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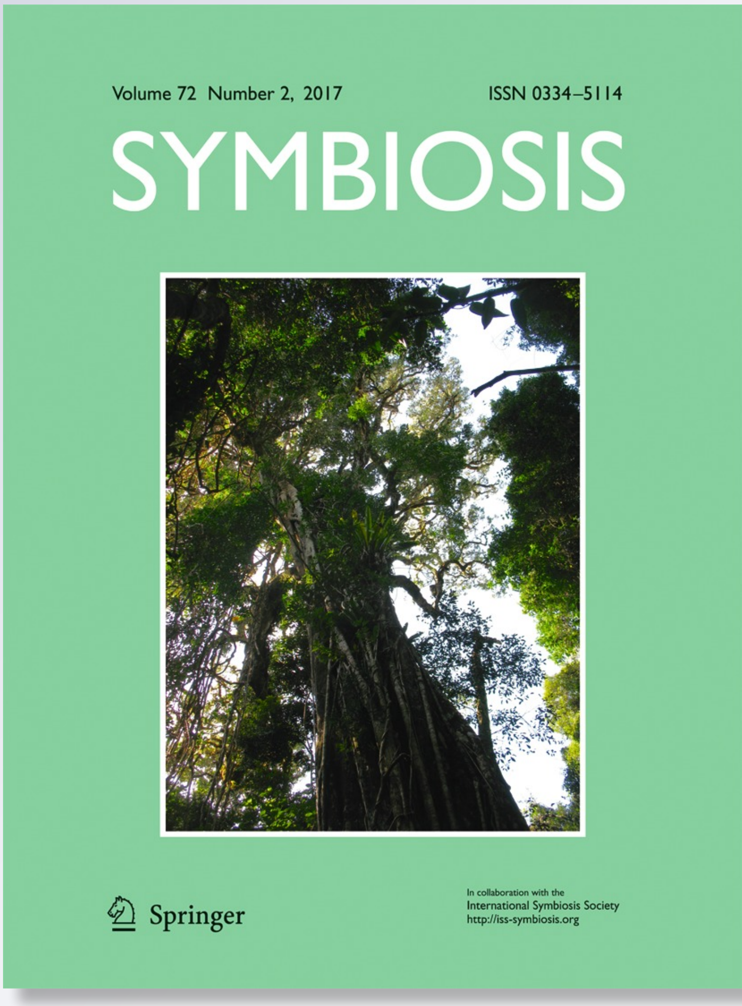

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Impact of elevated CO_2 in *Casuarina equisetifolia* rooted stem cuttings inoculated with *Frankia*

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Abstract Impact of different levels of elevated CO_2 on the activity of *Frankia* (Nitrogen-fixing actinomycete) in *Casuarina equisetifolia* rooted stem cuttings has been studied to understand the relationship between *C. equisetifolia, Frankia* and CO_2. The stem cuttings of *C. equietifolia* were collected and treated with 2000 ppm of Indole Butyric Acid (IBA) for rooting. Thus vegetative propagated rooted stem cuttings of *C. equisetifolia* were inoculated with *Frankia* and placed in the Open top chambers (OTC) with elevated CO_2 facilities. These planting stocks were maintained in the OTC for 12 months under different levels of elevated CO_2 (ambient control, 600 ppm, 900 ppm). After 12 months, the nodule numbers, bio mass, growth, and photosynthesis of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* were improved under 600 ppm of CO_2. The rooted stem cuttings of *C. equisetifolia* inoculated with *Frankia* showed a higher number of nodules under 900 ppm of CO_2 and cuttings without *Frankia* inoculation exhibited poor growth. Tissue Nitrogen (N) content was also higher under 900 ppm of CO_2 than ambient control and 600 ppm levels. The photosynthetic rate was higher (17.8 μ mol CO_2 m^{-2} s^{-1}) in 900 ppm of CO_2 than in 600 ppm (13.2 μ mol CO_2 m^{-2} s^{-1}) and ambient control (8.3 μ mol CO_2 m^{-2} s^{-1}). This study showed that *Frankia* can improve growth, N fixation and photosynthesis of *C. equietifolia* rooted stem cuttings under extreme elevated CO_2 level conditions (900 ppm).

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Keywords *Casuarina equisetifolia* · *Frankia* · CO_2 · Nodulation · N fixation

1 Introduction

Increase of carbon dioxide (CO_2) and other green house gases in atmosphere due to burning of fossil fuels, clearing forests and converting lands for industrial purpose results in global warming and climate change. It was predicted that the amount of CO_2 in the atmosphere is rising by approximately 3 Pg carbon per year (UNESCO/UNEP 2011). The recent report of NOAA (2016) stated that at present (May 2016) the CO_2 concentration in the atmosphere is 407.70 ppm. To mitigate the global warming through carbon sequestration, studies are being undertaken worldwide particularly on afforestation, reforestation and reclamation of waste lands with suitable tree species. However, studies on microorganisms are equally important to reduce the CO_2 levels as the soil microorganisms contribute significantly in the consumption of greenhouse gases such as CO_2, methane (CH_4), nitrous oxide (N_2O), and nitric oxide (NO) (Wiley et al. 2009). For e.g. it was reported that a bacteria *Methylokorus infermorum* consuming methane about 11 kg/year for their energy and multiplication (Jenkinson et al. 1991). Similarly, mycorrhizal fungi consumes 10–20 % of photosynthetically fixed carbon from plants for their survival in plant roots (Staddon et al. 1999) particularly under elevated CO_2 conditions (Quoreshi et al. 2003). These microbial symbionts associated with plants contribute to carbon sequestration by increasing nutrient uptake in plants (Garcia et al. 2011). Plants rely upon microbial symbionts like mycorrhizal fungi and symbiotic nitrogen fixing bacteria to acquire nutrients such as phosphorus (P) and nitrogen (N) for their growth and metabolism. These microbial symbionts scavenge nutrients from soils and transfer to the host

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plant and in turn the symbionts obtain carbohydrates from the host plant (Hodge 1996). In an experimental work total biomass, root biomass and mycorrhizal colonization of *Quercus alba* and *Pinus echinata* seedlings were increased under elevated CO_2 (O'Neill et al. 1987). It was also reported that N fixing plants respond positively to elevated CO_2 than other plants due to their high nutrient demand (Temperton et al. 2003) and the N- fixing plants improved their nutrient supply through N fixing bacteria under elevated CO_2 (Arnone and Gordon 1990; Vogel et al. 1997). Trees under elevated CO_2 also showed increased growth and photosynthesis due to high nutrient supply through microbial symbionts (Ceulemans et al. 1999). Hence it was understood that the microbial symbionts facilitate to sequestrate the carbon in plants. Microbial symbionts also stimulate host plant photosynthesis to a greater extent at elevated CO_2 than at ambient CO_2 (Staddon et al. 1999). This was also confirmed by Tissue et al. (1997) as they found increased photosynthetic rates and carbon storage in *Gliricidia sepium* inoculated with *Rhizobium* sp. under elevated CO_2. Based on these informations, a study has undertaken to determine the inoculation effect of *Frankia* in *Casuarina equisetifolia* rooted stem cuttings under elevated CO_2 to find out the response of N fixation and biomass improvement in *C. equisetifolia*.

Frankia is a symbiotic actinomycete which associates with *C. equisetifolia* and form N fixing root nodules. As part of the symbiotic relationship with *Frankia*, *C. equisetifolia* can fix N up to 300 kg ha^{-1} $year^{-1}$ (Wheeler and Miller 1990) and in return for the fixed N, the tree supply carbon to the symbiotic bacteria (Santi et al. 2013). This tree is used in agro forestry system along with vegetable and pulse crops in India. It grows up to 50 m height with 50 cm girth and the final yield is within 3.5 to 4 years. The annual production of pulp wood alone from *C. equiseitifolia* is 10 million tonnes that worth of $ 300,000 (Karthikeyan et al. 2009). At present the poles of *C. equisetifolia* costs $ 100–120 /tonne in India. It is also used as fuel wood, poles for services like shelterbelts, windbreaks, rehabilitating mine spoils and nutrient poor areas (Diagne et al. 2013). This tree was also recorded for good nutrient turnover through litter decomposition (Uma et al. 2014). However, there are no earlier reports on effect of CO_2 on *Frankia* and *C. equisetifolia* association. The relationship between elevated CO_2 and *C. equisetifolia* in the presence and absence of *Frankia* will be helpful to understand the impact of elevated CO_2 on the growth and photosynthesis of *C. equisetifolia*.

2 Materials and methods

2.1 Culture of *Frankia*

Root nodules of *C. equisetifolia* were collected from the mature trees at farm fields of Coimbatore, India. The nodules were transported in an ice box and stored at −4 °C and surface sterilized with 30 % H_2O_2. Later, the nodules were kept at room temperature for 30–40 min. Under aseptic conditions the nodules were rinsed in sterile distilled water and 0.2 g of nodule was ground manually in a sterile mortar and pestle. The nodule solution was centrifuged at 1000 rpm for 20 min and the supernatant was filtered through Whatman No.1 filter paper. The suspension was then spread on P media* (Shipton and Burgraff 1983) plates and incubated at 25 °C for 3–4 weeks. *(One litre of P medium contained: 10 g $CaCl_2$,$2H_2O$, 20 g $MgSO_4$, 0.46 g propionic acid, 0.15 g H_3BO_3, 0.15 g $ZnSO_4$.7H2O, 0.45 g $MnSO_4$. H2O, 0.004 g $CuSO_4.5H_2O$, 0.028 g $Na_2MoO_4.2H_2O$, 0.009 g $CaCl_2.6H_2O$, 0.04 g Biotin, 100 g K_2HPO_4, 67 g $NaH_2PO.2H_2O$, 0.1 g FeNa EDTA, and 8.g agar; pH of the medium is 6.8). After 25 days of incubation, the *Frankia* growth appeared as fluffy white cloudy colonies. These colonies were transferred in to P media broth for scaling up the inoculum.

2.2 Propagation of rooted stem cuttings

The stem cuttings of *C. equisetifolia* were obtained from the Casuarina germplasm bank at Model Nursery of Institute of Forest Genetics and Tree Breeding, Coimbatore, India. Uniform sized (5 cm length: 1 mm girth) stem cuttings with 10 g (±0.8) of total biomass were treated with 0.1 % carbendazim fungicide for 3 min. The cuttings were later treated with 2000 ppm of IBA (40 mg of IBA + 20 g of talcum powder) by immersing the basal end of the cuttings in the hormonal solution for 0.5 min. The treated cuttings were then placed in 100 cm^3 root trainers containing the inert vermiculite. The rooted stem cuttings were thereafter placed in polytunnels made of polythene sheets (180 cm × 90 cm) and maintained under a temperature range of 32–35 °C and 60–65 % relative humidity for 30 days for the development of roots. Previously the stem cuttings of *C. equisetifolia* were analysed for major reserved tissue nutrients according Jackson (1973).

2.3 Inoculation of *Frankia*

After the development of adventitious roots, *Frankia* was inoculated at the rate of 10 ml /rooted stem cutting. The rooted stem cuttings of *C. equisetifolia* grown in 100 cm^3 root trainers with or without *Frankia* inoculation were placed in Open Top Chambers (OTC) and maintained for 12 months from April 2014 to March 2015. These OTC are cubical structures of 3 × 3 × 3 m dimension fabricated with galvanized iron pipe frame and covered with polyviny chloride sheet. The upper part of the chamber was uncovered to maintain the atmospheric conditions. A software facility called supervisory control and data acquisition (SCADA) was used to control the CO_2 supply.

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The control and *Frankia* inoculated rooted stem cuttings were replicated at 10 times consists of 5 rooted stem cuttings/replicate (Totally 50 rooted stem cuttings/treatment). The rooted stem cuttings of *C. equisetifolia* were watered daily however, no fertilizers were added. Three OTC were used for this study viz., (i) OTC with 600 ppm CO_2 supply /day (ii) OTC with 900 ppm CO_2 supply/day (iii) and an ambient CO_2 controlled chamber. 598 (±2.2) ppm of CO_2 was provided throughout the day in 600 ppm chamber and 899 (±1.7) ppm of CO_2 was provided in 900 ppm chamber. These CO_2 levels were supplied using CO_2 cylinder in the chambers for the entire study period and monitored through SCADA. The ambient CO_2 chamber showed 380 (±1.1) ppm of CO_2. All the chambers were built in the premises of Institute with an espacement of 4 × 4 m. The average temperature in the chambers was 36.8 (±1.00) and the average relative humidity was 65 % (±1.2). The mean annual rainfall was recorded in Coimbatore; India during the period of study was 796.8 mm.

2.4 Harvest and analyses

The rooted stem cuttings of *C. equisetifolia* were harvested after 12 months from the OTC chambers and measured for their growth characteristics like shoot length, root length, number of nodules, root collar diameter and biomass. The tissue N content of rooted stem cuttings was analyzed according to Jackson (1973).

2.5 Photosynthetic rate

At the end of the study period the light saturated photosynthetic rate ($A_{sat,}$ μ mol CO_2 m^{-2} s^{-1}) was measured on the 15 days old needle leaves of *C. equisetifolia* rooted stem cuttings from the top of the stem using photosynthetic meter (Li 6400 XT, Licor linc, USA). These needle leaves are usually will be matured after 10 days as they emerged from the matured rooted stem cuttings. The leaf chamber of photosynthetic meter was set at 380 ppm of CO_2 concentration, 24 °C temperature and saturating photosynthetic rate of 1500 μ mol CO_2 m^{-2} s^{-1}. All the rooted stem cuttings of *C. equisetifolia* with/without inoculation of *Frankia* placed in OTC were measured for determination of photosynthetic rates under ambient, 600 ppm and 900 ppm CO_2 conditions.

2.6 Statistical analyses

Each measured variable in the OTC experiments were statistically analyzed using Duncan's multiple range test (SPSS ver. 17). Standard error (±SE) was also applied on the data of photosynthetic rate and tissue N content.

3 Results

3.1 *C. equisetifolia* Rooted stem cuttings

At the end of 12 months (Mar 2015), the effect of elevated CO_2 on *Frankia* inoculated rooted stem cuttings of *C. equisetifolia* showed that the growth and biomass were improved under 900 ppm of elevated CO_2 conditions. The shoot biomass includes needle leaves and stem (65.3 g plant $^{-1}$), root biomass (44.5 g plant $^{-1}$) and number of nodules (24.3 plant $^{-1}$) were significantly ($P = 0.05$) increased in *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* under 900 ppm of elevated CO_2 conditions than 600 ppm and ambient CO_2 conditions (Table 1). Root nodules were observed in the rooted stem cutting of *C. equisetifolia* inoculated with *Frankia* and grown in the inert media (vermiculite) under elevated CO_2 conditions (Fig. 1) Nodule numbers were significantly ($P = 0.05$) higher under 600 ppm of elevated CO_2 conditions due to inoculation of *Frankia* than ambient CO_2 conditions. However, the uninouclated control plants grown under 900 ppm and 600 ppm of elevated CO_2 had poor growth, biomass than ambient elevated CO_2 conditions. *Frankia* inoculation significantly *(*$P = 0.05$) increased the collar diameter under 600 ppm and 900 ppm of elevated CO_2 conditions. Under ambient CO_2 conditions, seedlings inoculated with *Frankia* showed significantly ($P = 0.05$) higher growth and biomass and number of nodules than control plants (Table 1).

In overall, the results showed that the rooted stem cuttings inoculated with *Frankia* had improved growth and biomass under elevated CO_2 conditions, whereas, the uninoculated control plants had poor performance under elevated CO_2 conditions particularly under 900 ppm.

3.2 Photosynthetic activity

C. equisetifolia rooted stem cuttings showed increased photosynthetic rates in 600 ppm and 900 ppm of elevated CO_2 conditions in the presence of *Frankia*. The photosynthetic rate was significantly ($P = 0.05$) increased in 900 ppm level (17.8 μ mol CO_2 m^{-2} s^{-1}) of elevated CO_2 conditions than 600 ppm (13.2 μ mol CO_2 m^{-2} s^{-1}) and ambient control (8.3 μ mol CO_2 m^{-2} s^{-1}) conditions. The control plants had poor photosynthetic rates compared to *Frankia* inoculated seedlings particularly under 900 ppm of elevated CO_2 conditions (Fig. 2).

3.3 Tissue nutrient content

Low major tissue nutrients (N, P, K) were showed in the stem cuttings of *C. equisetifolia* that considered as reserved food material (Fig. 3). However, the tissue N content (mg/g) was significantly ($P = 0.05$) higher for *C. equisetifolia* rooted stem

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Table 1 Response of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* to elevated CO_2 conditions (mean of 10 replicates) after 12 months (mean of 10 replicates)

Treatment	Ambient control						CO_2-600 ppm						CO_2 - 900 ppm					
	Shoot length (cm) plant^{-1}	Root length (cm) plant $^{-1}$	Collar diameter (cm)	No. of nodules plant^{-1}	Biomass Shoot (g) plant^{-1}	Biomass Root (g) plant^{-1}	Shoot length (cm) plant-^{1}	Root length (cm) plant-^{1}	Collar diameter (cm) plant^{-1}	No. of nodules plant-^{1}	Biomass Shoot (g) plant^{-1}	Biomass Root (g) plant^{-1}	Shoot length (cm) plant^{-1}	Root length (cm) plant^{-1}	Collar diameter (cm) plant-^{1}	No. of nodule plant^{-1}	Biomass Shoot (g) plant^{-1}	Biomass Root (g) plant^{-1}
1	36.4b	44.5b	3.8a	6.5	24.6b	22.3b	55.5b	54.5b	4.4a	12.4a	42.3b	32.3b	81.5b	66.3b	5.6b	24.3	65.3b	44.5b
2	30.6a	38.5a	3.1a	00	20.3a	18.5a	24.2a	32.6a	1.8a	00	14.8a	12.5a	9.8a	11.2a	1.1a	00	8.4a	6.2a

1. *Frankia* inoculated; 2. Control

Means followed by same letters are not significantly different at 5 % level of DMRT

Fig. 1 *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* showed root nodules under 900 ppm of elevated CO_2 conditions (*White arrow* indicate root nodules)

cuttings inoculated with *Frankia* at 600 and 900 ppm of elevated CO_2 conditions. Further, *Frankia* inoculated *C. equisetifolia* rooted stem cuttings showed significantly ($P = 0.05$) higher N content (3.2 mg g-^{1}) under 900 ppm of elevated CO_2 conditions than ambient and 600 ppm of elevated CO_2 conditions (Fig. 4).

4 Discussion

Global CO_2 levels are rising and it is anticipated that by the year 2100 these levels could reach 815 ppm (UKCIP 2011). The microbial symbionts like *Frankia* can contribute to carbon sequestration by increasing nutrient uptake by plants (Garcia et al. 2011) as found in this study. In this study elevated CO_2 greatly influenced the growth, biomass, nutrient content and photosynthesis in *C. equisetifolia* inoculated with *Frankia*. The rooted stem cuttings of *C. equisetifolia* grown in soilless media (vermiculite) without any fertilization the plants have responded well in growth and biomass under elevated CO_2 due to inoculation of *Frankia*. It was also confirmed that the inoculation of *Frankia* has only promoted the growth of

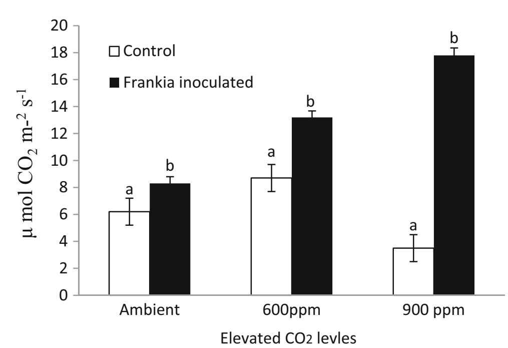

Fig. 2 Photosynthetic rates of *C. equisetifolia* rooted stem cuttings under elevated CO_2 conditions (mean of 10 replicates). Bars indicating same letters are not significantly different according to DMRT ($p < 0.05$). Error bard indicating SE (±) of mean

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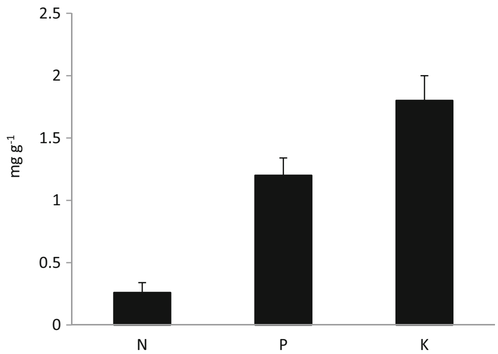

Fig. 3 Major tissue nutrients (N, P, K) content in stem cuttings of *C. equisetifolia* (mean of 10 replicates). Error bard indicating SE (±) of mean

C. equisetifolia rooted stem cuttings through N fixing root nodules as the other microbes were absent in the inert media. In earlier studies, the N_2 fixing microbes have been attempted in legume or actinorhizal plants at the seedling stage with inoculation of N fixing bacteria to find out the response under elevated CO_2 conditions (Arnone and Gordon 1990; Vogel and Curtis 1995; Ryle et al. 1992; Tissue et al. 1997). It was reported that the symbiotic N fixers (*Frankia, Rhizobium*) promoted the growth and biomass of N fixing trees under elevated CO_2 conditions (Norby 1987). Inoculation of *Frankia* mitigate the temperature and nutrient stress of the *C. equisetifolia* under the elevated CO_2 (AbdElgawad et al. 2015) which may be the reasons of growth improvement in *C. equisetifolia*. The rooted stem cuttings of *C. equisetifolia* in the present study responded positively to elevated CO_2 in growth, bio mass, photosynthetic rates and nutrient accumulation which is in accordance with an earlier study (Xu et al. 2014). The supply of carbon to nodules was used in the nitrogenase enzyme system as source of energy to fix N and development of root nodules (Hartwig and Nosberger 1994). An increase in the number of root nodules might have increased the nitrogenase activity of *Frankia* / nodule biomass that led to higher fixation of N in *C. equisetifolia* rooted stem cuttings. The inoculated *Frankia* with P medium contains N free and low amount of and also contains propionic acid which is the main carbon source for *Frankia* growth. Though N or P fertilizers were not applied in control and *Frankia* inoculated *C. equisetifolia*, root nodules still developed on roots of inoculated cuttings which could be attributed to the reserve food material present in the rooted stem cuttings of *C. equisetifolia.* This may be the reason in extreme CO_2 elevated conditions (900 ppm) the rooted stem cuttings of *C. equisetifolia* in the absence of *Frankia* had poor growth due to deficient N supply. Nigom et al. (2016) also reported the successful tolerance of casuarinas to environmental stress in the presence of *Frankia* through N fixation. Some of earlier studies have shown that the inoculation of microbial symbionts under elevated CO_2 conditions could improve the efficiency of nutrient uptake by plants (Tang et al. 2012; Song et al. 2013). Song et al. (2014) found enhanced growth and biomass in *Lolium perenne* inoculated with *Trichoderma* under ambient CO_2 conditions. Elevated CO_2 conditions (900 ppm) increased collar diameter compared to ambient CO_2 which is in agreement with the earlier studies (Yazaki et al. 2004). Higher elevated CO_2 (900 ppm) increased nodulation by *Frankia* which indicates an increased availability of carbon in form of carbohydrate to the bacterial symbiont. Similar results were also reported for *Alnus hirsuta* inoculated with *Frankia* under elevated CO_2 conditions (Tobita et al. 2005). It was also reported in earlier studies that CO_2 positively correlated with the amount of N acquired through N fixing bacteria in plants (Vogel et al. 1997). Nasser et al. (2007) found an increased leaf area index in Lentil under 700 ppm of elevated CO_2 level. They also found higher nodule numbers in response to rhizobial inoculation at 700 ppm of elevated CO_2 conditions. Tissue et al. (1997) reported an increased photosynthetic rates and carbon storage in *Gliricidia sepium* inoculated with *Rhizobium* under elevated CO_2 conditions which are coherent with the present study. Increased photosynthetic rates observed in the present study might have enhanced the rate of N fixation as evidenced by higher concentration of N in *Frankia* inoculated plants (Thomas et al. 1991). In overall this study showed that *C. equisertifolia* along with *Frankia* responded positively at 900 ppm of elevated CO_2 conditions.

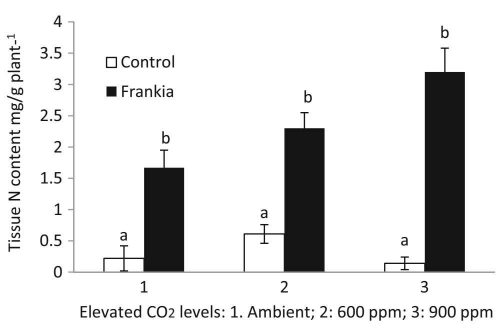

Fig. 4 Tissue N content in rooted stem cuttings of *C. equisetifolia* inoculated with *Frankia* under elevated CO_2 conditions (mean of 10 replicates). Bars indicating same letters are not significantly different according to DMRT ($p < 0.05$). Error bard indicating SE (±) of mean

5 Conclusion

Nitrogen fixing microbial symbiont, *Frankia* plays important roles in improvement of Casuarinas. In this present study under high atmospheric CO_2 conditions the *Frankia* facilitate the *C. equisetfolia* rooted stem cuttings for growth and biomass improvement. Universally, *C. equisetifolia* is propagating through rooted stem cuttings from genetically superior clones for establishing commercial plantations to make paper and pulp. These commercial plantations of *C. equisetifolia* may be inoculated with *Frankia* to improve the growth and

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biomass and to mitigate the increasing CO_2 levels by carbon sequestration.

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