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Interactive comment on "Interactions between uptake of amino acids and inorganic nitrogen in wheat plants" *by* E. Gioseffi et al.

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We thank Referee #2 for the very thorough reading and commenting on our paper. We have carefully tried to address the concerns raised and to revise the paper accordingly. Some of the concerns of Referee #2 seem to be based on the fact that the different nitrogen treatments in Experiments I to III had been misinterpreted - partly because we had not described the treatments sufficiently clear. We have tried to improve this in order to prevent uncertainties and misunderstandings.

Referee comment #1. The subject matter of the paper is interesting and of importance to understanding the acquisition of N by plants but, unfortunately, the value of the manuscript and its conclusions would have been much greater if the experiments had been better designed for their stated purpose. There is also a need for much clari-

fication of the description of the experimental methodology and there are some aspects of the data, which give me cause for concern.

Response: The overall concerns of the Referee are repeated in the specific points listed below and our response is specified in connection with these points.

Referee comment #2. In the Abstract, it is stated that the underlying hypothesis was that amino acids may lead to the down-regulation of inorganic N uptake. If this truly was the hypothesis, the experiments are poorly designed to test it. In my opinion, the only effect of one form of N on the uptake of another that is really tested is the effect of NO3- on glycine uptake. Only at this point is a single concentration of a solute applied with and without the presence of the potentially competing form of N. As the authors themselves point out, rates of solute uptake by roots are generally concentration-dependent and the effect of concentration varies between solutes. Thus, if the comparison of uptake rate with and without the competing N form is not carried out at the same concentration, it is hard to know how to interpret the results.

Response: Although in the Abstract it might seem that the overall aim was to study the down-regulation of inorganic N uptake by amino acids, the three experiments tested three different hypotheses, which are explained in the introductory part of the Materials and methods section. We have clarified this in the Abstract. It might have been useful to have more combinations of different treatments and concentration levels but this was not feasible in our experimental setup, so we focused on total N uptake in Experiment I and on down-regulation in Experiment II. We do not fully agree with the Referee that it would have been necessary to combine all different levels of concentration in order to arrive at safe conclusions. There is already a lot of information available on the uptake kinetics of nitrate and ammonium in plant roots and in our opinion this knowledge can be exploited to arrive at valid conclusions even though the concentration levels were not exactly the same in the different experiments. We already think that this aspect was addressed in the Discussion section. Referee comment #3. In Fig. 4, uptake of NO3- alone is shown and alluded to in the Abstract, but I am unclear what this refers to

as I can find no description of the methodology for this. I am also confused by the fact that uptake of NO3- is shown in this Fig. for a treatment where in the methods it states that glycine alone was used.

Response: It is clearly stated in Materials and Methods that NO3- in Experiment II (Fig. 4) was added in a concentration of 3 mM with or without glycine. Uptake of NO3- (and NH4+) was calculated alone according to its depletion in the nutrient solution, as thoroughly explained in the calculation sub-section. Then it was added to the rate for amino acids in the mixture treatments and so the total N uptake rate was calculated. This was done for all treatments including those having only amino acids, also to monitor the possible deamination and nitrification of amino acids in the solution. At all time points the NO3- level in solutions containing amino acids was zero, and this is also what it is shown in Fig. 4. What is alluded in the Abstract refers to our results in Experiment I, which are shown in Fig. 2.

Referee comment #4. There is no straightforward test of the effect of the presence of amino acids on uptake of NH4+ or the effect of the presence of NH4+ on amino acid uptake. It is also stated in the Abstract that NH4+ was taken up at about twice the rate of organic N. According to the statistical information shown in Fig. 3, glycine N was taken up at the same rate as NH4+. This kind of fundamental error gives me serious cause for concern about the quality of this investigation.

Response: Generally, uptake of inorganic N was about twice as high as that of organic N. As also stated in our response to reviewer 1, comment #4, we have now specified the differences between uptake of organic and inorganic forms.

Referee comment #5. P11313 L2. adsorbed?

Response: 'adsorbed' changed to 'absorbed'

Referee comment #5. P11314 L8 and various places in the Introduction. I do not understand why Experimental is capitalised.

Response: 'Experimental' changed to 'experimental'

Referee comment #6. Materials and Methods Experiment 1. It is not clear to me exactly what the experimental set up was here. It sounds as though four replicate containers were used for each N treatment, each with four pseudo-replicate plants. Is this correct? How large were the plants? In the description of the basic nutrient solution, NO3- is a counter ion for Mg, Ca, K and Fe. How was this altered in the N treatments? There are also questions regarding the concentrations of other ions. A complete breakdown of the nutrients in the different treatments would be helpful information for the reader.

Response: The four plants contained in each container were analyzed together as if it was one experimental unit, so the 4 replicates refer to 4 completely independent containers within each treatment. The weight of the plants at harvest did not vary between the different treatments and the average biomass weight has now been added in the Methods section. Apart from NO3- and NH4+, the only nutrient concentrations differing between the experiments were those of Ca2+ and SO42- since the uptake of these two ions are known to be rather conservative across different external concentrations. This was already specified in line 7 p. 11316 and has now been further clarified in the text. We will be happy to provide the complete breakdown of the nutrients for all treatments if the Editor thinks this is necessary; however, in our opinion this is not meaningful for the purpose of the paper.

Referee comment #7. Experiment 2. As I mention above, it is not at all clear what was done in this experiment. Was NO3- added alone? If so, at what concentration was it supplied? Why does Fig. 4 show NO3- uptake in a treatment where only glycine was supplied? Please clarify the replication as above.

Response: It is clearly stated in Materials and Methods that NO3- was added in a concentration of 3 mM with or without glycine.

Referee comment #8. Experiment 3. The description of this is not clear. What was the composition of the nutrient solutions without NO3-? What is the source of the data

shown for NO3- in Fig.7? Please clarify the replication.

Response: The experimental conditions of experiment 3 is detailed in Materials and Methods, stating that: 'Under the same light and temperature conditions as exp I and II, wheat plants were grown for 7 days in complete nutrient solutions with NO3- as N source, followed by a 17-day period in which 1 mM unlabelled glycine, glutamine, arginine or asparagine were provided as the only N source (4 replicates).' NO3- constituted also one of the treatments, and this has been added in the sub-section. As stated before, we will be happy to provide the full composition of the nutrient solutions if required by the Editor, but we do not think it gives added value to the understanding of the experimental treatments.

Referee comment #9. P11317 L6-8 Please give more detail of the use of 15NH4+ and 15NO3-. I can find no other mention of this.

Response: We did not use labelled NO3- or NH4+ in our experiments, but only labelled amino acids. However, we did measure 15NH4+ and 15NO3- in a few samples from treatments containing only amino acids were we had found NH4+ formation in solution. This was done in order to see whether the ammonium was amino acid-derived or not.

Referee comment #10. Table 1. It would help the reader if data for the whole plant were supplied as this is the real focus of the manuscript. Partitioning between root and shoot is interesting, but of secondary importance in my opinion.

Response: In our opinion the partitioning between roots and shoots are of methodological importance in order to asses the metabolic fate of 13C,15N double labelled amino acids and the errors associated with using only shoot values to estimate uptake rates and recoveries of absorbed amino acids as is often the case in experiments with soil grown plants. Our data clearly demonstrate the differences in 13C and 15N enrichment in roots and shoots, warranting that the data are presented separately.

Referee comment #11. P11319 L4-6 It seems contradictory that plants receiving NO3-

and glutamine should have the highest N content when much is made of the fact that glutamine was taken up at a lower rate than NO3-. This requires some comment at least.

Response: The N content of plants receiving NO3- and glutamine is not significantly different from the other treatments, as shown in Table 1. They only differed from the pre-treated plants. This has now been emphasized.

Referee comment #12. P11319 L8. The C to N ratios for glutamine and glycine are the wrong way round. Fig.2 To which form of N do the error bars refer in the mixed treatments?

Response: We thank Referee #2 for pointing out the mistake. The theoretical C:N ratio has been switched. The error bars in Fig. 2 refer to the total N uptake, therefore considering the SE in both inorganic and organic N uptake. This has now been specified in the figure legend.

Referee comment #13. P11319 L16-18 As I mentioned above, Fig. 4 shows the uptake of glutamine N to be the same as NH4+ and all mixed N treatments.

Response: We assume that Referee #2 was referring to Fig. 2 and not Fig. 4, since Fig. 4 does not show uptake rates of neither glutamine nor NH4+. We disagree with the statement that the uptake of glutamine N is the same as NH4+ and all mixed N treatments, as there are significant differences between gln and both NH4+ and all mixed N treatments except for gln+NH4+. We have specified these differences in the text.

Referee comment #14. P11319 L25-26 What form of statistical analysis is being referred to? How was this test conducted?

Response: The statistical analysis mentioned in P11319 L25-26 is an ANOVA between two statistical models - both mixed linear models with plants and blocks as random factors and organic and inorganic form of N as fixed factors which contribute to the

output variable, i.e. total N uptake rate – where the first model predicted an interaction between the fixed factors and the second one included the two factors, but not their interaction. At 95% CI, the two models were statistically different, hence the more complex model has to be accepted, i.e. the interaction model. We modified the statistical analysis sub-section in order to make this process clearer.

Referee comment #15. P11320 L2 Pre-starvation is not mentioned in the methods.

Response: The explanation of the plant pre-starvation has been included in the methods section under Experiment II.

Referee comment #16. Fig.6. I find it rather worrying that recovery of the 15N is so low. There seems to be no obvious reason for this. There also appears to be a tendency for the recovery to be lower in the glutamine treatments. I think that some explanation is necessary. I also wonder what effect there was of more-or-less completely depleting the solutions of organic N on the results i.e. the concentration of the solutes changes dramatically over the experimental period. Further, I wonder how there can be almost complete depletion of organic N from solution, yet have greater uptake of inorganic N (Fig.2). This does not seem possible and gives me severe reservations about the quality of the presented data. Could differences found between the rates of uptake of different forms of N be influenced by the different ways of measuring them utilised?

Response: The recovery of 15N was around 80%. We agree that this is lower than one would anticipate. Isotopic mass balance data are actually only available in very few of the published studies on amino acid uptake and we therefore included them in the present work to illustrate the large differences between for 15N and 13C. Possible reasons for 15N and 13C losses were already extensively discussed in section 4.2. Differences in recovery between glutamine and glycine treatments were generally not significant and we have therefore not discussed them in further details.

The reviewer asks how uptake rates of inorganic N can be higher than those for organic N despite almost complete depletion of the organic N from the solution. The explana-

tion is that in cases where nitrate or ammonium in Experiment I came close to being depleted, as revealed by analysis of samples taken at 45 h after the beginning of the treatment period (line 10-11, p. 11316) uptake rates were calculated over this shorter time interval. This has now been specified in section 2.3 by insertion of the sentence: “All calculations of NO3- and NH4+ uptake rates were based on the initial linear part of the depletion curves extending up to at least 45 h after start of the experiment”. The analyses of the nutrient solutions also showed that the amino acids were not depleted before at the end of the experiment (66 h; Fig. 6). In experiment II, nitrate was applied in a higher concentration than in Experiment I and was not depleted at the end of the experiment.

Referee comment #17. Fig.7. Why is data for shoots presented alone?

Our response: Root data were not measured in Experiment III.

Referee comment #18. Discussion. In my opinion, the interpretation of the data needs completely re-evaluating with a much more critical eye. Because of the design of the experiments, I think that what can be said about the effect of organic and inorganic forms of N on each other is limited. Perhaps if the inconsistencies in the data are attended to and the description of the experiments is clarified, a clear message with scientific merit can be discussed.

Response: Through answering all comments of both Referees and by clarifying and modifying some parts of the manuscript, including more comprehensive methodological considerations in the Discussion section, we think that we have managed to address all discussion points and to improve our paper.

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