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Protein as a sole source of nitrogen for *in vitro* grown tobacco plantlets

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Abstract

We tested the capability of plants to utilize protein as the exclusive source of nitrogen. The aim of this study was to find out how such a nutrition affected plantlet growth, photosynthetic performance, and N assimilation metabolism in tobacco (*Nicotiana tabacum* L., cv. Petit Havana SR1) grown *in vitro*. Plantlets grown in a casein-supplemented (CA) medium were compared to plantlets grown in a complete Murashige-Skoog (MS) medium, plantlets grown in an ammonium-deficient medium (N1), or plantlets grown in a nitrate-reduced medium (N2). In addition, the plantlets were grown in the presence or absence of 1.5 % (m/v) saccharose as an additional carbon source. Casein, similarly as inorganic N limitation, reduced generally plantlet growth, whereas no significant effects were observed on photosynthetic parameters evaluated by chlorophyll *a* fluorescence. Although addition of saccharose stimulated the plantlet growth particularly in the MS, it showed a rather negative influence both on the growth and on the photochemical efficiency of photosystem II in the plantlets grown in the CA and N1. The activities of enzymes involved in N assimilation, such as nitrate reductase (NR) and glutamine synthetase (GS), were lower in the plantlets grown in the CA, N1, and N2, both in leaves and in roots. On the other hand, glutamate synthase and glutamate dehydrogenase were employed by the plantlets grown in the CA. The presence of saccharose in the growth medium stimulated mainly NR and GS activities in the MS grown plantlets, whereas enzyme activities of the plantlets grown on the N1, N2, and CA were not significantly influenced. We proved that the tobacco plantlets can utilize casein as the sole source of N particularly during their photoautotrophic cultivation. Contrary to positive effects of photomixotrophic nutrition for the MS grown plantlets, exogenous sugar seemed to diminish the ability of the casein-supplemented plantlets to utilize efficiently the additional C source.

Additional key words: ammonium, casein, chlorophyll fluorescence, nitrate, photosynthesis.

Introduction

Soil contains organic-bound nitrogen in the form of proteinous and non-proteinous amino acids, nucleotides, and their polymers including large proteins above 100 kDa (Warren 2014). Soil organic N is considered as source exclusively for microbes and animals (Schmidt *et al.* 2014). Until recently, only little attention was devoted to research the role of complex organic N as the N source for plants, despite a strong discrepancy between measured rates of production of inorganic N forms and annual plant N uptake (Nasholm *et al.* 2009). The current view is that in ecosystems, where the rate of microbial mineralization is slow, such as in boreal forest and heathlands, plants rely on mycorrhizal symbioses to break down soil proteins, whereas in ecosystems with a high microbial activity, such as grasslands and tropical forests, plants use mostly inorganic N (Paungfoo-Lonhienne *et al.* 2008). However, a wide variety of plants shows the ability to accept organic N in field or laboratory experiments. Nevertheless, the degree to which organic N serves as an important N source for plants is still under

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Abbreviations: CA - casein supplemented medium; Chl - chlorophyll; F_v/F_m - maximal efficiency of photosystem II photochemistry; FM - fresh mass; GDH - glutamate dehydrogenase; GOGAT - glutamine:2-oxoglutarate aminotransferase (glutamate synthase); GS - glutamine synthetase; MS - complete Murashige-Skoog medium; N1 - ammonium deficient medium; N2 - nitrate reduced medium; NR - nitrate reductase; PS - photosystem; q_N - nonphotochemical quenching coefficient; q_P - photochemical quenching coefficient; 1 - g_P - the number of closed PS II centres; S - sucrose; S/R - shoot/root ratio; Φ_{PSII} - actual efficiency of photosystem II.
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debate (Gruffman *et al.* 2014).

Plants have evolved various inorganic and organic N uptake systems to cope with a heterogeneous N availability in the soil. For nitrate and ammonium, two types of uptake system have been described: low-affinity transport systems operating at a high nutrient concentration (> 1 mM), and a high-affinity transport system that predominates in a micromolar range; the transporters belong to several gene families (Kraiser *et al*. 2011, Xu *et al.* 2012). The crucial transporters are regulated at the transcriptional level by nitrate, nitrite, ammonium, glutamine, N starvation, irradiance, sucrose, diurnal rhythm, and pH (Wang *et al.* 2012, Krapp *et al*. 2014). Some of the nitrate transporters are also involved in nitrate sensing, plant development, and pathogen defense (Wang *et al.* 2012, Ruffel *et al.* 2014). Moreover, it has been proved that plants can take up organic N of a low molecular mass such as amino acids and peptides (Rentsch *et al.* 2007, Thornton *et al.* 2007, Komarova *et al*. 2008, Hill *et al.* 2013, Gruffman *et al.* 2014, Warren 2014). Some types of transporters are capable of amino acid uptake by roots from the environment (Miller *et al*. 2007, Tegeder 2012, Pratelli and Pilot 2014). It seems that proteins and amino acids can be acquired also by plants without being in a symbiotic association with other organisms (Adamczyk *et al*. 2008, 2010, Paungfoo-Lonhienne *et al*. 2008, 2012, Stoelken *et al*. 2010, Lonhienne *et al*. 2014).

In many plant species, nitrate (NO_3^-) is the main source of N and its assimilation is essential for normal plant growth and development (Cruz *et al*. 2004). The reduction of NO_3^- to ammonium (NH_4^+) involves the sequential action of nitrate reductase (NR, EC 1.7.1.1, nitrite:NAD^+ oxidoreductase) and nitrite reductase (NiR, EC 1.7.7.1, ammonia:ferredoxin oxidoreductase). The resulting NH_4^+ is then assimilated by glutamine synthetase [GS, EC 6.3.1.2, L-glutamate:ammonia ligase (ADP-forming)] and glutamate synthase [GOGAT, glutamine:2-oxoglutarate aminotransferase, EC 1.4.1.14, L-glutamate:NAD^+ oxidoreductase (transaminating)] to organic forms such as glutamine (Gln) and glutamate (Glu). Glutamate provides the amino group to other amino acids *via* aminotransferase reactions (Forde and Lea 2007). Glutamate can be also metabolized *via* glutamate dehydrogenase [GDH, EC 1.4.1.2, L-glutamate:NAD^+ oxidoreductase (deaminating)], which is able to catalyze a reversible reductive amination / oxidative deamination and may participate either in synthesis or in catabolism of Glu (Dubois *et al*. 2003, Masclaux-Daubresse *et al.* 2006, Purnell and Botella 2007, Wang *et al*. 2007, Gajewska and Sklodowska 2009).

Although the fate and conversions of absorbed organic N compounds following root uptake have not been thoroughly studied, amino acid glycine has become a model compound for organic N uptake by plants (Wang *et al.* 2014). It seems that metabolism of absorbed amino acid N may not primarily depend on de-amination followed by incorporation of released N within the GS/GOGAT cycle, but rather on transaminations (Nasholm *et al*. 2009). Recent results showed that dipeptides, tripeptides, and even oligopeptides can be also used by various plant species without prior digestion (Paungfoo-Lonhienne *et al*. 2008, Adamczyk *et al*. 2010, Farrell *et al.* 2011).

The availability of C and N is crucial for plant growth and development and the C/N ratio is sensitively controlled (Coruzzi and Zhou 2001, Zheng 2007, Tschoep *et al*. 2009). A sugar-sensing mechanism enables plants to decrease photosynthesis when C-skeletons are abundant, whereas the N-sensing mechanism enables plants to turn off nitrate uptake and reduction when amounts of reduced or organic N are high (Coruzzi and Zhou 2001).

Beside its importance for a commercial plant propagation, *in vitro* cultivation can be used as a universal model system for plant nutrition experiments, where soil symbiotic organisms are excluded on purpose. However, *in vitro* plants grow usually under a high relative humidity and low air turbulence; photosynthesis, and in consequence biomass production, is also often limited by a low irradiance [*ca*. 50 - 60 μmol(photon) m^{-2} s^{-1}] and a low CO_2 concentration, particularly during the light period (Pospisilova *et al*. 2007). The most commonly used growth medium for *in vitro* propagation is that based on the original formulation of Murashige and Skoog (1962) (MS). The MS medium contains relatively high concentrations of N in a form of NO_3^- (40 mM) in combination with NH_4^+ (20 mM); this mixture was found to be superior to promote growth of *in vitro* plantlets (Sweby *et al*. 1994). *In vitro* cultivation often includes also sugar feeding (photomixotrophic nutrition) that can induce both positive and negative effects on plantlet growth, sometimes in dependence on plant species. Nevertheless, in *in vitro* grown tobacco, sugar presence increases not only photosynthetic potential but also prevents occurrence of photoinhibition symptoms under a higher irradiance (Ticha *et al.* 1998).

The aim of the study was to find out if tobacco plantlets are able to utilize protein as the exclusive source of N. In our experiment, we examined effects of casein as the sole source of N on growth, photosynthetic performance, and N assimilation metabolism in tobacco plantlets grown *in vitro*. To compare effects of casein with a low inorganic N nutrition, the plantlets were grown also under a limited inorganic N and with or without saccharose as an additional source of carbon.

Material and methods

Plants: The plantlets of *Nicotiana tabacum* L. (cv. Petit Havana SR1) were grown on an agar medium in ventilated *Magenta* (*GA-7*, *Sigma*, Diesenhofen, Germany) containers in a growth chamber under a 16-h photoperiod,

an irradiance of *ca*. 100 μmol(photon) m^{-2} s^{-1}, day/night temperatures of 22/18 °C, and a relative humidity of 80 %. For N nutrition experiments, the basic MS medium was used and changed as mentioned below only in its N compound content, all other elements were maintained the same. The plantlets were grown usually for *ca*. four weeks under the above conditions. At the end of the growth period, the plantlets were divided into shoots and roots and their fresh masses (FM) were recorded. For photosynthetic parameters and enzyme activities, leaves were separated from stems, frozen in liquid nitrogen, and kept at -80 °C until analyses.

Nutrition experiment design: Plantlets were grown under the above mentioned environmental conditions on 0.8 % (m/v) agar in the absence (photoautotrophic nutrition) or in the presence of 1.5 % (m/v) saccharose (photomixo-trophic nutrition). They were divided into four groups: *1*) control plantlets were grown on a basic MS medium containing 20.6 mM NH_4NO_3 and 18.7 mM KNO_3 (MS); *2*) plantlets grown on a medium containing 0.5 % (m/v) casein as the sole form of N (CA); *3*) plantlets grown on an ammonium-deficient medium, *i.e*., the ammonium salt was absent and the concentration of KNO_3 was 3.74 mM (5-times lower compared with the MS) (N1); and *4*) plantlets grown on a nitrate-reduced medium supplemented only with 4.12 mM NH_4NO_3 (a 5-times lower concentration compared with the MS) (N2). Deficiency of K^+ in the CA, N1, and N2 media was compensated by addition of 3 mM KCl. The total N-content of the cultivation media were 0.85, 1.00, 0.05, and 0.12 g(N) dm^{-3} in the MS, CA, N1, and N2, respectively.

Chlorophyll *a* fluorescence characteristics: Photosynthetic performance of photosystem (PS) II was evaluated by chlorophyll (Chl) *a* fluorescence using a pulse-amplitude modulated fluorometer (*PAM*, *Walz*, Elffetrich, Germany) in detached leaves similarly as described by Pospisilova *et al*. (2009). Maximum quantum yield of PS II photochemistry was calculated as the variable to maximum fluorescence ratio (F_v/F_m) in leaves dark-adapted for 20 min. Effective quantum efficiency of PS II photochemistry (Φ_{PSII}) was determined in light-adapted leaves. Nonphotochemical (q_N) and photochemical (q_P) quenching coefficients were calculated according to Van Kooten and Snel (1990).

Enzyme extraction and activity assays: For enzyme activity assays, 0.5 g of leaf or root tissue was homogenized in 1.5 cm^3 of a 100 mM Tris-HCl buffer (pH 7.8) containing 1 mM dithiothreitol, 1 mM EDTA, and 5 mM $MgCl_2$. Then, 0.02 g of polyvinylpyrrolidone was added and the homogenate was centrifuged at 23 000 *g* and 4 °C for 15 min. Activity assays for enzymes of N metabolism were carried out according to Debouba *et al*. (2006) and Manchenko (1994) with slight modifications. The volume of all reaction mixtures was 1 cm^3. Determination of NR activity was done in the reaction mixture containing a 100 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4), 10 mM EDTA, 0.1 M KNO_3, and 0.15 mM NADH. The enzyme reaction was initiated by addition of 0.1 cm^3 of the plant extract and stopped after 30 min with 0.1 cm^3 of 5.8 mM sulfanilamide and 0.8 mM N-(1-naphtyl)ethylenediamine dihydrochloride. After diazotation of nitrite ions, absorbance at 540 nm was measured by a spectrophotometer (*Helios α Thermo Spectronic*, Waltham, USA) against a blank, where KNO_3 and NADH were substituted with distilled water. Standard solutions of KNO_2 were used to create a calibration curve for determination of a product concentration in a nanomol range.

The reaction mixture for determination of GS activity contained a 100 mM Tris-HCl buffer (pH 7.6), 5 mM KCl, 5 mM $MgCl_2$, 10 mM L-glutamate, 2.5 mM ATP, 10 mM NH_4Cl, 0.3 mM NADH, 1 mM phosphor-*enol*pyruvate, 6.6 U of pyruvate kinase, and 13.5 U of lactate dehydrogenase. After initiation of the reaction with 0.1 cm^3 of the plant extract, GS activity was determined spectrophotometrically at 340 nm by a kinetic method based on releasing NAD^+ yielded by a double coupled reaction. Activity of GOGAT was also determined at 340 nm by a kinetic method. The reaction mixture contained 100 mM Tris-HCl (pH 7.6), 5 mM 2-oxoglutarate, 5 mM L-glutamine, and 0.3 mM NADH. The reaction was started with 0.1 cm^3 of the plant extract. Simultaneously with the GS and GOGAT activity assays, a blank consisting of 100 mM Tris-HCl (pH 7.6), 0.3 mM NADH, and 0.1 cm^3 of the plant extract was processed to control nonspecific reactions; the blank value was subtracted to obtain the final absorbance. The activities of GS and GOGAT were proportional to the amount of yielded NAD^+. The molar coefficient of absorbance of NAD^+ equal to 6200 M^{-1} cm^{-1} was used for calculation.

Since reactions catalyzed by NAD/NADP-GDH are highly reversible, the colorimetric method of activity determination was used rather than the kinetic method. Activity assays of NAD/NADP-GDH were carried out in the reaction mixture with a 100 mM Tris-HCl buffer (pH 7.1), 10 mM L-glutamate, and 0.2 mM $NAD^+/NADP^+$. The reaction was initiated with 0.1 cm^3 of the plant extract. The NADH/NADPH was visualized with 0.2 cm^3 of a detection solution (5 mg cm^{-3} iodonitrotetrazolium chloride and 5 μg cm^{-3} phenazine methosulfate). After 30 min, absorbance at 500 nm was measured against a blank, where L-glutamate was omitted. A calibration curve with standard solutions of NADH/NADPH served for activity calculation.

Statistical analysis: Experiments were repeated in three independent series, where each plantlet cultivation type was represented by 10 plantlets. Data were analyzed by the *Sigmaplot, v. 11* protocol for two-way analysis of variance and differences between samples were tested by all pairwise multiple comparison of the *Holm-Sidak*'s method. Differences were considered significant at $P \leq 0.05$.

Results

The N nutrition affected plantlet growth significantly and it also changed a biomass distribution between shoots and roots (Fig. 1). The plantlets *in vitro* with different N supplies also showed a significant dependence on saccharose (Fig. 1 Suppl. and Table 1 Suppl.). The MS grown plantlets showed a superior growth (Fig. 1*A*) compared to all other plantlets; saccharose promoted particularly their root growth (Fig. 1*C-D*). Exclusively, the organic N supply (CA) reduced severely plantlet growth without any particular biomass redistribution (Fig. 1*C*). The ammonium-deficiency (N1) reduced the total plantlet and shoot sizes less than the CA medium, whereas a root size increased compared with the MS grown plantlets. The nitrate-limited (N2) grown plantlets

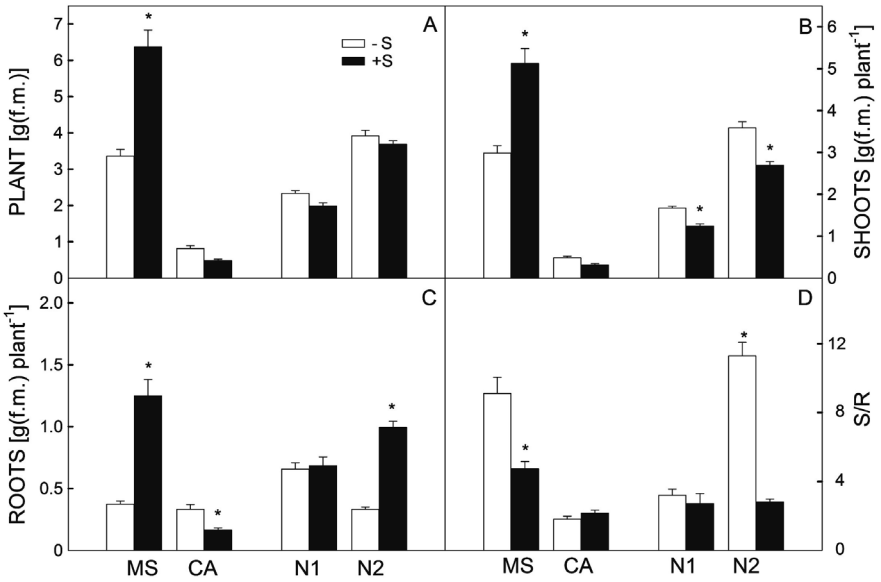

Fig. 1. Fresh masses of the whole tobacco plantlets (*A*), shoots (*B*), and roots (*C*), and shoot/root ratio (S/R; *D*) of plantlets grown in control (MS), ammonium deficient (N1), nitrate deficient (N2), and casein supplemented (CA) media. All the plantlet groups were cultivated without saccharose (*white columns*) or with saccharose (*black columns*). Means ± SEs, $n = 10$. *Asterisks* denote significant differences ($P \leq 0.05$) among the plantlet groups with respect to saccharose.

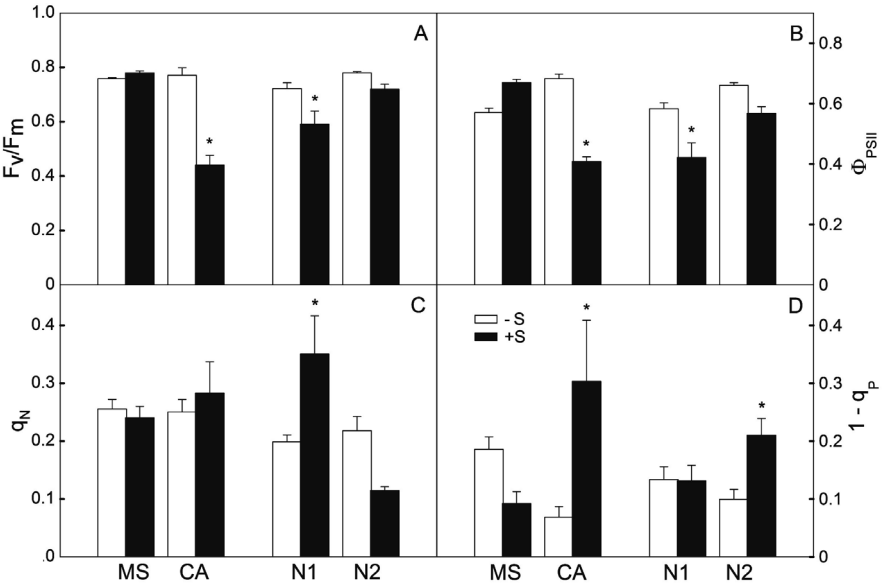

Fig. 2. Maximal efficiency of photosystem (PS) II photochemistry, F_v/F_m (*A*), actual efficiency of PS II, Φ_{PSII} (*B*), nonphotochemical quenching coefficient, q_N (*C*), and the number of closed PS II centres characterized by $1 - q_P$ (*D*) of leaves of plantlets grown on MS, N1, N2, and CA media without saccharose (*white columns*) or with saccharose (*black columns*). Means ± SEs, $n = 5$. *Asterisks* denote significant differences ($P \leq 0.05$) among the plantlet groups with respect to saccharose.

maintained the total size similar to that of the MS grown plantlets (Fig. 1*A*), whereas root growth was significantly stimulated by the low nitrate nutrition (Fig. 1*C-D*). Contrary to the MS and CA grown plantlets, the N1 and N2 grown plantlets showed an advanced senescence namely on the bottom leaves in the saccharose-supplemented media (Fig. 1 Suppl.). Saccharose caused a slight reduction of plant mass and a significant reduction of shoot mass in the plantlets grown on the N1 and N2 media, but a significant stimulation of root mass was observed in the plantlets grown in the N2 medium. Saccharose affected negatively all the growth parameters in the CA medium (Fig. 1).

Plantlet fitness was also evaluated according to Chl *a* fluorescence parameters, which characterize namely PS II performance (Fig. 2). Although some differences were found among the plantlets with different N nutrition, the effect of both the N nutrition and saccharose on photosynthesis was generally statistically insignificant (Table 1 Suppl.). The F_v/F_m and Φ_{PSII} were independent on N nutrition and values were similar in all the *in vitro* grown plantlets (Fig. 2*A,B*). The photomixotrophic nutrition lowered significantly both F_v/F_m and Φ_{PSII} in the plantlets grown in the CA and N1 media. The q_N showed some differences between the plantlets, but it was not significantly affected by N nutrition (Fig. 2*C*) similarly as the number of closed PS II centers (1 - q_P) (Fig. 2*D*). Saccharose increased q_N in the N1 grown plantlets, whereas (1 - q_p) was enhanced in the CA and N2 grown plantlets. The significantly negative effects of saccharose, particularly on F_v/F_m, Φ_{PSII}, and 1 - q_P, were found in the CA grown plantlets.

The activity of NR was significantly dependent on the N nutrition, whereas the effect of saccharose was significantly positive only in leaves of the MS grown plantlets (Table 1 Suppl., Fig. 3*A*), where the highest activity of NR was also found. The CA, N1, and N2 grown plantlets exhibited a similar reduction in the NR activity in shoots (Fig. 3*A*). In roots, the highest NR activity was found in the N2 grown plantlets without saccharose (Fig. 3*B*), however, it was statistically insignificant.

The activity of GS in leaves showed a similar pattern as NR in all the treatments (Fig. 3*C*, Table 1 Suppl). Saccharose stimulated the GS activity only in the MS grown plantlets, both in leaves and roots (Fig. 3*C-D*). A significantly lower GS activity was found in the CA, N1, and N2 grown plantlets, the only exception was the GS activity in roots of the plantlets grown in the N1 without saccharose (Fig. 3*D*).

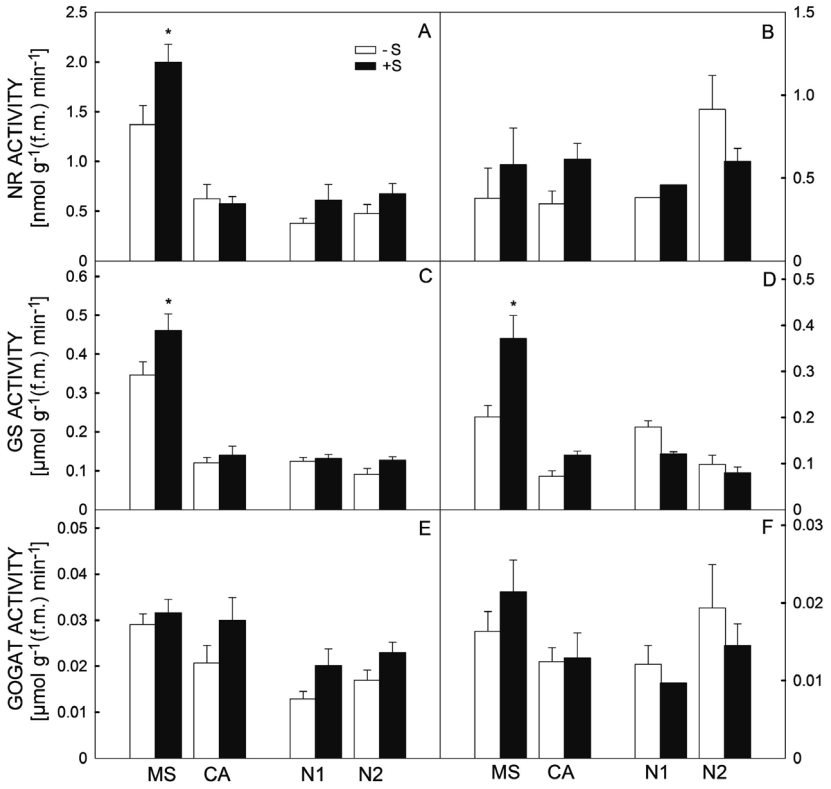

Fig. 3. Activities of nitrate reductase (NR) (*A,B*), glutamine synthetase (GS) (*C,D*), and glutamate synthase (GOGAT) (*E,F*) in leaves (*A,C,E*) and roots (*B,D,F*) of plantlets grown on MS, N1, N2, and CA media without saccharose (*white columns*) or with saccharose (*black columns*). Means ± SEs, $n = 3$. *Asterisks* denote significant differences ($P \leq 0.05$) among plantlet groups with respect to saccharose.

The activity of GOGAT was higher in leaves than that in roots (Fig. 3*E-F*). In leaves, the GOGAT activity seemed to be the highest in the MS and CA grown plantlets, lower in the N2, and the lowest in the N1 grown plantlets (Fig. 3*E*), but the differences were statistically insignificant. In roots, differences in the GOGAT activity were not statistically significant among all the plantlet groups, and no effect of saccharose was proved (Fig. 3*F*).

Activity of GDH, measured with both NAD and NADP as cofactors, was significantly dependent on N nutrition both in leaves and roots (Table 1 Suppl.). It was mostly stimulated by saccharose, particularly in leaves of the CA gown plantlets (Fig. 4*A*), where it was significantly higher compared to that in the N1 and N2 grown plantlets. In leaves, the highest activity was found in the MS grown plantlets independently on saccharose supply. In roots, the activity of NAD-GDH seemed to be the highest in the plantlets grown in the N2 without saccharose (Fig. 4*B*), but it was statistically insignificant.

Generally, the differences in the activity of NAD(P)-GDH were not statistically significant in leaves (Table 1 Suppl.) although saccharose seemed to stimulate its activity in the CA grown plantlets (Fig. 4*C*). In roots, the activities were significantly dependent on the N nutrition, and the presence of saccharose caused a considerable inhibition, namely in the MS and CA grown plantlets, where the highest activities were found under the photoautotrophic nutrition (Fig. 4*D*). The opposite trend was found in both the N1 grown plantlets and the N2 grown plantlets (Fig. 4*D*) although the effect of saccharose was not statistically significant. Generally, the effect of saccharose on NAD(P)-GDH was opposite in roots compared to leaves of the MS and CA grown plantlets and the N1 and N2 grown plantlets.

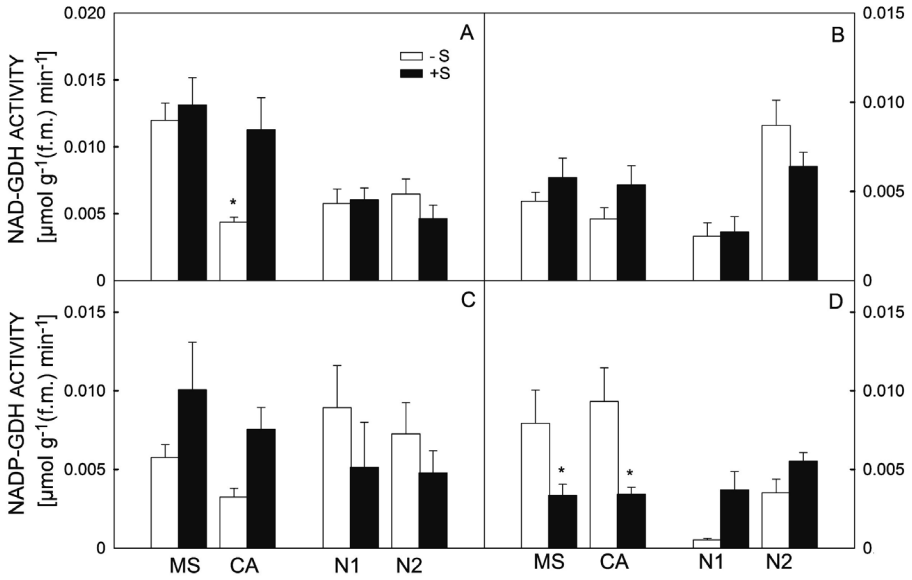

Fig. 4. Activities of NAD-glutamate dehydrogenase (NAD-GDH) (*A,B*) and NAD(P)-GDH (*C,D*) in leaves (*A,C*) and roots (*B,D*) of plantlets grown on MS, N1, N2, and CA media without saccharose (*white columns*) or with saccharose (*black columns*). Means ± SEs, $n = 3$. *Asterisks* denote significant differences ($P \leq 0.05$) among plantlet groups with respect to saccharose.

Discussion

Until recently, the opinion prevailed that organic compounds must be degraded by soil microorganisms to amino acids and, particularly, to inorganic ions prior being absorbed by plant roots and used further in metabolism. Several research groups changed the view on spectrum of N sources that can be accessed by plants and on N cycling (Adamczyk *et al*. 2008, Paungfoo-Lonhienne *et al*. 2008). In addition to amino acids and oligopeptides, also peptides and proteins, such as casein, bovine albumin, or green fluorescent protein, were shown to enable plant growth when supplied as a sole N source in sterile media without symbiotic bacteria. Wheat, *Hakea actites*, and *Arabidopsis thaliana* plants are able to exude proteases, incorporate proteins and use them as N source (Adamczyk *et al*. 2008, Paungfoo-Lonhienne *et al*. 2008, 2012, Lonhienne *et al*. 2014). Two mechanisms of N assimilation from proteins were suggested: *1*) exudation of proteolytic enzymes from roots and digestion of exogenous proteins, and *2*) endocytosis (Paungfoo-Lonhienne *et al.* 2008, Adamczyk *et al.* 2010). However, a biochemical insight into the metabolism of plants dependent only on proteins is still lacking, and our present experiments can contribute to solve this question.

Our experiments proved that also the tobacco plantlets were able to grow successfully when casein was the sole N source in the growth medium under sterile conditions. In accordance with previously published model systems (Paungfoo-Lonhienne *et al*. 2008), we found that also

casein did not promote plantlet growth to the same extent as the inorganic N sources. The growth of tobacco with 0.5 % (m/v) casein was much slower compared to the plantlets supplied with the complete MS medium and also slower than that of the plantlets with reduced inorganic N (the N1 and N2 plantlets, Fig. 1), although the total amounts of N in the MS and CA were similar [0.85 and 1 g(N) dm^{-3}, respectively], and higher than that in the N1 and N2 plantlets [0.05 and 0.12 g(N) dm^{-3}, respectively]. On the contrary, Adamczyk *et al*. (2008) found that wheat plants can use casein as N source and they exhibit even a higher plant fresh mass in the presence of 0.01 to 1 % (m/v) casein compared to plantlets grown in the complete MS medium. It was suggested that the ability to utilize soil proteins is strongly affected, in addition to inorganic N availability in the soil, by species-dependent differences in the activity of root-secreted proteases (Eick and Stohr 2009, Adamczyk *et al*. 2010). Actually, the basic MS medium contains higher concentrations of inorganic N than plants can find available under natural conditions, which was useful for salt-tolerance studies (Sweby *et al.* 1994) and probably also as stimulation of fast growth demanded for commercial plant propagation techniques.

Saccharose is a common component of *in vitro* plant cultivation media although various negative and positive effects have been observed (Ticha *et al*. 1998). In our experiment, saccharose influenced positively plantlet fresh mass accumulation and photosynthetic capability only in the plantlets grown in the full MS medium, whereas in the other plantlets, the photomixotrophic nutrition even retarded growth or did not show any significant effect (Fig. 1). As the C/N ratio is important for plant growth and root morphology (Zhang *et al*. 2007), the high content of saccharose could speed-up the N-depletion rate and thereby promote the N shortage in roots and older leaves. In our experiment, such conditions might occur in the plantlets with a reduced N supply (*i.e*., in the N1 and N2 plants) and also in the plantlets with a low N availability (with casein).

The photosynthetic capacity of leaves correlates positively with their N content presumably because most N is used for synthesis of major components of the photosynthetic apparatus and Rubisco (Foyer *et al.* 1994). Despite the small plantlet size, the CA grown plantlets showed the PS II parameters comparable with the MS grown plantlets under the photoautotrophic conditions (Fig. 2). Under the low irradiance, the CA grown plantlets were able to utilize radiation energy in a similar way as the MS grown plantlets. Nevertheless, a notable photoinhibition was caused by the same irradiance in the plantlets grown in the CA medium with saccharose, which was indicated by a decline in F_v/F_m and Φ_{PSII} and the increased number of closed PS II centres. This means that these plantlets were not able to utilize fully the absorbed radiation for photosynthetic electron transport. Excess energy was partly dissipated by nonphotochemical mechanisms as heat to lower the efficiency with which excitation energy is transferred to PS II reaction centres. This was observed to some extent also in the N1 and N2 grown plantlets; saccharose mostly enhanced the negative effect (Fig. 2). Similarly, leaves of grape vine use only a smaller portion of absorbed radiation energy for photosynthetic electron transport when grown under a low N supply compared to a high N supply (Chen and Cheng 2003a,b).

As the main aim of our study was to obtain more information on N metabolism of the CA grown plantlets, we focused on the enzymes involved in N assimilation: NR, GS/GOGAT, and GDH. Nitrate reductase is known as an important regulation point. The expression of NR is regulated by irradiance, nitrate, and saccharides; nitrate and saccharides also inhibit NR kinase because phosphorylation enables binding of inhibiting 14-3-3 protein and inactivation of NR (Lambeck *et al.* 2012). Our results proved that the reaction catalyzed by NR was more pronounced in leaves than in roots; the NR activity in leaves apparently responded to the amount of available N in the cultivation medium, *i.e*., in the plantlets grown at the N1, N2, and CA, the NR activity was low. The stimulatory effect of saccharose was found only in the plantlets at the MS. The plantlets grown with the N-limited supplies showed a rather negative effect of saccharose; it means that the C/N ratio was not favourable to stimulate further N assimilation. In this case, the availability of N played probably a more important role than the total N amount present in the media. Nitrate reductase is known to catalyze also formation of NO, which is an important signalling molecule for regulation of many physiological processes and stress responses (Romero-Puertas *et al*. 2013, Yu *et al.* 2014). However, to find out if a low NR activity influences metabolism and signalling in the CA-supplied plantlets, further experiments are required.

The metabolism of plants grown with proteins as the sole source of N has not been investigated yet. Our results showed that although the NR and GS activities declined (Fig. 3*A,C,D*), the GOGAT and GDH activities (Fig. 3*E*,*F*, Fig. 4) in the plantlets at the CA were comparable with those at the MS. Thus, a subsequent metabolic transformation of amino acids rather than primary deamination and amino acid degradation could be expected in the plantlets grown at the CA. Nevertheless, further experiments are needed to prove it.

Glutamate dehydrogenase can operate either in an anabolic direction to assimilate NH_4^+ or in a catabolic direction where it may help to alter the cellular C/N ratio and replenish tricarboxylic acid cycle intermediates under a C deficit (Purnell and Botella 2007). The activity of NAD-GDH is known to be carefully regulated by various metabolites, particularly in animal cells (Li *et al.* 2011), but the ratio of C/N is important for the expression of NAD-GDH in plant cells. The gene expression is induced in darkness and in a low C/N ratio in sinks, and it is repressed by a high C/N ratio occurring during photosynthesis (Purnell and Botella 2007). Our results prove that the highest activity was in leaves of the MS grown plantlets supplied sufficiently with N, whereas it

was lower in the N1 and N2 grown plantlets where the effect of added saccharose was not significant. The CA grown plantlets experienced a different situation: the activity of NAD-GDH was the lowest under the photoautotrophic C nutrition, whereas in the saccharose presence, the activity significantly increased and was comparable to that in the MS grown plantlets (Fig. 4*A*). It seems that there was a very low N available for metabolism in the CA grown plantlets, and beside the C/N ratio, actual concentrations of some metabolites, which are sensed by a plantlet, are also important. Less attention was devoted in literature to NAD(P)-GDH. Our experiments prove that the tobacco plantlets involved this enzyme in their N metabolism also under the conditions of a low N available. More C available seemed to have the opposite effects in leaves and roots of the experimental plantlets, and the effect also varied among the MS and CA grown plantlets and those grown on the limited inorganic N nutrition.

Finally, we could hypothesize what might be a reason for the slower growth of the tobacco plantlets with casein as the sole source of N when the total N in the cultivation medium was higher than that in the MS grown plantlets. The first reason, discussed above, is the substantially lower availability of N for plant metabolism; the protein must be degraded into smaller peptides or amino acids and transported to root cells and other tissues of shoots. The second reason might be the total absence of nitrate in the cultivation medium because nitrate is not only the major source of N but also an important signalling molecule regulating expression of many genes and thus affecting many physiological events including root and leaf development (Forde 2002, Medici and Krouk 2014). However, the surprisingly competent photosynthetic apparatus of the *in vitro* plantlets was probably able to ensure energy and reducing equivalents for the slow growth of the CA-supplemented plantlets.

Conclusion

The tobacco plantlets were able to grow on the sterile modified MS medium where the only source of N was casein. The growth of the casein-supplemented plantlets was slower compared to the plantlets grown in the complete MS medium with a high content of inorganic N, but similar to the plantlets growing on the inorganic N-limited medium. No significant difference was observed in photosynthesis evaluated by Chl *a* fluorescence. Saccharose as the additional carbon source positively influenced only the plantlets on the MS medium. Enzymes involved in N assimilation, such as NR and GS, showed the reduced activities in the plants grown on the casein-supplemented and N-limited media, but the activities of GOGAT and GDH were similar to the MS grown plantlets. Thus, it seems that the latter enzymes were more important for the casein-supplemented and inorganic N-limited plantlets. Although the total amount of N in the casein-supplemented medium was slightly higher than that of the complete MS medium, the availability of N was lesser, nevertheless, the plantlets were able to utilize N in the form of protein, even better under the photoautotrophic conditions.

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