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ORIGINAL ARTICLE

The $\delta^{15}N$ response and nitrate assimilation of *Orychophragmus violaceus* and *Brassica napus* plantlets in vitro during the multiplication stage cultured under different nitrate concentrations

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Abstract Natural nitrogen isotope composition ($\delta^{15}N$) is an indicator of nitrogen sources and is useful in the investigation of nitrogen cycling in organisms and ecosystems. $\delta^{15}N$ is also used to study assimilation of inorganic nitrogen. However, the foliar $\delta^{15}N$ of intact plants, which is a consequence of nitrate assimilation occurring in the roots and shoots, is not suited for studying nitrate assimilation in cases where nitrate is the sole nitrogen source. In this study, *Orychophragmus violaceus* (*Ov*) and *Brassica napus* (*Bn*) plantlets, in which nitrate assimilation occurred in the leaves, were used to study the relationship between foliar $\delta^{15}N$ and nitrate assimilation. The plantlets were grown in vitro in culture media with different nitrate concentrations, and no root formation occurred for the plantlets during the multiplication stage. Nitrogen isotope fractionation occurred in both the *Ov* and the *Bn* plantlets under all treatments. Furthermore, the foliar nitrogen content of both the *Ov* and *Bn* plantlets increased with increasing nitrate concentration. Foliar nitrogen isotope fractionation was negatively correlated with foliar nitrogen content for both the *Ov* and *Bn* plantlets. Our results suggest that the foliar nitrogen isotope fractionation value could be employed to evaluate nitrate assimilation ability and leaf nitrate reductase activity. Moreover, high external nitrate concentrations could contribute to improved foliar nitrogen content and enhanced nitrate assimilation ability.

Keywords $\delta^{15}N$ · Nitrate assimilation · Nitrogen isotope fractionation · Nitrogen content · Nitrate reductase activity

1 Introduction

Nitrogen is essential in the growth and development of plants. It is a vital component of proteins, nucleic acids, enzymes, and chlorophyll (Raven et al. 2004; George et al. 2008; Hawkesford et al. 2012). Nitrogen exerts the greatest nutrient influence (up to 50%) on the growth and yield of plants under different environmental conditions (Stewart et al. 2005).

Nitrate is an important nitrogen source for all plants due to its contributions to plant nutrition and physiological regulation (Raven 2003; Wang et al. 2012). There are two transport systems for the uptake of nitrates, namely, high-affinity transport systems (HATS) and low-affinity transport systems (LATS) (Crawford 1995; Britto and Kronzucker 2006). HATS are responsible for nitrate uptake at low concentrations, while LATS are responsible for nitrate uptake at high concentrations. The transition concentration that determines the switch between HATS and LATS typically occurs around 1 mM for nitrate ions (Crawford 1995). Nitrate is assimilated by nitrate reductase in leaves and roots (Crawford 1995; Evans et al. 1996; Robinson et al. 1998; Comstock 2001; Kaiser and Huber 2001). During the multiplication stage of the in vitro plantlets, we regulated the concentration ratio of cytokinin and auxin to ensure that no root formation occurred in this experiment. As a consequence, nitrate was only assimilated in the leaves. Generally, the uptake of nitrates belongs to LATS

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in the tissue culture. In addition, nitrate is regarded as the principal form of nitrogen for most plant cultures and has served as the sole source of nitrogen in several studies (George et al. 2008). Hence, we chose nitrate as the sole source of nitrogen in this study.

An optimum concentration of nitrogen increases leaf area and photosynthetic capacity (Munir et al. 2007; Ahmad et al. 2009), and ultimately contributes to higher yield (Cheema et al. 2001, 2008; Rafiq et al. 2010). Therefore, the optimization of nitrogen concentration is an extremely important aspect of plant cultivation. In addition, nitrogen assimilation ability is an important consideration in choosing plant species with high nitrogen use efficiency. Kalcsits et al. (2015) found that high assimilatory demand may reduce nitrogen isotope fractionation, which indicates that isotope fractionation could be used to evaluate nitrogen assimilation. Since we employed nitrate as the sole source of nitrogen in this study, nitrogen isotope fractionation mainly depended on the assimilation of nitrate; interference in nitrogen isotope fractionation due to the assimilation of NH_4^+ was eliminated. Consequently, the degree of nitrogen isotope fractionation may indicate nitrate reductase activity.

The nitrogen isotope composition of plants may give insight into nitrogen uptake and assimilation (Evans 2001; Robinson 2001; Kalcsits et al. 2014). Deviations of $\delta^{15}N$ values in plants from those of the substrate arise through isotopic discrimination—the consequence of kinetic or thermodynamic processes that result in the lighter isotope (^{14}N) being favored. Hence, the heavier isotope (^{15}N) is depleted in the product (Handley and Raven 1992; Comstock 2001; Evans 2001; Robinson 2001; Dawson et al. 2002; Pritchard and Guy 2005; Kalcsits et al. 2014). In addition, the $\delta^{15}N$ in plant tissues is connected to preference for the inorganic nitrogen source (Kalcsits et al. 2015).

Denton et al. (2001) found that cannabis $\delta^{15}N$ strongly reflects the $\delta^{15}N$ of the growth substrate. In addition, foliar $\delta^{15}N$ can be an indicator of nitrogen source (Choi et al. 2002). Nitrogen isotope fractionation cannot be observed if the substrate is fully consumed. However, there are many circumstances under which nitrogen isotope fractionation does occur (Dawson et al. 2002). Kalcsits and Guy (2013) found that nitrogen availability and nitrogen demand exert considerable influence on the degree of nitrogen isotope discrimination.

The assimilation of nitrate occurs in shoots and roots (Crawford 1995; Evans et al. 1996; Robinson et al. 1998; Comstock 2001; Kaiser and Huber 2001), and nitrogen isotope fractionation occurs during assimilation by nitrate reductase (Robinson et al. 1998; Evans 2001). Hence, nitrogen isotope fractionation occurring in leaves, for which the sole nitrogen source is nitrate, is due to the assimilation by nitrate reductase of shoots and roots in hydroponic or sand cultures. Consequently, the relationship between leaf nitrogen isotope fractionation and leaf nitrogen assimilation ability cannot be quantified. Pritchard and Guy (2005) chose to study $\delta^{15}N$ discrimination in the *Picea glauca* (Moench) Voss, and both NH_4^+ and NO_3^- assimilated strictly in the roots in this species. However, researchers have encountered difficulty determining the relationship between foliar nitrogen isotope fractionation and nitrate assimilation in leaves for plants that were grown in the fields or hydroponic cultures, due to the nitrate assimilation occurring in the roots and shoots (Evans et al. 1996). Therefore, to study the relationship between nitrogen isotope fractionation and nitrate assimilation in leaves, plant tissue culture was employed in this study. Plant tissue culture has many advantages: (1) culture conditions are uniform, (2) the use of cloned plantlets in vitro eliminates individual differences, and (3) no root formation occurs in vitro during the multiplication stage.

Plantlets of *Orychophragmus violaceus* (*Ov*) and *Brassica napus* (*Bn*), which belong to brassicaceae, were selected for this experiment. The *Ov* and *Bn* plantlets in vitro are easy to culture and have frequently been employed as experimental materials in plant tissue culture (Li et al. 1995; Luo et al. 1995). Furthermore, *Ov* has adapted to grow in karst regions (Wu et al. 2004), whose characteristics include rocky desertification (Wang et al. 2004), nutrient-poor soils (Piao et al. 2000), drought, high calcium content (Yuan 1988), high pH value, and high bicarbonate content (Yan et al. 2012). *Bn* plantlets in vitro were used as a control. In this study, our primary objectives were to study whether foliar nitrogen isotope fractionation could be employed to evaluate foliar nitrogen assimilation ability, and whether nitrate concentration had an influence on foliar nitrogen isotope fractionation and foliar nitrogen content. Furthermore, the foliar nitrogen isotope fractionation of *Ov* and *Bn* plantlets in vitro might provide new insights into the adaptation mechanisms used by plants in karst regions.

2 Materials and methods

2.1 Plant materials and experimental treatments

Ov and *Bn* plantlets were cloned and grown in vitro. Single shoots, each with four unfolded leaves excised from in vitro cultures of *Ov* and *Bn* plantlets, were employed as explants. The average fresh weight (FW) per shoot was 0.08 g for *Bn* plantlets and 0.11 g for *Ov* plantlets. The culture consisted of 50 ml of Murashige and Skoog (MS) (1962) medium, supplemented with 2.0 mg · L^{-1} 6-benzylaminopurine (6-BA), 0.2 mg · L^{-1} α-naphthylacetic

acid (NAA), 3% (w/v) sucrose, and 7.5 g · L^{-1} agar. The concentrations of cytokinin and auxin in this experiment precluded root formation for the plantlets in vitro. All culture media were adjusted to pH 5.8 and then sterilized by autoclaving at 121 °C for 20 min. The plantlets in vitro were maintained in a growth chamber with a 12-h photoperiod (50 μmol m^{-2} s^{-1} PPFD) at 25 ± 2 °C. Sodium nitrate was employed as the sole nitrogen source at five concentrations (10, 20, 40, 80, and 120 mM).

2.2 Determination of growth parameters

A 150-ml erlenmeyer flask containing 50 mL culture substrate was weighed before cultivating the plantlet in vitro. Next, a single shoot was cultivated in the medium and then the whole erlenmeyer flask was weighed again. The FW of the shoot was the difference of the first weighing and second weighing.

After 5 weeks of culturing, the plantlet in vitro was taken out of the erlenmeyer flask. The biomass of *Ov* and *Bn* plantlets grown under different nitrate concentrations was measured. Meanwhile, the leaf FW was measured individually for each *Ov* and *Bn* plantlet in vitro. Then, the leaves of the *Ov* and *Bn* plantlets were dried at 60 °C. Moreover, the leaf dry weight (DW) of each *Ov* and *Bn* plantlet was also measured. Finally, the dried leaves were ground to a fine powder.

2.3 Nitrogen accumulation content and nitrogen utilization coefficient of leaves

The nitrogen accumulation content (NAC) value of the leaves was the absolute nitrogen content in the dried leaves, and was calculated using the following equation:

$$\mathrm{NAC} = \mathrm{DW} \times \mathrm{N\,content} \quad (1)$$

where the N content of the dried leaves was determined using an elemental analyzer.

The nitrogen utilization coefficient (NUC) is the ratio of the total NAC of the dried leaves relative to the nitrate content of the medium, and was calculated using the following equation:

$$\mathrm{NUC}\,(\%) = \frac{(\mathrm{NAC/M})}{\mathrm{n_{nitrate}}} \times 100 \quad (2)$$

where M is the molar mass of nitrogen, and $\mathrm{n_{nitrate}}$ is the number of moles of nitrate in the medium.

2.4 Elemental analysis and $\delta^{15}N$

The total nitrogen and carbon contents of the dried leaves were determined using an elemental analyzer (vario MACRO cube, Germany). Stable nitrogen isotope ratios ($^{15}N/^{14}N$) were measured using a gas isotope ratio mass spectrometer (MAT-253, Germany) with a continuous flow mode. Samples containing approximately 50 μg of nitrogen and reference materials were weighed into tin capsules, sealed, and loaded into an automatic sampler. The nitrogen isotope results are expressed in δ notation:

$$\delta^{15}\mathrm{N}\,(‰) = (R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1) \times 1000 \quad (3)$$

where R_{sample} refers to the N isotope ratio of plant material and R_{standard} refers to the isotope ratio of a known standard (N_2 in air). The instrument was calibrated using the IAEA N_1, IAEA N_2, and IAEA NO_3 reference materials (Yousfi et al. 2013). The analytical precision of the $\delta^{15}N$ values was 0.2‰. The nitrogen isotope discrimination ($\Delta^{15}N$) relative to the source was calculated as (Evans et al. 1996):

$$\Delta^{15}\mathrm{N} = \delta^{15}N_{\mathrm{substrate}} - \delta^{15}\mathrm{N_{product}} \quad (4)$$

where $\delta^{15}\mathrm{N_{substrate}}$ was 8.08‰.

2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA). The means of the different groups were compared via Tukey's test ($p < 0.05$). Data are shown as the mean ± the standard error (SE).

3 Results

3.1 Growth parameters

The biomass of the *Ov* plantlets in vitro was significantly affected by nitrate concentration, reaching a maximum at 20 mM nitrate, and being clearly suppressed at high nitrate concentration (80 and 120 mM). Compared to the biomass of the *Ov* plantlets, the biomass of the *Bn* plantlets was relatively low at the highest and lowest nitrate concentrations. However, the biomass of the *Bn* plantlets showed no significant changes and was relatively high at concentrations of 20, 40, and 80 mM nitrate (Table 1).

The leaf FW of the *Ov* plantlets in vitro gradually increased from 10 to 20 mM of nitrate, and reached a maximum value at 20 mM nitrate. After that, the leaf FW of the *Ov* plantlets declined with increasing nitrate concentration. However, the leaf FW of the *Bn* plantlets rose with increasing nitrate concentration, except at 120 mM nitrate. The leaf/stem ratio of the *Ov* and *Bn* plantlets increased with increasing nitrate concentration. However, the leaf/stem ratio of the *Bn* plantlets was higher than that of the *Ov* plantlets for all treatments (Table 1).

Table 1 The growth parameters of the *Ov* and *Bn* plantlets in vitro

Treatments (mM)	Biomass (g)		Leaf FW (g)		Leaf/stem ratio (%)	
	Ov	*Bn*	*Ov*	*Bn*	*Ov*	*Bn*
10	4.00 ± 0.25b	2.18 ± 0.29b	0.44 ± 0.03b	0.40 ± 0.04c	12.28 ± 0.42c	22.41 ± 0.71c
20	5.23 ± 0.16a	3.33 ± 0.17a	0.70 ± 0.03a	0.88 ± 0.05ab	15.32 ± 0.32c	35.76 ± 1.12b
40	4.42 ± 0.23ab	3.54 ± 0.26a	0.60 ± 0.05a	1.06 ± 0.04a	15.77 ± 0.58bc	43.13 ± 3.17ab
80	2.42 ± 0.24c	3.21 ± 0.19a	0.45 ± 0.02b	1.01 ± 0.07a	23.37 ± 2.35ab	46.28 ± 2.19a
120	1.58 ± 0.12c	2.08 ± 0.15b	0.36 ± 0.03b	0.71 ± 0.05b	29.87 ± 2.69a	51.56 ± 1.80a

Each value represents the mean ± SE (n = 3). Values signed with the same letter are not significantly different by Tukey's test ($p < 0.05$)

3.2 Elemental analysis of the *Ov* and *Bn* plantlets in vitro

The *Ov* and *Bn* plantlets had similar leaf nitrogen contents (expressed as a percent of DW) across the five treatments and showed a rising trend with increasing nitrate concentration. The carbon contents of the *Ov* and *Bn* plantlets showed no significant changes at low nitrate concentrations. However, both the *Ov* and *Bn* plantlets had relatively low carbon contents at 80 and 120 mM nitrate. The C/N ratios of the *Ov* and *Bn* plantlets showed no significant changes at 10 and 20 mM nitrate. Beyond that, the C/N ratios of the *Ov* and *Bn* plantlets decreased with increasing nitrate concentration (Fig. 1).

3.3 Nitrogen accumulation content and nitrogen utilization coefficient

The NAC values of the *Ov* plantlets showed no significant difference with increasing nitrate concentration, except at the lowest concentrations. In contrast, the NAC value of the *Bn* plantlets was strongly affected by nitrate concentration, increasing with increasing nitrate concentration except at a nitrate concentration of 120 mM. However, the NAC value was still relatively high at 120 mM nitrate compared with the maximum NAC value of the *Bn* plantlets (Table 2).

The NUC values of both the *Ov* and *Bn* plantlets showed obvious changes with increasing nitrate concentration. The *Ov* NUC values declined with increasing nitrate concentration, with the maximum value recorded at the lowest nitrate concentration. However, the *Bn* NUC values showed no distinct change at low nitrate concentrations. Meanwhile, the NUC values of the *Bn* plantlets declined from 40 to 120 mM nitrate (Table 2).

3.4 Foliar nitrogen isotope ratio

Isotope discrimination occurred in both the *Ov* and *Bn* plantlets cultured under different nitrate concentrations (Fig. 2). The $\delta^{15}N$ values of both the *Ov* and *Bn* plantlets showed no significant differences at low nitrate concentrations. However, the $\delta^{15}N$ values of the *Ov* plantlets were higher than those of the *Bn* plantlets at low nitrate concentrations. The $\Delta^{15}N$ of the *Bn* plantlets was relatively high compared with the *Ov* plantlets at low nitrate concentrations. The $\Delta^{15}N$ of the *Ov* plantlets gradually decreased with increasing nitrate concentration, while the $\Delta^{15}N$ of the *Bn* plantlets declined from 40 to 120 mM nitrate. Meanwhile, good correlation between $\Delta^{15}N$ and nitrogen content were observed for both *Ov* and *Bn* plantlets, with higher $\Delta^{15}N$ values corresponding to lower nitrogen content (Fig. 3).

4 Discussion

In traditional in vitro cultures, nitrate ions are a major source of nitrogen for most plant cultures. In this study, increasing the NO_3^- concentration had positive effects on the biomass of *Ov* and *Bn* plantlets at low nitrate concentration, which agrees with the findings of Poothong and Reed (2016). Compared to other nitrate concentrations, the *Ov* and *Bn* plantlets had higher biomass at 20 mM nitrate. In addition, some studies suggest that a nitrogen concentration of 60 mM is excessive in MS medium for some cultures (Nowak et al. 2007; George et al. 2008). At high nitrate concentrations, the observed decrease in biomass of *Ov* plantlets in vitro might be the consequence of salt stress. This salt stress may result in stomatal closure, which reduces CO_2 availability in the leaves, which then inhibits the carbon fixation. Net photosynthesis was reduced by 35% in a cotton cultivar (Guazuncho) that was subjected to salt stress (Meloni et al. 2003). To some extent, the *Bn* plantlets in vitro performed better in terms of resisting the salt stress than the *Ov* plantlets.

Nitrate also had an important effect on leaf FW for both the *Ov* and *Bn* plantlets in vitro. Hunt et al. (1985) found that total leaf area and shoot mass decreased with decreasing nitrate concentration. In the present study,

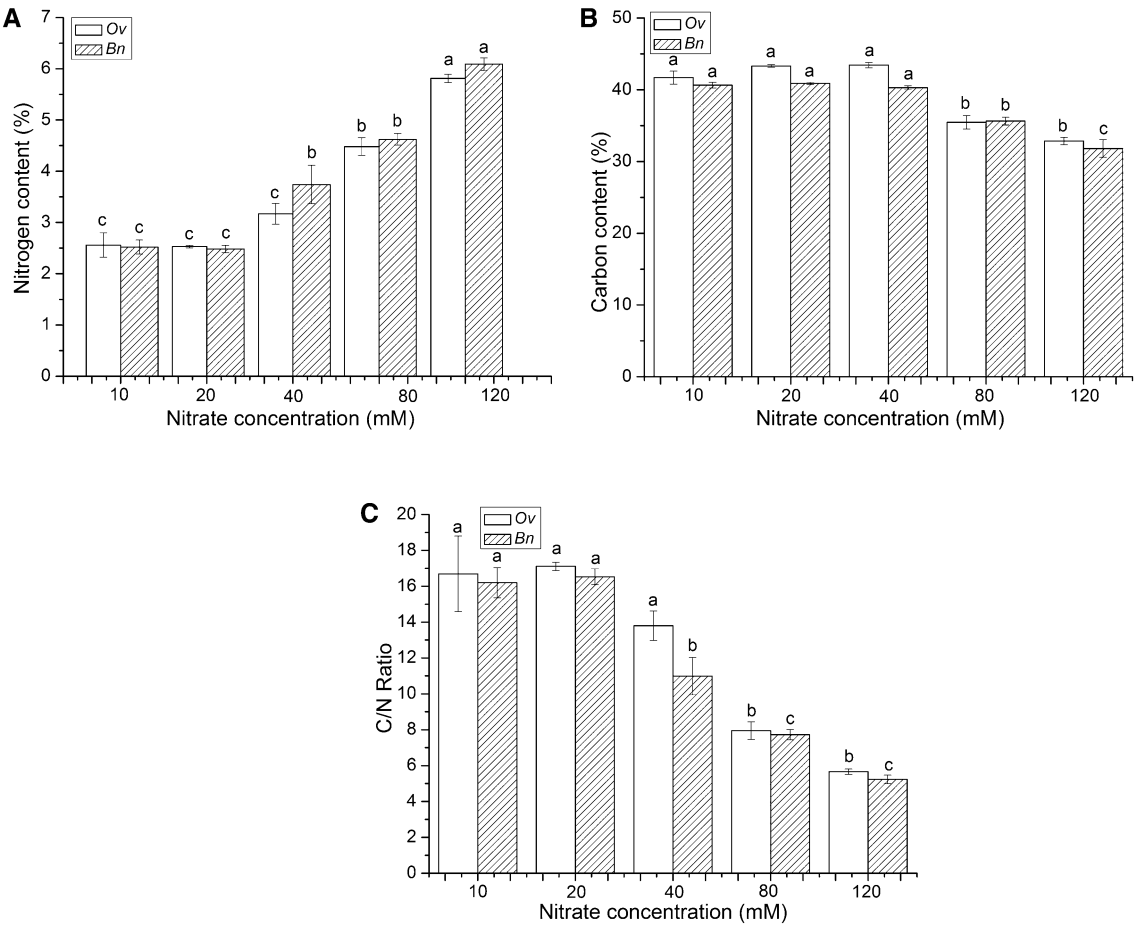

Fig. 1 Nitrogen content (**a**), carbon content (**b**), and C/N ratio (**c**) of the *Ov* and *Bn* plantlets in vitro. *Note* The mean ± SE (n = 3) followed by *different letters* in the same plant species differ significantly (Tukey's test, $p < 0.05$)

Table 2 Leaf nitrogen accumulation contents and leaf nitrogen utilization coefficients of the *Ov* and *Bn* plantlets in vitro

Treatments (mM)	Leaf DW (mg)		NAC (mg)		NUC (%)	
	Ov	*Bn*	*Ov*	*Bn*	*Ov*	*Bn*
10	51.6 ± 5.1bc	29.5 ± 2.7c	1.32 ± 0.19b	0.74 ± 0.03d	18.9 ± 2.7a	10.5 ± 0.4a
20	80.0 ± 4.6a	59.6 ± 3.2b	2.03 ± 0.11a	1.48 ± 0.10c	14.5 ± 0.8a	10.5 ± 0.7a
40	62.3 ± 2.1b	74.7 ± 2.7a	1.97 ± 0.07ab	2.78 ± 0.17ab	7.1 ± 0.2b	9.9 ± 0.6a
80	42.3 ± 2.7 cd	73.2 ± 3.3a	1.90 ± 0.16ab	3.38 ± 0.19a	3.4 ± 0.3b	6.0 ± 0.3b
120	32.2 ± 3.4d	42.2 ± 2.3c	1.86 ± 0.18ab	2.56 ± 0.09b	2.2 ± 0.2b	3.0 ± 0.1c

Each value represents the mean ± SE (n = 3). Values signed with the same letter are not significantly different by Tukey's test ($p < 0.05$)

lower nitrate concentration corresponded to reductions in leaf FW. In addition, Kiba et al. (2010) found a close correlation between cytokinin content and nitrogen supply. Moreover, nitrate induces biosynthesis of endogenous cytokinin in leaves (Miyawaki et al. 2004), thus accelerating leaf growth. These findings were supported by the leaf/stem ratio of the *Ov* and *Bn* plantlets rising with increasing nitrate concentration.

Foliar nitrogen content increases significantly with increasing nitrogen availability in most plants (Gulmon and Chu 1981). Nitrogen usually constitutes 1.5%–5% of the DW of higher plants (Novoa and Loomis 1981). In this study, the foliar nitrogen content of both the *Ov* and *Bn* plantlets increased with increasing nitrate concentration. The maximum foliar nitrogen content was achieved at the highest external nitrate concentrations for both the *Ov* and

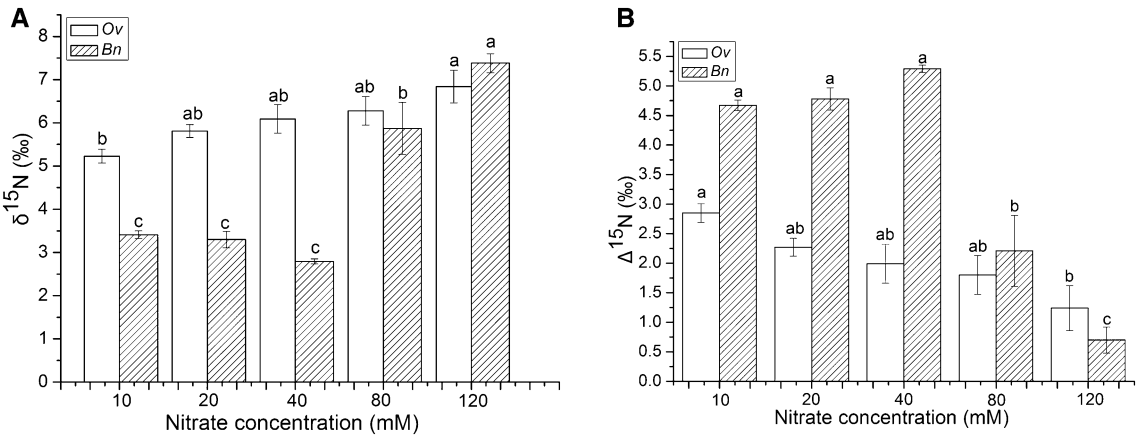

Fig. 2 The $\delta^{15}N$ (**a**) and $\Delta^{15}N$ (**b**) values of the *Ov* and *Bn* plantlets in vitro. *Note* The mean ± SE (n = 3) followed by *different letters* in the same plant species differ significantly (Tukey's test, $p < 0.05$)

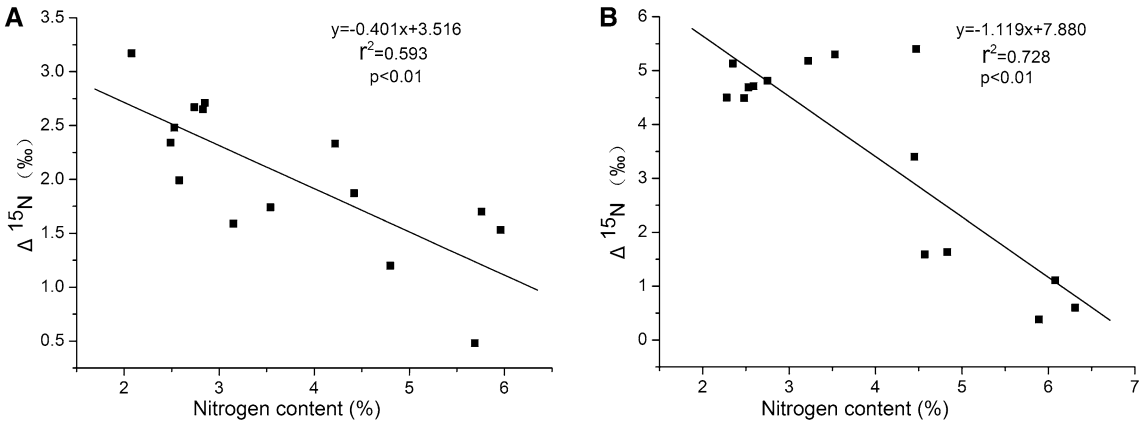

Fig. 3 The relationship between $\Delta^{15}N$ and nitrogen content of the *Ov* (**a**) and *Bn* (**b**) plantlets in vitro, based on five treatments ($n = 15$)

Bn plantlets. The results suggested high external nitrogen concentration responded to high nitrogen assimilation ability. Generally, nitrate reductase activity (NRA) was induced by the addition of nitrate (Mendel et al. 1982; Campbell 1999; Kaiser and Huber 2001). Hence, foliar nitrogen assimilation ability depended on NRA in this experiment. The decline in foliar carbon content of both the *Ov* and *Bn* plantlets in response to salt stress might be related to proline biosynthesis, which is a highly energy-demanding process (Cuin and Shabala 2005). Under salt stress, proline concentrations increased significantly in three rice cultivars (Moradi and Ismail 2007). The decrease in the C/N ratios of the *Ov* and *Bn* plantlets at high nitrate concentrations was a consequence of high nitrogen content and low carbon content.

At high nitrate concentrations, the *Bn* plantlets had better NAC values compared with the *Ov* plantlets. However, *Ov* NAC values were higher than those of *Bn* plantlets at low nitrate concentration. The NUC values of both the *Ov* and *Bn* plantlets suggest that the nitrate was partially consumed at all treatment levels.

Many studies have found that N isotope fractionation occurs during NO_3^- assimilation (Mariotti et al. 1982; Robinson 2001; Kalcsits et al. 2015). The current results show that nitrogen isotope fractionation occurred in both the *Ov* and *Bn* plantlets cultured under different nitrate concentrations. However, the degree of fractionation was different for the *Ov* and *Bn* plantlets (Fig. 2). Kalcsits et al. (2014) found that nitrogen isotope discrimination increases with increasing nitrate concentrations in the substrate at HATS. Interestingly, the nitrogen isotope fractionation of both *Ov* and *Bn* plantlets decreased with increasing nitrate concentration in this experiment at LATS. Our observed results might be related to the growth status of plantlets in vitro. No root formation occurred in either *Ov* or *Bn* plantlets in this experiment.

A salinity-induced depletion of foliar ^{15}N under the same nitrogen supply has been widely reported (Handley et al. 1997; Yousfi et al. 2009; Del Amor and Cuadra-Crespo 2011). Handley et al. (1997) suggested that "stress" makes plant $\delta^{15}N$ more negative than in controls, due to down-regulation of the nitrate reductase. In addition, under

salt stress, leaf NRA decreases with increasing salinity (Kaiser and Huber 2001). Yousfi et al. (2009) found that the total nitrogen content of the shoot significantly decreases with increasing salt stress. Therefore, the reduced assimilatory demand of the plant results in increased nitrogen isotope fractionation (Kalcsits et al. 2014, 2015). However, the nitrate concentration was also increased with increasing salinity in this experiment. The NRA was inducible with available nitrate (Campbell 1999; Kaiser and Huber 2001) and leaf NRA increased with increasing nitrate supply (Kaiser and Huber, 2001; Black et al. 2002). The foliar nitrogen content of both *Ov* and *Bn* plantlets increased with increasing nitrate. Hence, the effect of salt stress on nitrogen assimilation might be offset by increasing nitrate supply. High nitrogen assimilation ability at high nitrate concentration contributes to less nitrogen isotope fractionation (Kalcsits et al. 2014, 2015).

Plant $\delta^{15}N$ depends on the nitrogen source, as well as the relationship between enzymic demand and external concentration (Mariotti et al. 1982). In the present study, nitrate was the sole source of nitrogen for the *Ov* and *Bn* plantlets. Hence, the discrimination against ^{15}N might depend on NRA. Pate et al. (1993) found that high $\delta^{15}N$ values are associated with high shoot NRA levels. The degree of isotope fractionation might be reduced, given high assimilatory demand, because most of the cytoplasmic nitrogen will assimilate and little will be lost back to the substrate (Kalcsits et al. 2015). As a result, $\Delta^{15}N$ can be an indirect indicator of nitrogen assimilation ability. Foliar $\Delta^{15}N$ was negatively correlated with foliar nitrogen content for both *Ov* and *Bn* plantlets (Fig. 3), which suggests that higher $\Delta^{15}N$ values correspond to low nitrogen contents and lower $\Delta^{15}N$ values correspond to high nitrogen contents. Our results also suggest that the nitrogen assimilation ability of *Ov* plantlets in vitro was higher than that of *Bn* plantlets in vitro at low nitrate concentrations.

This study confirms that the *Ov* plantlets in vitro perform better in terms of biomass and nitrogen assimilation compared with the *Bn* plantlets in vitro at low nitrate concentrations. Therefore, growing *Ov* plantlets in vitro may be adaptive in karst regions where the available nutrients for plants are usually scarce, which is consistent with the results of Wu et al. (2004).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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