10mM, a value considered to be the threshold of toxicity for some species (Cao and Tibbitts, 1998). Most plant species show a stunted root system, smaller leaves and reduced growth when exposed to high nitrogen concentrations, and in severe cases this leads to the death of the plant. The fronds grown in the medium containing 20mM ammonium formed clusters and even after the fronds were returned to N-free medium neither the growth rate nor the nitrogen-fixing activity recovered for at least several weeks. The reason behind the decrease in growth of the association under higher concentrations of NH_4NO_3 is due to the inhibition of de novo synthesis of nitrogenase (Mahl and Wilson, 1968), or due to the inactivation of the nitrogenase system (Bone 1972) thereby reducing the nitrogen supply by the endophyte. However, it is possible to consider that in the *Azolla-Anabaena* association, the reduction of the nitrogen fixation capacity by high ammonium concentrations is a result of the decrease in the number of endophyte cells.

The association like any other plant systems has several mechanisms to withstand conditions of nutrient starvation. One among them is by modifying the root architecture. Roots being the primary site of nutrient absorption can be increased in surface area by increasing number, length and lateral hairs to increase the area of nutrient absorption. The varying concentration of NH_4NO_3 caused modifications in root morphology. The root length of the association reduced with increase in NH_4NO_3concentrations.The root growth and other characteristics such as the rate of growth to depth, root density and maximum rooting depth indicate a supply of potentially available mineral nitrogen and water (King *et al*., 2003, Svoboda *et al*., 2000). According to Svoboda and Haberle (2006) high doses of nitrogen decreases root growth to depth (length) and density as the plant demand for N is saturated from the top soil layer. In case of hydroponics increased nitrogen supply increased shoot growth than root thereby increasing shoot/root ratio, indicating a negative effect on root length (Robinson *et al*., 1994). Jampeetong *et al*. (2016) in his study with *Azolla* observed a decrease in the growth rate and root length in higher concentration of ammonia than 5mM. It is also reported that the N status of plants influences their root mass fraction (Ingestad and Agren, 1991). Furthermore, biomass allocation to shoots and roots has been shown to be influenced by the NO_3^- content of leaves, implying that NO_3^- may act as a signal for shoot allocation (Scheible *et al*., 1997) as well as root branching (Zhang and Forde, 1998).

Effect of NH_4NO_3 on GS specific activity, Chlorophyll content, total and soluble sugars

Glutamine synthetase is the primary ammonium assimilating enzyme in the plants and plays a crucial role in plant growth and development. GS assimilates ammonia as amino acids by incorporating it into carbon skeletons and plays a vital role in balancing C/N metabolism (Lea and Milfin 2003; Sood *et al*., 2002). The incorporation of NH_4^+ into amino acids involves the glutamine synthetase/glutamate synthase pathway and glutamate dehydrogenase. The GS/GOGAT cycle is the primary N assimilation pathway under normal growth conditions and operates at low N concentrations with a high affinity for NH_4^+ (Bedell *et al*., 1999). GS specific activity is the measure of ammonium intake which determines the rate of ammonium assimilation. In the association the GS specific activity increased with the increasing concentration of NH_4NO_3 till 5mM. The highest GS activity was observed on the 7^{th} day which decreased drastically on the 14^{th} and then increased after the 21^{st} day. On 14^{th} day the GS specific activity reduced as a result of increased internal concentration of N due to increased assimilation on the 7^{th} day. The NH_3 accumulation significantly increased the GS activity to assimilate NH_3 in *Arabidopsis* (Ishiyama *et al*., 2004). A similar response was observed in sandal plants which exhibited

higher GS activity on the 7th day in 0.5mM NH_4NO_3 indicating concentration and time dependent up regulation of GS enzyme (Yusuf and Deepa, 2017). The NH_4NO_3concentrations above 5mM reduced GS specific activity due to the self regulatory N balancing mechanisms. In 1mM and 2.5mM NH_4NO_3 the GS specific activity increased on the 14th day and again on the 21st day indicating that a concentration below 1mM and 2.5mM is the permissible limits.

The N supply has direct positive effect on chlorophyll content, leaf growth and photosynthesis (Bojovic and Markovic, 2009). In the association the increasing concentrations of NH_4NO_3increased the biosynthetic activities and increased photosynthetic efficiency which is supported by the increased levels of chlorophyll content. Chlorophyll content of the association supplied with combined NH_4NO_3 was less when compared to control plants grown with or without any added combined nitrogen. The chlorophyll content of the association is a cumulative effect of the chlorophyll content in *Azolla* and in the cyanobacteria. A higher concentration of NH_4NO_3 inhibits the endophyte growth; leading to decrease in chlorophyll contribution by the endophyte. Thus total chlorophyll content will be less in association with combined nitrogen than control. In the association under N starvation the chlorophyll content was lesser compared to other growth condition. The chlorophyll content was found to decrease on the 14th day as a result of higher internal N status within 7days in NH_4NO_3 treated plants. However, on the 21st day the deficiency of N was compensated by induced NH_4^+ uptake by plant signals which in turn increased chlorophyll content with better quantity in 2.5mM and 5mM NH_4NO_3. The concentration dependent NH_4^+ uptake and increased GS specific activity induced better N assimilation resulted in higher quality of chlorophyll pigments which positively influenced the photosynthetic efficiency and C assimilation (Dai *et al*., 2009). Sanchez-zabala *et al*. (2015) studied the NH_4^+ induced chlorophyll biosynthesis that provides positive relation between NH_4^+nutrition and leaf pigment biosynthesis in *Arabidopsis* which is in support with the present study. The decrease in chlorophyll content at higher concentrations of NH_4NO_3suggests the requirement of optimum concentration of N nutrient for better production of chlorophyll pigments.

The influence of NH_4NO_3on GS specific activity and photosynthetic pigments without any doubt has influence on total and soluble sugar contents in association. The sugar status of a plant is modulated by photosynthesis which determines the overall growth and development (Smeekens, 2000). The sugar accumulated through photosynthesis serves as carbon skeleton for N assimilation during GS/GOGAT pathway. Higher sugar content induces mechanisms of N assimilation so as to maintain a C/N balance (Oliveira and Coruzzi, 1999). The assimilation of inorganic N into amino acids, proteins, and other macromolecules requires a constant supply of carbon skeletons. The N assimilation is dependent on the product of recent photosynthesis or endogenous carbohydrate reserves. Turpin *et al*. (1991) observed that NH_4^+ assimilation is greatly reduced in cells lacking readily metabolizable carbohydrate reserves. Various reports suggest that NH_4^+enrichment, causes a decrease in the flow of recent photosynthates to sucrose and starch, and that NH_4^+assimilation is regulated by the supply of reducing equivalents and carbon skeleton (Turpin *et al*., 1988). In the present study increased NH_4^+uptake led to higher chlorophyll contents and photosynthetic efficiency contributing to increased soluble and total sugar production. The increased GS specific activity at higher concentrations of NH_4^+can assimilate the excess sugar to amino acids so that a stable C/N balance can be maintained. The increased sugar content induces the conversion of stored $NO_3^-$to NH_4^+which is assimilated by GS using the carbon source. Thus in the association an increased sugar content can induce the activity of endophyte to increase the supply of NH_4^+.

Effect of NH_4NO_3 on C/N ratio

The concentration gradient of NH_4NO_3 affecting the N assimilation have direct influence on the C/N ratio as N and carbon (C) metabolism are tightly co regulated (Tomohiro *et al*., 2011; Stitt *et al*., 2002; Oliveira and Coruzzi, 1999). The formation of organic matter requires a certain quantity of N and other nutrients in a relatively fixed ratio with C, in accordance with biochemical stoichiometry (Sterner and Elser, 2002). The C and N quantity is maintained by the concerted mechanisms of CN sensing and signalling and affected by different internal and external C/N nutrient status (Zheng 2009). In the present study it was shown that the CN ratio decreased with an increasing supply of N under a stable C accumulation. The association under N limitation showed the highest values of C/N ratio as the N intake was less than any other experimental condition. However, association under ambient conditions maintained a C/N ratio of 7.9-8 after 7 and 14 days. A gradient concentration of NH_4NO_3 increased the N content in *A. pinnata* the total N content also increased. Thus the CN ratio decreased with increasing intake of N till 5mM NH_4NO_3, which showed a CN ratio similar to that of ambient condition. Concentrations higher than 5mM caused an increase in CN ratio as the N intake decreased at extremely higher NH_4NO_3concentrations. The increasing concentrations of urea in the medium increased the total nitrogen content in the tissues of *Azolla* (Watanabe, 1982). In medium containing ammonium a concentration up to 10mM, the N content increased but decreased with a further increase of the ammonium concentration. The total nitrogen content was less affected by the increase of the nitrate concentration except for a moderate increase below 5mM.

Conclusion

Azolla pinnata showed better growth and doubling time at 5mM NH_4NO_3 and was optimized as the ideal concentration. While comparing with the in vivo cultures, the growth parameters were less satisfactory as the additional supply of ammonia might have exerted an inhibitory effect on the endophyte. Increase in NH_4NO_3 concentration above 5mM affected the association due to ammonium toxicity. The measure of GS activity, chlorophyll content, sugar and C/N ratio proved concentration dependent N assimilation in the association.

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