Foliar nitrogen supply enhances the recovery of photosynthetic performance of cold-stressed coffee (*Coffea arabica* L.) seedlings

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Abstract

Cold-stressed leaves possess an elevated risk of photodamage due to an inefficient photosynthetic apparatus during winter conditions. Recovery of cold-stressed coffee seedlings treated with different concentrations of nitrogen applied as foliar urea sprays [control (0), 5, 10, 20, and 40 mM], was investigated under optimum growth chamber conditions (25/20°C) for three months. Concentrations of nitrogen and photosynthetic pigments in the leaves increased with increasing concentration of the foliar sprays up to 20 mM. This was accompanied with a recovery of the photosynthetic apparatus and increased net carbon assimilation rate. In addition, 10 and 20 mM-treated plants also had the highest maximal efficiency of PSII compared to their lower or higher nitrogen concentration treated counterparts. Conversely, these plants contained lower concentrations of 5-caffeoylquinic acid, mangiferin, trigonelline, and caffeine than control plants. We concluded therefore that foliar sprays of the appropriate nitrogen concentration were adequate for recuperating the photosynthetic apparatus and improved the photosynthetic performance of the cold-stressed coffee seedlings.

Additional key words: alkaloids; chlorophyll fluorescence; phenolic compounds; photochemical efficiency.

Introduction

Coffee (*Coffea* spp.) evolved as an understorey shrub species in the tropical rainforest of the mainland Africa and the island of Madagascar under moderate light and temperature conditions (Charrier and Berthaud 1985). Due to its economic value, however, coffee cultivation has expanded to various geographical zones with more severe seasonal climatic conditions and fluctuations such as low night temperatures and high light intensity during winter seasons in Brazil, which is the largest coffee producing country globally (DaMatta and Ramalho 2006). The ability of the coffee plants to adapt to such adverse conditions is owed to the plasticity of its photosynthetic apparatus and a number of photoprotection mechanisms (Chaves *et al.* 2008, Matos *et al.* 2009, Fortunato *et al.* 2010, Ramalho *et al.* 2014). Nevertheless, the typical low

nonfreezing temperatures (between 18 and 4°C) during winter conditions even in *C. arabica*, which is a seemingly more cold-tolerant species, result in diminished vegetative growth as a result of low photosynthetic productivity instigated by both biochemical and diffusivity limitations (DaMatta *et al.* 1997, Ramalho *et al.* 2003, 2014).

Low nonfreezing temperatures can cause significant degradation of chlorophyll (Chl) pigments and breakdown of the photosynthetic apparatus (Adams *et al.* 2004). This is instigated by low photosynthetic efficiency that leads to overproduction of reactive oxygen species (ROS) (Mittler 2002, Asada 2006). ROS in chloroplasts are produced as a result of photoinhibition caused by excess excitation energy in the photosynthetic apparatus (Asada 1999, DaMatta 2004, del Río *et al.* 2006, Demmig-Adams and Adams 2006). Plants are equipped with an elaborated ROS defence mechanism, which is comprised of both

enzymatic and nonenzymatic antioxidant systems (Logan *et al.* 2006, Fortunato *et al.* 2010). Antioxidant enzymes such as superoxide dismutase catalyse the breakdown of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). Consequently, the H_2O_2 is neutralized by catalase and ascorbate peroxidase (Asada 1999). In addition, a number of low molecular mass compounds including both lipophilic and hydrophilic compounds have been characterised as antioxidant scavengers (Das and Roychoudhury 2014). Recently, both alkaloids and phenolic compounds have been implicated in ROS scavenging in coffee plants during oxidative stress conditions (Ramalho *et al.* 2018). Profound systematic accumulations of high amounts of alkaloids such as caffeine and trigonelline together with phenolic compounds such as chlorogenic acids and mangiferin have been reported in all the cells of the young leaf blades in sun-grown plants compared to their shade counterparts (Campa *et al.* 2012, 2017). The surge in these secondary metabolites provides the evidence for their role in defence against ROS that are produced in conditions of excess excitation energy to which either the coffee plants is not usually adapted or instigated by abiotic stress conditions. Moreover, because of a repressed enzymatic antioxidant system during cold stress conditions, coffee plants rely on the nonenzymatic antioxidant scavengers for restoration of ROS to homeostatic conditions (Ramalho *et al.* 2018, Acidri *et al.* 2020a).

The inherent inability of the rapid remobilization of nitrogen on resumption of favourable temperatures further causes oxidative stress due to a mismatch between biochemical and diffusivity aspects of photosynthesis (DaMatta *et al.* 1999, Pompelli *et al.* 2010). Nevertheless, adequate nitrogen supply such as irrigating with 250 mL containing 23 mM N every after two weeks for 45 d has been reported to improve photosynthesis and overall metabolism of coffee plants during winter conditions and also in the growing season during the summer (Ramalho *et al.* 2000, DaMatta *et al.* 2002, Carelli *et al.* 2006, Pompelli *et al*. 2010, Bote *et al.* 2018). In addition, nitrogen (nutrition) has also been reported to facilitate the recovery of plants such as rice after exposure to low temperatures (Liu *et al.* 2019). The use of foliar fertilizers such as urea as a corrective relief for physiological stress is commended for its efficiency and instantaneous assimilation into plant tissues compared to conventional nitrogen application in the soil normally used in coffee production (Gray and Akin 1984, Carelli *et al.* 2006, Bhuyan *et al.* 2012, Oad *et al*. 2018). Soil fertilization generally requires high application rates that result into NO_x diminution by soil bacteria, which lead to greenhouse gas production (Shepherd *et al.* 1991). On the other hand, the application rates during foliar fertilization are normally lower with no risk of NO_x diminution while inducing quick responses in plants (Fageria *et al.* 2009). Nevertheless, foliar fertilization comes together with challenges that might arise when toxic concentration levels of foliar sprays are applied causing burns and cell mortality (Krogmeier *et al.* 1989). Moreover, the effect of foliar urea sprays of varying nitrogen concentrations on the recovery of the photosynthetic apparatus, together with several gas exchange and PSII chlorophyll fluorescence parameters as well as the content of selected secondary metabolites in coffee leaves remain unknown. We anticipated that an appropriate concentration of nitrogen applied as foliar urea sprays would improve the biochemical composition resulting into the recovery of the photosynthetic efficiency of the previously cold-stressed coffee plants.

This hypothesis was tested by measuring the changes in gas exchange and associated chlorophyll fluorescence parameters of the coffee plants treated with serial concentrations of nitrogen applied as foliar urea sprays. In addition, the contents of photosynthetic pigments and selected leaf alkaloid and phenolic compounds in coffee leaves were analysed.

Materials and methods

Plant material and growing conditions: The experiment was conducted between August 2017 and December 2018 at Tottori University (35°30'32.39"N, 134°10'10.80"E; 20 m a.s.l.) in Japan. *C. arabica* L. seeds imported from Bali, Indonesia were germinated by soaking them in running water for 3 d and thereafter transferred to an incubator on a paper towel. Germination occurred in the dark at 30°C in 14 d (Gebreselassie *et al.* 2010). After sprouting, each seedling was transplanted into a 10×10 cm (length $\times$ diameter) pot containing a mixture of peat moss, perlite, and humus at a ratio of 5:3:2, respectively. The seedlings were raised under shade provided by black cheesecloth in a vinyl-house from the summer season (August 2017) with an irrigation regime of 5 mL of half strength nutrient solution applied every other day. The nutrient solution was prepared according to Hoagland and Arnon (1950) with a few modifications. The concentration of the nutrient solution was in mmol L^{-1}: 2.9 N – NO_3^-, 0.5 N – NH_4^+, 0.05 P – $H_2PO_4^-$, 1.2 Ca^{2+}, 0.3 Mg^{2+}, 0.4 S – SO_4^{2-}; and in µmol L^{-1}: 17.5 Fe (III) EDTA, 0.4 Cu, 0.8 Zn, 3 Mn, 9 B, and 0.05 Mo. The coffee seedlings were cultivated until the onset of winter season (December 2018) under naturally fluctuating environmental conditions (Fig. 1). The average minimum winter temperatures caused visual signs of cold stress including stunted growth and yellowing of the leaves (Fig. 2).

For experimentation, visually uniform cold-stressed seedlings were transferred into the growth chamber and grown under PPFD of 250 µmol m^{-2} s^{-1} provided by white luminescent lamps at a 12-h photoperiod, temperature of 25/20°C, (day and night, respectively), and a 70% relative humidity. Five treatments were thereafter established by adjusting the concentration of nitrogen applied as foliar sprays in form of urea. The treatments included: Control (0), 5, 10, 20, and 40 mM N. Isopropyl alcohol (*Kao Global Chemicals*, Tokyo, Japan) solution (1%) was used as a surfactant in all the treatments. The sprays were administered just before darkness, one time per week for 90 d on the whole shoot until runoff on the leaves occurred. The sprays were applied on all the experimental units at once using a volume of 20 mL of the urea solution per treatment. All the seedlings were continuously irrigated with the previously described Hoagland solution but without nitrogen throughout the experimental term.
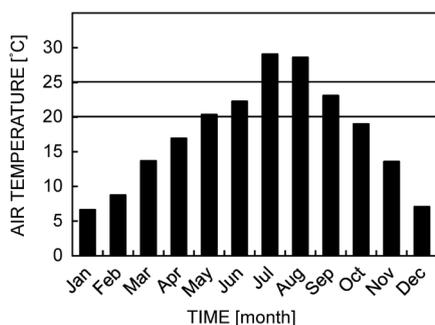
Fig. 1. Time-course changes in the monthly average of the ambient temperatures during a one-year period in the naturally conditioned greenhouse at Tottori University, western Japan. *Horizontal lines* indicate the adequate minimum and maximum temperatures for ideal cultivation of *Coffea arabica* L. according to Partelli *et al*. (2009).

Fig. 2. Coffee seedlings exhibiting visual signs such as yellowing of the leaves induced by cold stress conditions.

Gas exchange and Chl fluorescence: Photosynthetic responses and Chl fluorescence parameters were measured on fully expanded leaves of the first pair using *Li-6400 XT* open gas-exchange system (*LICOR Biosciences*, Lincoln, NE, USA). The system was checked for leaks and calibrated each time before the measurements were taken. Gas exchange and fluorescence measurements at the light-adapted state were done after the 6-h illumination at a 400 ppm CO_2 concentration and PPFD of 500 µmol m^{-2} s^{-1} (10% blue) according to Pompelli *et al*. (2010). The dark-adapted state fluorescence parameters were measured 6 h after the onset of darkness using the same system. Nonphotochemical quenching, proportion of open PSII centres and the maximum quantum efficiency of the PSII photochemistry for both the dark-adapted and light-adapted states were calculated according to Murchie and Lawson (2013).

Biochemical analysis

Sample preparation: At the end of the experiment, fully expanded leaves corresponding to first and the second pair from the top were collected after 6-h illumination (corresponding to midday) for biochemical analysis. The leaves were thoroughly washed and rinsed with distilled water to remove surface contaminations. The samples were thereafter immediately flash frozen in liquid nitrogen and stored under –80°C for further analysis. For all the assays, the samples were freeze-dried (*Eyela DRC 1000-FDU 1110*, Tokyo, Japan) and thereafter milled into a fine powder using a blender (*Wonder blender*, Osaka, Japan).

Total nitrogen in the leaf samples was measured using an organic elemental analyser (*CN corder JM1000CN*, Tokyo, Japan).

Photosynthetic pigments extraction and quantification: The concentrations of Chls and carotenoids were determined according to Porra *et al*. (1989) with a few modifications. Extraction was made from the freeze-dried powder using 80% chilled acetone in 50-mL Falcon tubes (*Thermo Fisher Scientific*, Massachusetts, USA) in the dark. The mixture was sonicated in ice-cold water bath in three cycles of 15 min each and centrifuged at $29,300 \times g$ at a temperature of 4°C (*Hitachi* high-speed refrigerated centrifuge *CR21N*, *Hitachi Koki Co. Ltd*., Tokyo, Japan). Extraction was repeated until the green residue turned white. All the extracts were pooled together for analysis in a known volume of the solution. Absorbance was read using a spectrophotometer (*Hitachi* ratio beam spectrophotometer, *U-5100*, Japan). Chls and carotenoid concentrations were calculated using equations developed by Porra *et al*. (1989) and Lichtenthaler and Buschmann (2001), respectively.

HPLC quantification of the selected metabolites: Alkaloids and phenolic compounds were analysed according to Acidri *et al*. (2020b). The analytes were determined simultaneously using a high-performance liquid chromatography (HPLC) system equipped with a UV detector at a wavelength of 270 nm (*Hitachi L-2490*, *Hitachi*, Tokyo, Japan) from a 10-µL sample extract. Separation of the analytes was performed on a *TSKgel ODS-100* C18 column (5-µm particles size, 4.6×150 mm) in a thermostatic oven at a temperature of 40°C (*Sigma Aldrich*, Tokyo, Japan) with a binary phase mobile gradient at a total flow rate of 0.4 mL min^{-1}. The mobile phase consisted of two filtered (0.22 µm *Millipore*), sonicated and degassed solvents A (methanol, 100%) and B (acetic acid:H_2O, 98:2, v/v) with linear evolution of the gradient profile according to a set elution program. Calibration curves were obtained from three replicate points for the standard compounds (trigonelline, 5-CQA, caffeine, and mangiferin). The selected compounds were reported to increase in the leaves of coffee plants during oxidative stress conditions (Campa *et al*. 2017, Ramalho *et al*. 2018, Acidri *et al*. 2020a). Each sample or standard was eluted for 25 min followed by idling for 20 min interval before proceeding to the next. Each analyte from the samples was then identified by peak position and thereafter quantified by peak area measurement using regression equations developed from calibration curves for the standard compounds. All the analytical standards and the organic solvents used were of HPLC grade (*Sigma Aldrich*, Tokyo, Japan).

Statistical analyses: Experimental data were analysed using a one-way analysis of variance (*ANOVA*) by comparing variation in the parameters among the treatments. Comparison for statistical differences were made using the *Newman-Keuls*'s test at $p \leq 0.05$. Normality tests were done using the *Kolmogorov-Smirnov*'s test and data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using *Stata 12.0* statistics and data analysis program (*Stata Corp.*, Texas, USA). Data are expressed as means ± SD, $n \geq 3$.

Results

Total nitrogen content in the leaves increased considerably with increasing concentration of the urea foliar sprays (Table 1). Compared to control treatment plants, the concentration of nitrogen in the leaves increased with increasing concentration of nitrogen in the foliar sprays for 5, 10, and 20 mM treatments. However, despite showing no statistical significance, a further increase of 100% in the concentration of foliar nitrogen supply to 40 mM caused a 3.5% decline in the concentration of leaf nitrogen compared to the preceding (20 mM) treatment.

Photosynthetic pigments: Variation in the concentration of Chls and carotenoids is indicated in Table 1. The concentrations of the respective Chl components *a* and *b* and their corresponding total (*a*+*b*) were the least in the leaves of control treatment plants. These increased with the increasing concentration of the nitrogen supply in the treatments. This increasing trend climaxed in the 20 mM treatment plants while further increase in the concentration of the foliar sprays to 40 mM caused a consistent decline of 16.5, 13.7, and 15.9% in the concentrations of Chl *a*, *b* and their corresponding total (*a*+*b*), respectively.

Although not significantly different, the concentration of the total carotenoids determined as a sum of xanthophylls and beta carotene (*x*+*c*), also showed a similar trend to that of Chls in variation amongst the treatments. Total carotenoids were the least in the control treatment plants and thereafter increased in the 5, 10, and 20 mM treated plants. Similarly, a further increase to 40 mM of foliar sprays instigated a decline in the concentration of total carotenoids.

The ratio of Chl *a*/*b* was the lowest in the control plants and increased with the increasing nitrogen concentration in the 5, 10, and 20 mM treated plants, respectively. Thereafter, the ratio of the Chl *a*/*b* indicated a slight decline despite an increase in the concentration of foliarly applied nitrogen in the 40 mM treatment. On the other hand, although the ratio of total chlorophylls to total carotenoids was the least in the control plants, it was the highest in 5 mM treatment plants and thereafter consistently declining despite the increase in the concentration of nitrogen treatment (10, 20, and 40 mM). Nevertheless, the ratios of the concentration of the pigments to that of nitrogen in the leaves was generally constant amongst that treatments despite slight declines in the respective ratios in the 40 mM treatment plants compared to the rest of the plants.

Taken together, the current results generally indicated that foliar application of nitrogen up to 20 mM improved the nitrogen status in the leaves of the coffee plants compared with control counterparts. This was accompanied by an increase in the accumulation of the photosynthetic pigments especially Chl. On the other, a further increase in the concentration of nitrogen in the urea treatments caused low concentrations of both leaf nitrogen and photosynthetic pigment.

Improved gas exchange and Chl fluorescence: The net CO_2 assimilation rate (P_N) was the lowest in the control plants with 5.9 $\mu mol(CO_2)$ m^{-2} s^{-1}, gradually increasing

Table 1. Effect of foliar nitrogen supply on the concentrations of leaf total nitrogen (N), chlorophylls *a* and *b* (Chl *a* and Chl *b*, respectively), total chlorophyll [Chl (*a*+*b*)], total carotenoids [Car (*x*+*c*)], ratios of Chl *a* to *b* (Chl *a*/*b*), chlorophyll to carotenoids (Chl/Car), chlorophyll *a* to nitrogen (Chl *a*/N), chlorophyll *b* to N (Chl *b*/N), total chlorophyll to N Chl/N) and that of total carotenoids to N (Car/N) in the leaves of coffee seedlings recovering from cold stress treated with different concentrations of foliar nitrogen in form of urea weekly for three months in a growth chamber. *Different letters* in a row denote significant differences between the treatment means (p<0.05, *Newman-Keuls*'s test). Data are expressed as means ± SD (n = 4).

Parameter	Foliar nitrogen supply [mM]				
	0	5	10	20	40
Total N [mg g⁻¹(DM)]	19.3 ± 4.3ᶜ	25.3 ± 3.0ᵇ	29.4 ± 2.6ᵃᵇ	34.4 ± 1.0ᵃ	33.2 ± 3.8ᵃ
Chl *a* [mg g⁻¹(DM)]	3.77 ± 0.59ᵇ	5.30 ± 1.00ᵃᵇ	6.58 ± 1.60ᵃ	6.84 ± 2.07ᵃ	5.71 ± 0.74ᵃᵇ
Chl *b* [mg g⁻¹(DM)]	1.47 ± 0.19ᵇ	1.92 ± 0.38ᵃᵇ	2.30 ± 0.44ᵃ	2.34 ± 0.59ᵃ	2.02 ± 0.19ᵃᵇ
Chl (*a*+*b*) [mg g⁻¹(DM)]	5.25 ± 0.78ᵇ	7.23 ± 1.38ᵃᵇ	8.88 ± 2.04ᵃ	9.18 ± 2.66ᵃ	7.72 ± 0.93ᵃᵇ
Car (*x*+*c*) [mg g⁻¹(DM)]	1.03 ± 0.23ᵃ	1.13 ± 0.18ᵃ	1.52 ± 0.32ᵃ	1.59 ± 0.37ᵃ	1.42 ± 0.14ᵃ
Chl *a*/*b*	2.55 ± 0.08ᵇ	2.76 ± 0.11ᵃ	2.84 ± 0.19ᵃ	2.90 ± 0.14ᵃ	2.82 ± 0.10ᵃ
Chl/Car	5.20 ± 0.81ᵇ	6.35 ± 0.35ᵃ	5.81 ± 0.16ᵃᵇ	5.72 ± 0.56ᵃᵇ	5.43 ± 0.18ᵇ
Chl *a*/N	0.20 ± 0.06ᵃ	0.21 ± 0.04ᵃ	0.22 ± 0.05ᵃ	0.20 ± 0.06ᵃ	0.17 ± 0.03ᵃ
Chl *b*/N	0.08 ± 0.02ᵃ	0.08 ± 0.01ᵃ	0.08 ± 0.01ᵃ	0.07 ± 0.02ᵃ	0.06 ± 0.01ᵃ
Chl/N	0.28 ± 0.08ᵃ	0.29 ± 0.06ᵃ	0.30 ± 0.06ᵃ	0.27 ± 0.08ᵃ	0.23 ± 0.04ᵃ
Car/N	0.06 ± 0.02ᵃ	0.05 ± 0.01ᵃ	0.05 ± 0.01ᵃ	0.05 ± 0.01ᵃ	0.04 ± 0.01ᵃ

with the increase in the concentration of the foliarly applied nitrogen in the treatments and peaking in the plants treated with 10 mM nitrogen at 8.3 μmol(CO_2) m^{-2} s^{-1} (Fig. 3A). Further increase in the concentration of nitrogen in the treatments was, however, associated with a steady decline in P_N from 8.3 to 6.7 and 7.2 μmol(CO_2) m^{-2} s^{-1} in the 20, and 40 mM nitrogen-treated plants respectively. Similarly, although stomatal conductance (g_s) was not statistically different between the treatments (Fig. 3B), g_s was the lowest in the control and 40 mM plant with 51.4 and 50.3 μmol(H_2O) m^{-2} s^{-1} and the highest in the 10 and 20 mM plants at 62.6 and 63.3 μmol(H_2O) m^{-2} s^{-1}, while plants under 5 mM exhibited g_s at 54.3 μmol(H_2O) m^{-2} s^{-1}.

Intrinsic water-use efficiency (WUE_i), estimated as a ratio of net CO_2 assimilation rate to stomatal conductance (P_N/g_s), was also low in the control and 40 mM plants at 0.12 for the two treatments. Plants under 5 mM had
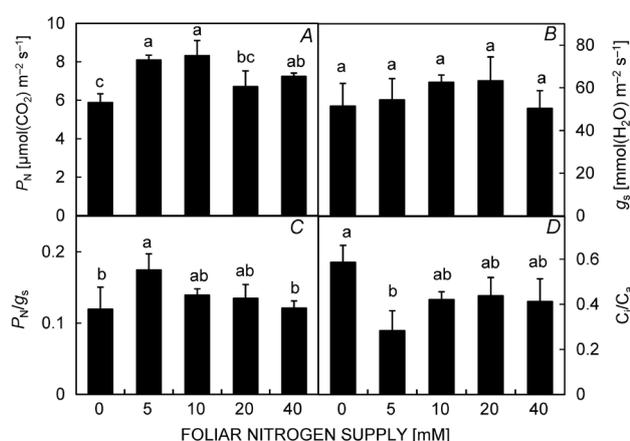
significantly higher WUE_i of 0.17, whereas 10, and 20 mM plants indicated moderate values of 0.14 and 0.13, respectively (Fig. 3C). In contrast, the ratio of internal to ambient CO_2 concentration (C_i/C_a) was significantly higher for the control plants at 0.59, whereas nitrogen treatments had relatively lower C_i/C_a of 0.28, 0.42, 0.44, and 0.41 for plants under 5, 10, 20, and 40 mM, respectively (Fig. 3D). The current results suggest therefore that nitrogen supply especially at lower application levels improved the rates of gas exchange in the leaves of the coffee plants. This was accompanied by a higher rate of CO_2 assimilation and hence resulting into low C_i/C_a in the treated compared with control plants.

The improved gas exchange in the treated plants was also accompanied with improved PSII photochemistry (Table 2). Despite showing no significant differences between the treatments, both the PSII photochemical efficiency (Φ_{PSII}) and the electron transport rate (ETR) were the highest in plants under 10 mM treatment. On the other hand, both control and 40 mM plants showed the least values of Φ_{PSII} and ETR, while plants under 5 and 20 mM treatments exhibited moderate Φ_{PSII} values. Similarly, the ETR values for the two treatments (5 and 20 mM) was moderate when compared to control and 40 mM plants.

Allocation of absorbed PAR to photochemistry indicated a consistent decline in the proportion of photochemical quenching (q_P) with the increase in the concentration of the nitrogen in treatments (Table 2). As a consequence, control and 5 mM plants exhibited the highest q_P, which was lowered in the 10 mM plants, whereas 20 and 40 mM showed the least q_P values. On the other hand, although the nonphotochemical quenching (NPQ) declined in the 5 and 10 mM plants compared with the control plants, a slight increment in NPQ was noticed in the 20 and 40 mM plants (Table 2). The proportion of PAR neither used in photochemistry nor dissipated as heat also known as fluorescence quenching (q_N) varied inversely to NPQ (Table 2). q_N was significantly higher in 5, 10, 20 mM plants and lower in the control and 40 mM plants. Similarly, more PSII reaction centres (q_L) also tended to remain open in plants treated with low concentration of nitrogen in the sprays (Table 2). On the contrary, both control plants

Fig. 3. Effects of foliar nitrogen supply on net photosynthetic rate (P_N) (A), stomatal conductance (g_s) (B), intrinsic water-use efficiency (P_N/g_s) (C), and the ratio of internal to ambient CO_2 concentration (C_i/C_a) (D). Nitrogen was applied as weekly foliar sprays in the form of urea on previously cold-stressed plants for three months in a growth chamber (25/20°C, day/night, respectively). Gas-exchange measurements were done at 6th hour of lighting. *Different letters* denote significant differences in the treatment means at $p<0.05$, *Newman-Keuls*'s test (foliar nitrogen effect). Error bars represent SD of the mean ($n = 3$).

Table 2. Effect of foliar nitrogen supply on the quantum yield of photosystem II photochemistry (Φ_{PSII}), electron transfer rate (ETR), photochemical quenching (q_P), fluorescence quenching (q_N), nonphotochemical quenching (NPQ), and the proportion of PSII open centres (q_L) in the leaves of coffee seedlings recovering from cold stress treated with foliar nitrogen weekly for three months in a growth chamber. *Different letters* in a row denote significant differences between the treatment means ($p<0.05$, *Newman-Keuls*'s test). Data are expressed as means ± SD ($n = 3$).

Parameter	Foliar nitrogen supply [mM]				
	0	5	10	20	40
Φ_{PSII}	0.10 ± 0.01^a	0.11 ± 0.01^a	0.12 ± 0.00^a	0.11 ± 0.02^a	0.11 ± 0.01^a
ETR [μmol m^{-2} s^{-1}]	22.8 ± 1.93^a	24.3 ± 3.10^a	25.5 ± 0.95^a	23.2 ± 3.94^a	20.2 ± 2.35^a
q_P	0.23 ± 0.02^a	0.23 ± 0.02^a	0.19 ± 0.02^a	0.17 ± 0.04^a	0.17 ± 0.04^a
NPQ	1.39 ± 0.32^a	1.06 ± 0.17^a	0.85 ± 0.21^a	0.87 ± 0.15^a	1.30 ± 0.21^a
q_N	1.97 ± 0.05^b	2.25 ± 0.07^a	2.45 ± 0.12^a	2.30 ± 0.18^a	1.98 ± 0.13^b
q_L	0.37 ± 0.03^a	0.40 ± 0.05^a	0.42 ± 0.01^a	0.38 ± 0.05^a	0.35 ± 0.02^a

and those treated with high concentrations of nitrogen sustained less open PSII reaction centres.

The maximum quantum efficiency of PSII during the dark-adapted state expressed as F_v/F_m and F_v/F_0 (Fig. 4*A,B*) and that during the light-adapted state, F_v'/F_m' and F_v'/F_0' (Fig. 4*C,D*), were the highest for the low concentration nitrogen-treated plants. Moreover, despite varying non-significantly amongst the treatments, F_v/F_m was the least for both control and 40 mM plants at 0.75, while the F_v/F_m values for the other treatments were 0.77 (Fig. 4*A*). Similarly, F_v/F_0 was the lowest for control and 40 mM plants at 3.0 for the two treatments while the low-nitrogen treatments exhibited F_v/F_0 values of 3.23, 3.44, and 3.26 for 5, 10, and 20 mM plants, respectively (Fig. 4*B*). The improvement in the maximum quantum
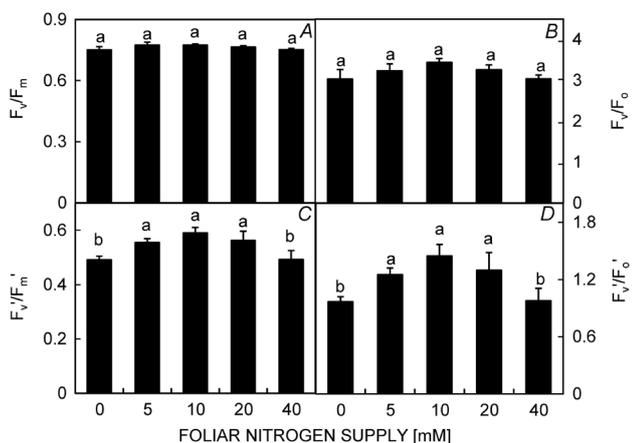
efficiency of PSII as a result of adequate nitrogen supply was more visible during the light-adapted state measurements with both control and 40 mM plants indicating significantly lower F_v'/F_m' values of 0.49, while 5, 10, and 20 mM plants showed F_v'/F_m' values of 0.56, 0.59, and 0.56, respectively (Fig. 4*C*). In addition, F_v'/F_0' were also significantly lower in the control and 40 mM plants at 0.97 and 0.98, while plants under 5, 10, and 20 mM indicated 1.25, 1.45, and 1.30, respectively (Fig. 4*D*). The current results suggest therefore that foliar nitrogen at lower concentrations improved the photochemical efficiency of PSII compared with the control plants whereas at the highest concentration (40 mM) caused the decline in the PSII quantum efficiency.

Content of the selected metabolites: Variations in the content of the selected metabolites amongst the treatments are indicated in Table 3. Caffeine showed neither significant differences nor peculiar trend in variation amongst the treatments. Nevertheless, plants under control, 10, and 20 mM treatments contained the highest content of caffeine while plants under 5 and 40 mM treatments contained the least caffeine content, respectively. On the other hand, trigonelline was significantly higher in control plants and lower in 40 mM plants. Plants under 5, 10, and 20 mM treatments contained moderate contents of trigonelline. Similarly, 5-CQA was significantly higher in control plants and lower in plants under 40 mM, while plants under 5, 10, and 20 mM treatments had moderate contents of the hydroxycinnamic acid ester, respectively. On the other hand, although mangiferin was not significantly different between the treatments, control plants had the highest content of the xanthonoid phenolic compound with 0.14 mg g^{-1}(DM), while the content of mangiferin in all the nitrogen treatments was well below 0.10 mg g^{-1}(DM).

Consequently, the total content of these selected metabolites was significantly higher in control plants and lower in plants under 40 mM, while 5, 10, and 20 mM contained median amounts, respectively. Similarly, the ratio of 5-CQA to caffeine despite showing no significant differences in variation between the treatments, was the highest in plants under control and lower in the nitrogen-treated plants.

Fig. 4. Effect of foliar nitrogen supply on the ratios of variable to maximum (F_v/F_m, F_v'/F_m') (*A,C*), and variable to initial (F_v/F_0, F_v'/F_0') (*B,D*) photosystem II maximum efficiency during the dark-adapted (F_v/F_m, F_v/F_0) (*A,B*) and the light-adapted (F_v'/F_m', F_v'/F_0') (*C,D*) chlorophyll states in the leaves of coffee plants. Nitrogen was applied as weekly foliar sprays in the form of urea on previously cold-stressed plants for three months in a growth chamber (25/20°C, Day/night, respectively). Light-adapted chlorophyll state was done at 6th hour of lighting whereas for dark-adapted state, the plants were measured after 6 h in total darkness. *Different letters* denote significant differences in the treatment means at *p*<0.05, *Newman-Keuls*'s test (foliar nitrogen effect). Error bars represent SD of the mean (*n* = 3).

Table 3. Effects of foliar nitrogen supply on the concentrations of caffeine, 5-caffeoylquinic acid, trigonelline, mangiferin, ratio of 5-caffeoylquinic acid to caffeine and the total content of these metabolites in the leaves of coffee seedlings recovering from cold stress treated with foliar nitrogen weekly for three months in a growth chamber. *Different letters* in a row denote significant differences between the treatment means (*p*<0.05, *Newman-Keuls*'s test). Data are expressed as mean ± SD (*n* = 3).

Parameter	Foliar nitrogen concentration [mM]				
	0	5	10	20	40
Caffeine [mg g^{-1}(DM)]	0.99 ± 0.17[a]	0.69 ± 0.10[a]	0.93 ± 0.11[a]	0.94 ± 0.08[a]	0.73 ± 0.19[a]
Trigonelline [mg g^{-1}(DM)]	0.61 ± 0.16[a]	0.41 ± 0.02[ab]	0.43 ± 0.08[ab]	0.45 ± 0.03[ab]	0.33 ± 0.07[b]
5-caffeoylquinic acid [mg g^{-1}(DM)]	5.89 ± 1.06[a]	3.45 ± 0.77[b]	4.07 ± 0.71[ab]	4.26 ± 0.16[ab]	3.19 ± 1.21[b]
Mangiferin [mg g^{-1}(DM)]	0.14 ± 0.04[a]	0.08 ± 0.00[a]	0.10 ± 0.02[a]	0.09 ± 0.03[a]	0.08 ± 0.04[a]
Total content [mg g^{-1}(DM)]	7.63 ± 0.93[a]	4.63 ± 0.82[b]	5.54 ± 0.79[b]	5.74 ± 0.24[b]	4.33 ± 1.46[b]
5-caffeoylquinic acid/caffeine	6.15 ± 1.74[a]	5.06 ± 0.99[a]	4.34 ± 0.29[a]	4.53 ± 0.22[a]	4.29 ± 0.85[a]

Discussion

The coffee plants used for this experiment developed visual symptoms of cold stress as a result of low temperatures from October as indicated in Fig. 1. Characteristically, the coffee plants had pale yellow leaves and stunted growth, which is normally associated with low concentrations of Chls (Fig. 2). Foliar nitrogen sprays elevated the leaf nitrogen status to levels above 23 mg g^{-1}(DM) (Table 1), a threshold under which nitrogen-deficiency symptoms are reported to occur in coffee plants (Pompelli *et al.* 2010). The control plants on the other hand, were still N-deficient at the end of the experiment and consequently accumulated lesser amounts of Chls and carotenoid pigments compared to the nitrogen supplied plants whose pigment concentration increased by 75 and 54%, respectively, with increasing nitrogen concentration up to 20 mM (Table 1). Low nitrogen nutrition generally results in low contents of both Chls and carotenoids, with the pigments increasing by over 135 and 125%, respectively, when coffee plants were fertilized with 23 mM N twice every after two weeks (Pompelli *et al*. 2010). Nevertheless, in this study, the high nitrogen concentration of the 40 mM treatment was associated with a remarked reduction in the concentration of the pigments. This is plausibly due to the burning effect associated with high concentration of foliar urea sprays. The negative effects of high concentration urea application on plant metabolism has been reported to result from accumulation of urea in plant tissues to toxic levels rather than its conversion to toxic substrates such as ammonia (Krogmeier *et al.* 1989). Therefore, at 40 mM, urea application was above the threshold and therefore its toxicity supressed plant metabolism although no burns were visually observed. Nonetheless, nitrogen supply in an appropriate concentration from foliar urea can be readily assimilated through combining with carbonyl chains form carbohydrates to form proteins and other important molecules such as Chls and carotenoids (Carelli *et al.* 2006, Pompelli *et al.* 2010) similar to observations made in the current study.

The low contents of photosynthetic pigments in the control plants were associated with low rates of gas exchange (Fig. 3). The seedlings used in the current study had been raised under a shaded vinyl house with irradiance levels similar to those used in the growth chamber (PPFD of 250 μmol m^{-2} s^{-1}). The current results agree with Praxedes *et al.* (2006) and Pompelli *et al.* (2010) who reported low net CO$_2$ assimilation in plants with low concentration of nitrogen and photosynthetic pigments. Chls play a vital role in both absorption and conversion of sunlight energy into chemical energy. In the current study, a consistent increase in the Chl concentration in the nitrogen-treated plants was accompanied with high rates of CO$_2$ assimilation (Fig. 3*A*). Stomatal conductance also tended to be the highest in plants with high CO$_2$ assimilation rates suggesting a positive effect of the improved water status in the coffee plants as a result of foliar nitrogen treatment on photosynthesis in general (Gimenez *et al.* 2005). Moreover, compared with control plants, nitrogen treatment improved WUE$_i$, indicating

a higher biomass gain per unit water loss that occurs through transpiration (Hatfield and Dold 2019). Control plants also indicated the highest C_i/C_a ratio compared to nitrogen-treated plants (Fig. 3*D*). This was possibly due to the inability of the mesophyll cells to assimilate the absorbed CO$_2$ in the intercellular air spaces (Farquhar *et al.* 1980). This phenomenon could have been induced by several biochemical limitations such as low photosynthetic enzymatic activities that were greatly improved by foliar nitrogen concentrations of up to 20 mM (Sun *et al.* 2016). On the other hand, plants under 40 mM had low rates of gas exchange (Fig. 3), empathizing the negative effects caused by high nitrogen concentration in this treatment. Similar results were obtained in coffee plants under conventional nitrogen supply (Bote *et al.* 2018) and in apple trees where plants grown under adequate nitrogen supply maintained higher photosynthetic rates even at low temperatures compared to those growing under soil nitrogen deficient conditions (Greer 2018).

The trend in the CO$_2$ assimilation rate was further supported by corresponding variations in the PSII photochemistry (Table 3). Both Φ_{PSII} and ETR showed similar quadratic variation amongst the treatments being lower in the both control and 40 mM plants whereas low concentration nitrogen-treated plants had moderate values. The current results suggest therefore that the PSII photochemical efficiency during steady-state photosynthesis tended to be downregulated in the photosynthetically depressed plants as a mechanism of photoprotection, which would otherwise result into photoinhibition due to an inefficient photosynthetic apparatus (Maxwell and Johnson 2000, Murchie and Lawson 2013). Moreover, control plants had both the highest q$_P$ and NPQ with the two parameters declining with increasing concentrations in the nitrogen treatments (Table 3). Nonetheless, a slight increase in the NPQ in plants under 40 mM indicated plausible damages to the photosynthetic apparatus resulting from the toxicity of the urea application in high concentrations. Moreover, plants under control and 40 mM treatments tended to close larger proportions of the PSII traps which otherwise could have caused photoinhibition due to biochemical limitations for high photosynthetic rates (Table 3). On the contrary, plants under moderate nitrogen concentration treatments had higher q$_L$ hence facilitating the flow of electrons from PSI to PSII, which resulted into higher photosynthetic rates (Murchie and Lawson 2013).

The maximum quantum efficiency of PSII under both dark and light conditions was also the lowest in the control and 40 mM-treated plants (Fig. 4). Both F$_v$/F$_m$ and F$_v$/F$_0$ for both the dark- and the light-adapted states (F$_v$'/F$_m$' and F$_v$'/F$_0$') are indicators of the degree of photoinhibition in PSII (Adams *et al.* 1995, Maxwell and Johnson 2000, Demmig-Adams and Adams 2006). The current results agree with Pompelli *et al.* (2010) who also reported lower F$_v$/F$_m$ ratio in low-N coffee plants especially under high-light conditions. Such low F$_v$/F$_m$ ratios have also been reported in coffee plants after exposure to cold stress conditions (Guo and Cao 2004, Batista-Santos *et al.* 2011). This decline in the maximum quantum efficiency is attributed to downregulation of PSII due to photoprotec-

tive energy dissipation, which is associated with nocturnal retention, and diurnal build-up of zeaxanthin in the xanthophyll cycle (Verhoeven *et al.* 1997, Ramalho *et al.* 2003, Partelli *et al.* 2009, Pompelli *et al.* 2010, Batista-Santos *et al.* 2011). This defence mechanism is, however, associated with inactive reaction centres and competes for energy with PSII photochemistry hence reducing its photochemical efficiency (Partelli *et al.* 2009).

The current results also indicated that in absence of an efficient photosynthetic system such as in control treatment plants, coffee plants accumulate relatively higher amounts of the secondary metabolites except caffeine (Table 3). Coffee plants contain two main alkaloids compounds whose roles in antioxidant defence are still unknown (Ashihara 2006). Accumulation of caffeine has been more associated with defence against herbivory, whereas trigonelline accumulates as a consequence of detoxification of nicotinic acid, a reservoir for biosynthesis of nicotinamide adenine dinucleotide (Shimizu and Mazzafera 2000). High contents of trigonelline [up to 0.61 mg g^{-1}(DM)] in the control plants compared to other treatments were observed in the current study. This is in consistence with Garg (2016) who recently indicated that trigonelline may have antioxidant roles in plant cells.

The main hydroxycinnamic acid ester, 5-CQA (Campa *et al.* 2012), and the xanthonoid, mangiferin, were also in the highest amounts in the control plants and decreased in the nitrogen-supplied plants (Table 3). The two phenolic compounds have been found to increase directly in response to oxidative stresses induced by high light intensities and cold stress conditions to which the coffee plant is not adapted to (Ramalho *et al.* 2014, Campa *et al.* 2017, Acidri *et al.* 2020a). The current results indicated a higher ratio of 5-CQA to caffeine in the photosynthetically limited plants compared to those treated with foliar sprays of nitrogen (Table 3). Chlorogenic acids such as 5-CQA have a higher metabolic plasticity compared to caffeine and act as storage forms of cinnamic acid derivatives which are used for lignification during cell wall development (Aerts and Baumann 1994). Therefore, the decline in the 5-CQA concentration could be attributed to a higher need for cell wall development than for defence against ROS in plants whose photosynthetic apparatus had been recovered by foliar nitrogen treatment. Yildirim *et al.* (2007) also observed a decline in ascorbic acid, a well-known antioxidant compound in nitrogen-treated broccoli plants compared to those under control conditions.

Nonenzymatic antioxidant compounds, such as phenolics, carotenoids, ascorbic acid, tocopherols and alkaloids, contain electrophilic carboxylic linkages and hydroxyl components, which neutralise the excess ROS generated during oxidative stress conditions (Das and Roychoudhury 2014). The presence of high contents of the secondary metabolites in the control treatment could therefore be due to the inability of the coffee plants to recover their photosynthetic apparatus in absence of foliar nitrogen sprays as indicated by contracted photosynthetic efficiency. On the other hand, the low photosynthetic rates in the high concentration foliar treatment, especially 40 mM, did not result in high antioxidant content possibly due

to accumulation of urea to toxic concentrations thereby supressing pigments and secondary metabolism in the leaves of the coffee plants (Krogmeier *et al.* 1989).

Conclusion: Foliar supply of different nitrogen concentrations (0, 5, 10, 20, and 40 mM) variably affected the recovery of both photosynthetic apparatus and its efficiency in the leaves of coffee seedlings with 10 mM causing the most significant effect. This recovery was associated with improved leaf nitrogen status and concentration of the photosynthetic pigments. On the contrary, nitrogen nutrition in the foliar urea sprays was associated with low concentrations of both alkaloids and phenolic compounds such as trigonelline and 5-CQA, respectively. Therefore, foliar urea supply containing 10 mM nitrogen was appropriate in recuperating the photosynthetic apparatus and hence enhanced the recovery of the photosynthetic performance of the previously cold-stressed coffee seedlings.

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