

STUDY OF A NITRATE SUPPLY ON THE REMOVAL TOXICITY OF AMMONIUM AND UREA NUTRITION IN WINTER OILSEED RAPE

M. ARKOUN[†], P. LAINE[†], P. ETIENNE[†], J-M. GARCIA-MINA^{*}, J-C. YVIN[‡] A. OURRY[†].

[†] UMR INRA-UCBN 950 EVA, Ecophysiologie Végétale, Agronomie, nutrition N, C & S, Esplanade de la paix, Université de Caen, 14032 Caen, France

^{*} CIPAV, Timac Agro Spain, Roullier Group. Poligono de Arazuri-Orcoyen, Calle C n°32, E-31160 Orcoyen, Spain

[‡] Timac Agro International, Roullier Group. 27 avenue Franklin Roosevelt, 35408 Saint-Malo, France

ABSTRACT

To study mechanisms of nitrate's beneficial effect on plants supplied with ammonium or urea, winter oilseed rape (*Brassica napus* L. var. Capitol) seedlings were grown with different nitrogen compounds supplied as sole (ammonium, nitrate and urea) or mixed forms (nitrate-ammonium and nitrate-urea). Nitrogen uptake and assimilation were studied at the physiological and molecular level.

Our results show that urea and ammonium grown-plants present symptoms of N starvation such as a decrease of shoot and root dry weights comparatively to nitrate fed plants. The use of ¹⁵N-labeled nitrogen demonstrates the capacity of oilseed rape to take up ammonium or urea but less efficiently than nitrate. A supply of nitrate to urea or ammonium fed plants increased root and shoot dry weights. However, urea and ammonium uptake was reduced by the presence of nitrate in the growth medium which seems to be preferentially taken up by plants. At the molecular level, *BnNRT1.1* and *BnNRT2.1*, genes encoding low and high-affinity NO₃⁻ transporters respectively, are strongly up-regulated in nitrate-urea and nitrate-ammonium fed plants, while shoot *BnDUR3* gene (encoding a high-affinity urea transporter) is down-regulated. Moreover, *BnGDH2*, a gene encoding glutamate dehydrogenase, an enzyme involved in ammonium assimilation, which was markedly up-regulated by the presence of ammonium fed plants and down-regulated by a nitrate supply. These data suggest a beneficial effect of nitrate in correcting the negative effects of ammonium and urea nutrition.

Keywords : assimilation, *Brassica napus* L. gene expression, mixed nitrogen nutrition, uptake

INTRODUCTION

Several authors have demonstrated that the negative effects associated with ammonium (A) and urea (U) nutrition are corrected by the supply of nitrate (N) in the nutrient solution (Aslam *et al.*, 1994 ; Cruz *et al.*, 2003; Mérigout *et al.*, 2008). However, the mechanism responsible for this beneficial effect of nitrate on ammonium and urea nutrition remains unknown. Many authors suggest that it might be related to changes in the physiological pH by the maintenance of appropriate carboxylate levels in plants (Feng *et al.*, 1998). Other experiments have shown that this beneficial effect does not seem to be mediated through the stimulation of glutamine synthetase or urease activities (Houdusse *et al.*, 2005). A possible effect of nitrate reducing ammonium accumulation through the activation of GDH cannot be ruled out. Moreover, the functional analysis of the expression profile of genes involved in nitrate uptake has not been reported in the presence of NA or NU. In this context, it becomes clear that a complementary study on the action of mixed feeding on the main nitrate (*NRT1.1* and *NRT2.1*) and urea (*DUR3*) transporters comparatively with nitrate, urea and ammonium as sole nitrogen source is of great interest. In order to verify these hypotheses, nitrogen uptake and its assimilation were studied at the physiological and molecular level by the use of ¹⁵N labelling and qPCR expression analysis of *BnNRT1.1*, *BnNRT2.1*, *BnDUR3* and *BnGDH2*, respectively.

MATERIAL AND METHODS

Experimental treatment, harvest and Isotope Analysis

Seeds of *Brassica napus* L. (var. Capitol) were sterilised and then germinated 72h in dark on perlite. Seedlings were then placed for 2 weeks in light then transferred on 10L tanks for different durations (0, 24h, 72h, and 15 days) and supplied with diluted Hoagland nutrient solution containing 2 mmol L⁻¹ of N-NO₃⁻, N-NH₄⁺, N-urea, N-NA, and N-NU. ¹⁵N labelling (atom % ¹⁵N, 2%), was used for measurement of cumulative N uptake at each time.

The experiment was carried out in green house, at 20°C with cycle day/night of 16/8h (Lamp Hortilux Schreder, 400 W, HS.TP4.23) and nutrient solution was renewed every 2 days.

At each harvest, fresh weight of root and shoot was determined before drying for 48 h at 60°C. After drying, organs were ground separately for 2 min to fine powder with inox beads of 0.4 mm diameter in an oscillating grinder (mixer mill MM301; Retsch) before isotope analysis. The ¹⁵N analyses were

performed using an analyzer (EA 300; Eurovector) coupled with a mass spectrometer (isoprime mass spectrometer; GV Instrument).

RNA Isolation and Quantitative RT-PCR Analysis

Total RNA was extracted from 200 to 400 mg of root and shoot fresh matter corresponding to three sets of seedlings for each treatment. Fresh root and shoot samples were ground in liquid nitrogen with a mortar. The resulting powder was suspended in 750 µL of extraction buffer (0.1 M Tris, 0.1 LiCl, 0.01 M EDTA, 1% SDS [w/v], pH8) and 750 µL of hot phenol (80 °C, pH 4). This mixture was vortexed for 30s. After addition of 750 µL of chloroform/isoamylalcohol (24:1), the homogenate was centrifuged (15000g, 5 min, 4 °C). The supernatant was transferred into 4 M LiCl solution (w/v) and incubated overnight at 4 °C. After centrifugation (15000g, 30 min, 4 °C), the pellet was suspended in 250 µL of sterile water. Fifty microliters of 3 M sodium acetate (pH 5.6) and 1 mL of 96% ethanol were added to precipitate the total RNA for 1 h at -80 °C. After centrifugation (15000g, 20 min, 4 °C), the pellet was washed with 1mL of 70% ethanol, then centrifuged at 15000g for 5 min at 4 °C. The resulting pellet was dried for 5min at room temperature and resuspended in sterile water containing 0.1% SDS and 20 mM EDTA. Quantification of total RNA was performed by spectrophotometer at 260 nm (BioPhotometer, Eppendorf) before RT-PCR analysis.

For RT, 1 µg of total RNA was converted to cDNA with an iScript cDNA synthesis kit using the manufacturer's protocol (Bio-Rad). The subsequent PCR reactions were performed with 4 µL of 200X diluted cDNA, 500 nM of the primers, 1X SYBR Green PCR Master Mix (Bio-Rad) in a total volume of 15 µL. The specificity of PCR amplification was examined by monitoring the melting curves after quantitative PCR reactions using the Chromo4 system (Bio-Rad) and by sequencing the quantitative PCR product to confirm that the correct amplicons were produced from each pair of primers (Biofidal). Comparative relative expression of the various genes was determined using the delta-delta Ct method employing the formula: relative expression = $2^{-[\Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}}]}$ where Ct refers to the threshold cycle, sample indicates the gene of interest, and control indicates the endogenous housekeeping gene (Livak *et al.*, 2001).

RESULTS AND DISCUSSION

Nitrate, ammonium and urea uptake

In order to study mechanisms of nitrate's beneficial effect, rapeseed seedlings were grown with different nitrogen compounds supplied as sole (ammonium, nitrate and urea) or mixed forms: nitrate-ammonium (NA) and nitrate-urea (N.U).

Plant growth (*i.e.* dry matter production) was not significantly affected during the first 24 and 72 hours of treatment (data not shown). After 15 days of treatment, the analysis of the accumulated dry biomass revealed very significant differences. Comparatively to nitrate fed plants, plants treated with urea or ammonium were characterized by a decrease of shoot and root dry weights (from approximately 4 and 2 fold respectively). Moreover, plants fed with urea presents symptoms of N starvation with an even lower efficiency. Our results also show that the addition of nitrate to urea or ammonium fed plants reduce significantly this negative effect compared with provision of either N source alone.

This difference of growth might reflect differences in N uptake or assimilation related to the N nutrition. The cumulative N uptake has been followed by the use of ¹⁵N labelling. Results demonstrate the capacity of oilseed rape to take up ammonium or urea but less efficiently than nitrate (data not shown). Surprisingly, urea and ammonium uptake was reduced by the presence of nitrate (from 43,3±5 to 29,6±2,15 mg ¹⁵N and from 1037,6±100 to 570±58,37 mg ¹⁵N, respectively) in the growth medium which seems to be preferentially taken up by plants, reinforcing the nitrophile character of Brassicaceae. However, other experiments perform on wheat have shown that nitrate increases their removal from the plant (Garnica *et al.*, 2009). Our study also reports the inhibitory effect of urea and ammonium (by 2,5 fold) on nitrate uptake. This observation is consistent with other studies, reporting that NO₃⁻ accumulation and assimilation are strongly repressed by NH₄⁺ or urea (Aslam *et al.*, 1994 ; Mériçout *et al.*, 2008).

Analysis of BnNRT1.1, BnNRT2.1 and BnDUR3 transcript levels

As demonstrated above, N treatments led to major changes in plants. To determine the relative expression of nitrate (*BnNRT1.1* and *BnNRT2.1*) and urea (*BnDUR3*) transporters, an analysis by qPCR on roots and shoots of plants has been undertaken. Our results show that root *BnNRT1.1* and *BnNRT2.1* are inducible by nitrate (figure 1A and B). Interestingly, *BnNRT1.1* is also up-regulated by ammonium at 24h and 72h after treatment (figure 1A), while no induction was observed in urea

treatment comparatively with starved conditions (S). As already described in other works, nitrate transporters are induced by its own substrate (Okamoto *et al.*, 2003). Surprisingly, our results show that, *BnNRT1.1* is strongly up-regulated in nitrate-urea (N.U) and nitrate-ammonium (N.A) fed plants, while *BnNRT2.1* is only up-regulated by nitrate-urea treatment. This could explain that there is more nitrate taken up when rapeseed is fed with mixed nutrition. The repressive effect of urea as the sole N source on nitrate uptake could be explained by a direct inhibition, but also by the high Glutamine content in root cells of urea-grown plants (Mérigout *et al.*, 2008).

It has been shown that *BnDUR3* represent a major transporter for high-affinity urea uptake in *Arabidopsis* (Kojima *et al.*, 2007). Our results show a slight induction of *BnDUR3* in roots supplied with urea (data not shown). Surprisingly, *BnDUR3* was also induced in shoots in urea fed plant after 72h of treatment (figure 1C), this suggests that a substantial part of urea may be directly translocated to the shoots. Indeed, 20% of ¹⁵N-labeled urea was translocated from roots to shoots in *Arabidopsis* (Mérigout *et al.*, 2008). Interestingly, *BnDUR3* is down-regulated in nitrate-urea fed plants (figure 1C), suggesting a beneficial effect of nitrate in reducing urea accumulation.

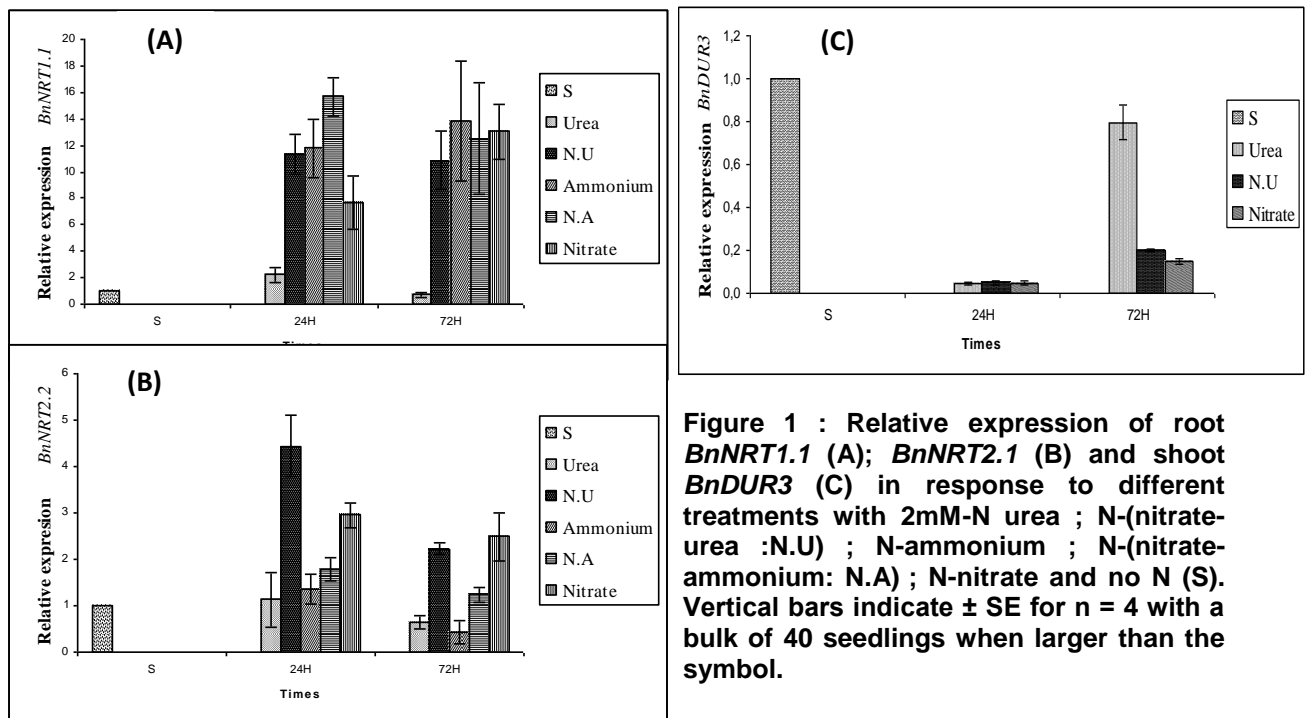


Figure 1 : Relative expression of root *BnNRT1.1* (A); *BnNRT2.1* (B) and shoot *BnDUR3* (C) in response to different treatments with 2mM-N urea ; N-(nitrate-urea :N.U) ; N-ammonium ; N-(nitrate-ammonium: N.A) ; N-nitrate and no N (S). Vertical bars indicate \pm SE for n = 4 with a bulk of 40 seedlings when larger than the symbol.

Ammonium assimilation: Analysis of *BnGDH2* transcript level

Many species, including rapeseed, develop symptoms of toxicity when subjected to high concentration of NH_4^+ (Britto and Kronzucker, 2002). GDH may play a potential role in ammonium assimilation under particular stress conditions (Dubois *et al.*, 2003). Indeed, our study shows that root *BnGDH2* is highly induced in ammonium fed-plant after 15 days of treatment, and repressed by nitrate-ammonium (NA) or nitrate (figure 2). No induction has been observed after 24 and 72 hours of treatment. The up-regulation of *BnGDH2* in response to elevated NH_4^+ levels suggests that GDH is important in detoxification of ammonium by assimilating some of the excess ammonium ions by catalysing the reductive amination of 2-oxoglutarate to glutamate (Terce-Laforgue *et al.*, 2004).

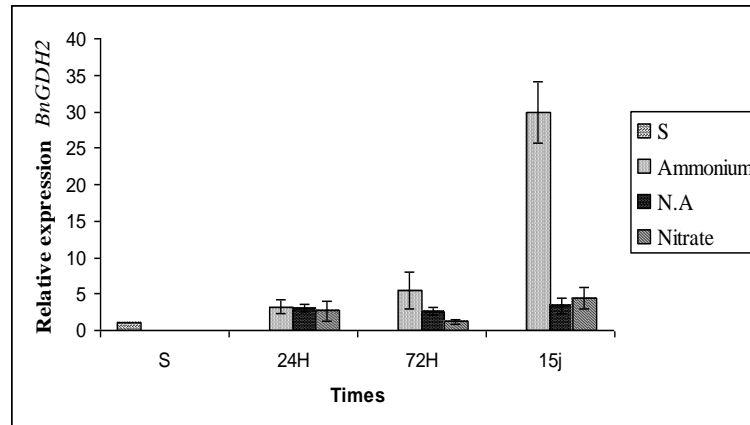


Figure 2 : Relative expression of root *BnGDH2* in response to different treatments with 2mM-N urea; N-(nitrate-urea: N.U); N-ammonium; N-(nitrate-ammonium: N.A), N-nitrate and no N (S). Vertical bars indicate \pm SE for $n = 4$ with a bulk of 40 seedlings when larger than the symbol.

CONCLUSIONS

Our data demonstrate:

1. The negative effect of ammonia and urea supply on the growth of oilseed rape, the effect being more pronounced when plants were supplied with urea.
2. *BnNRT1.1* and *BnNRT2.1* are strongly up-regulated in nitrate-urea (N.U) and nitrate-ammonium (N.A) fed plants, while *BnDUR3* is repressed. In consequence, there is more nitrate taken up by plants when they are fed with mixed nutrition. Indeed, urea and ammonium uptake was reduced by the presence of nitrate which seems to be preferentially taken up by plants, confirming the nitrophile character of Brassicaceae.
3. The up-regulation of *BnGDH2* under ammonium nutrition suggest the implication of this enzyme in ammonium detoxification.

ACKNOWLEDGEMENTS

The authors thank the « Pôle de compétitivité Mer-Bretagne » and the « Fond Unique Interministeriel » to support the AZOSTIMER project. We are grateful to technical team of UMR EVA 950 for IRMS analysis.

REFERENCES

- Aslam M, Travis RL, Huffaker RC (1994), *Plant Physiol.* 106: 1293–1301.
- Britto DT, Kronzucker HJ (2002), *J. Plant Physiol.* 159: 567–584.
- Cruz C, Castillo M, Domínguez CN, Juanarena N, Aparicio-Tejo P, Lamsfus C (2003), *Actas XV Reunión de la Sociedad Española de Fisiología Vegetal y VIII Congreso Hispano-Luso*, ed. by Sociedad Española de Fisiología Vegetal, Palma de Mallorca, p. 297.
- Dubois F, Tercé-Laforgue T, Gonzalez-Moro M.B, Estavillo M.B, Sangwan R, Gallais A. and Hirel B (2003), *Plant Physiol. Biochem.* 41: 565 – 576.
- Feng J, Volk RJ and Jackson W (1998), *Plant Physiol.* 118: 835–841.
- Garnica M, Houdusse F, Yvin JC and Garcia-Mina J.M (2009), *J Sci Food Agric.* 89: 55–62.
- Houdusse F, Zamarreño AM, Garnica M and Garcia-Mina JM (2005), *Funct. Plant Biol.* 32:1057–1067.
- Livak KJ, Schmittgen TD (2001), *Methods* 25: 402–408.
- Mérigout P, Lelandais M, Bitton F, Renou JP, Briand X, Meyer C, and Daniel-Vedele F (2008), *Plant Physiol.* 147: 1225–1238.
- Okamoto M, Vidmar JJ and Glass DMA (2003), *Plant Cell Physiol.* 44: 304-317.
- Terce-Laforgue T, Dubois F, Ferrario-Mery S, Pou de Crezenzo MA, Sangwan R, Hirel B (2004), *Plant Physiol.* 136: 4308–4317.