

Evaluation of Nitrogen Use Efficiency (NUE) of rice (*O. sativa*) under saline conditions

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Annexes

1 Protocols

Chlorophyll content measurement, Lichtenthaler (1987) (cri172):

- Crush 100 mg of fresh matter with 4 ml of acetone 80%.
- Collect crushed material inside a 15 ml falcon.
- Fill the volume of the falcon with acetone 80 % until 10 ml.
- Conserve falcon in ice and away from light.
- Centrifugation of the falcons at 3000 rpm during 10 min at 4°C.
- Directly after centrifugation, falcons are transferred in ice to quickly measure with the spectrometer.
- Read solution absorbance at 3 wave lengths: 663.2, 646.8, and 470.0 nm
- Blanco = acetone 80%

$$C_a \text{ (mg/L)} = 12.25W_{663.2} - 2.79W_{646.8}$$

$$C_b \text{ (mg/L)} = 21.5W_{646.8} - 5.1W_{663.2}$$

$$C_{\text{carot}} \text{ (mg/L)} = (1000W_{470.0} - 1.82C_a - 85.2C_b)/198$$

$$C_a \text{ (mg/gFM)} = [C_a \text{ (mg/L)} / \text{weight(g)}] \times [10 \text{ (ml)} / 1000]$$

$$C_b \text{ (mg/gFM)} = [C_b \text{ (mg/L)} / \text{weight(g)}] \times [10 \text{ (ml)} / 1000]$$

$$C_{\text{carot}} \text{ (mg/gFM)} = [C_{\text{carot}} \text{ (mg/L)} / \text{weight(g)}] \times [10 \text{ (ml)} / 1000]$$

Mineralization (cold process) for the Na-K-Cl measurements:

- Mash the dry matter sample.
- Collect 0.30 mg of the mashed dry sample in a falcon of 50 ml.
- Fill the falcon with 40 ml HNO₃ 0.5%.
- Shake the falcon for 24h.
- Centrifuge the falcon at 4000 rpm for 15 minutes.
- Filtrate the solution.
- Conserve the solution at 6°C before analysis.
- Analyze the solution by mass spectroscopy for Na and K content (iCAP 6500).
- Dilute 50X the solution and analyze it by ion chromatography for Cl content (Dionex ICS-2000).

Total protein content measurement, Bradford:

Sample preparation:

- Collect 70 mg of fresh material inside a 1,5 ml Eppendorf (beforehand crushed with liquid nitrogen).
- Fill the Eppendorf with 1 ml of phosphate buffer (100mM, pH = 7.4).

- Vortex the Eppendorf.
- Conserve Eppendorf in ice and away from light.
- Centrifugation of the Eppendorf at 10000 rpm during 15 min at 4°C.

Calibration and lecture at the spectrophotometer:

- Preparation of the calibration solutions with BSA (0.5µg/µl) in Eppendorf:

Concentration BSA [µg/µl]	0	2	4	6	8	10
<i>BSA [µl]</i>	0	4	8	12	16	20
<i>H₂O + NaOH 100mM [µl]</i>	100	96	92	88	84	80
<i>Bradford solution [µl]</i>	900	900	900	900	900	900

- Vortex and wait 15minutes.
- Preparation of the samples:
 - Take 100 µl of the root sample solution or 25 µl of the shoot sample solution in an Eppendorf.
 - Complete with 75 µl of H₂O + NaOH 100mM solution for the shoot sample.
 - Add 900 µl of Bradford solution and vortex.
 - Wait 15 minutes.
- Put 200 µl of the solution in a microplate.
- Read at the wavelength 595 nm on the spectrophotometer (SPECTROstar Nano).

2 Script of the Statistic Analysis with SAS 9.4

The information read between /* and */ are not taking into account while the script is computing.

The different parameters are:

- Gen: the genotype
- N: the nitrogen concentration
- NaCl: the salt condition

Procedure of data importation:

```
LIBNAME LLN "access_path";
PROC IMPORT OUT = LLN.data DATAFILE = "access_path\file_name.txt"
DBMS = TAB REPLACE;
GETNAMES = YES;
DATAROW = 2;
RUN;
```

Creation of Boxplot for raw analysis and visualization of the variable:

```
PROC SGPLOT DATA = LLN.data;
VBOX Name_of_the_variable / CATEGORY = NaCl GROUP = N; RUN;
PROC SGPLOT DATA = LLN.data;
VBOX Name_of_the_variable / CATEGORY = NaCl GROUP = Gen; RUN;
PROC SGPLOT DATA = LLN.data;
VBOX Name_of_the_variable / CATEGORY = N GROUP = Gen; RUN;
```

ANOVA script

Setting the generalized linear model of the variable with all parameters as discrete factors, calculating the type III or type IV Analysis of the variance, and measuring least squares means for the different parameter.

```
PROC GLM Data = LLN.data PLOTS=(DIAGNOSTICS);
CLASS Gen N NaCl;
MODEL Name_of_the_variable = Gen NaCl N N*NaCl N*Gen Gen*NaCl
N*NaCl*Gen / SOLUTION CLPARM; /*the command E4 can be placed after
the / in the model statement to calculate type IV ANOVA when lacking data are
present in the data file */
LSMEANS Gen / CL PDIFF ADJUST = TUKEY;
LSMEANS NaCl / CL PDIFF ADJUST = TUKEY;
LSMEANS N / CL PDIFF ADJUST = TUKEY;
LSMEANS NaCl*N / CL PDIFF ADJUST = TUKEY;
LSMEANS NaCl*Gen / CL PDIFF ADJUST = TUKEY;
LSMEANS N*Gen / CL PDIFF ADJUST = TUKEY;
RUN;
QUIT;
```

The triple interaction parameter N*NaCl*Gen can be removed of the model statement if it is not significant to explain the variability of the model (p value <0.05; interval of confidence is >95%).

Removal of data

The removal of an entire treatment or genotype was done through the following script:

```
Data LLN.Name_of_the_removed_treatment;
SET LLN.data;
WHERE Gen ne "Name_of_the_treatment "; /*N or NaCl; ne = not equal to */
Run;
```

The variable was proceeded with the specific new formed database.

The data from the experiment in Vietnam were imported the same way as the one for the experiment in Louvain-La-Neuve. The similar script was used to choose and analyze the variable from the Vietnamese database.

Pearson matrix

The Pearson matrix of correlation was built with the following script:

```
PROC CORR DATA = LLN.data;
Run;
QUIT;
```

3 Additional Results

Table 1: Results of the anions content analysis (preliminary test) for the hydronics experiment

Genotype	Salinity [dS/m]	N conc. [mM]	F [ppm]	Cl [ppm]	NO2 [ppm]	SO4 [ppm]	NO3 [ppm]	PO4 [ppm]
Pokkali	11,5	2,86	n.a.	55,65	n.a.	60,16	4824,68	n.a.
Pokkali	11,5	2,86	n.a.	52,49	n.a.	40,16	4721,28	n.a.
Pokkali	11,5	2,86	n.a.	61,68	n.a.	54,43	4840,31	n.a.
Ngoi	0	2,86	n.a.	n.a.	n.a.	56,88	4698,71	n.a.
Ngoi	0	2,86	n.a.	n.a.	n.a.	20,02	4744,73	n.a.
Ngoi	0	2,86	n.a.	23,53	n.a.	n.a.	4510,07	n.a.

Shoot Dry Weight at Heading Stage (Vietnam experiment)

The ANOVA (Table 2) of the complete model showed that the triple interaction parameter was significant on the shoot dry weight. The complete model was suitable for analysis ($R^2 = 0.97$). All the parameters were significant on the shoot dry weight. The total dry weight was highly correlated to this variable (99%). However, the results have to be taken cautiously as the number of repetitions was low due to the low survival rate under salinity stress.

Table 2: ANOVA model of the shoot dry weight at heading stage

<i>Type III ANOVA - Shoot dry weight</i>							
Parameters	Gen	NaCl	N	N*NaCl	Gen*N	Gen*NaCl	Gen*N*NaCl
Pr > F	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0016

Genotype influence: The genotype has a significant effect on the shoot dry weight (Table 3 and Figure 1). Cuom had the highest dry weight, Ngoi was the second highest one but the difference between them was not significant. FL478 followed and finally, IR28 had the lowest weight.

Table 3: LSMEANS of the shoot dry weight at heading stage

	<i>LSMEANS - Shoot dry weight [g]</i>
Cuom	25.61 ± 2.25
FL478	20.90 ± 1.81
IR28	18.11 ± 1.81
Ngoi	23.80 ± 2.09

NaCl influence: The salt had a significant negative influence over the shoot dry weight (Figure 1). The means dry weights of the medium salt level and strong salt level corresponded to 68% and 26% of the control, respectively.

N influence: The nitrogen had a significant positive effect on the shoot dry weight (Figure 1). The means dry weight of the 0.125N, 0.25N and 0.5N concentrations corresponded to 11%, 18% and 43% of the standard nitrogen one, respectively.

Interaction N-NaCl: The interaction had a significant effect on the shoot dry weight (Figure 1a). The trends observed for the separated parameters (the positive effect of N and the negative of NaCl) were present but they were greatly attenuated for the strong NaCl level. The 0.125N and 0.25N concentration dry weights were not significantly different under the medium salt and the control.

Interaction genotype-N: The interaction had a significant effect on the dry shoot weight (Figure 1b). Only the dry weights of the 1N were significantly different with Cuom and Ngoi being the highest ones, then FL478 was the third and finally IR28.

Interaction genotype-NaCl: The interaction had a significant effect on the dry shoot weight (Figure 1c). The effects were difficult to characterize. Cuom and Ngoi were the highest ones under the medium salinity and the control, with FL478 and IR28 being similarly lower. Under the strong salinity, FL478 was the highest with Ngoi, the two others were less productive.

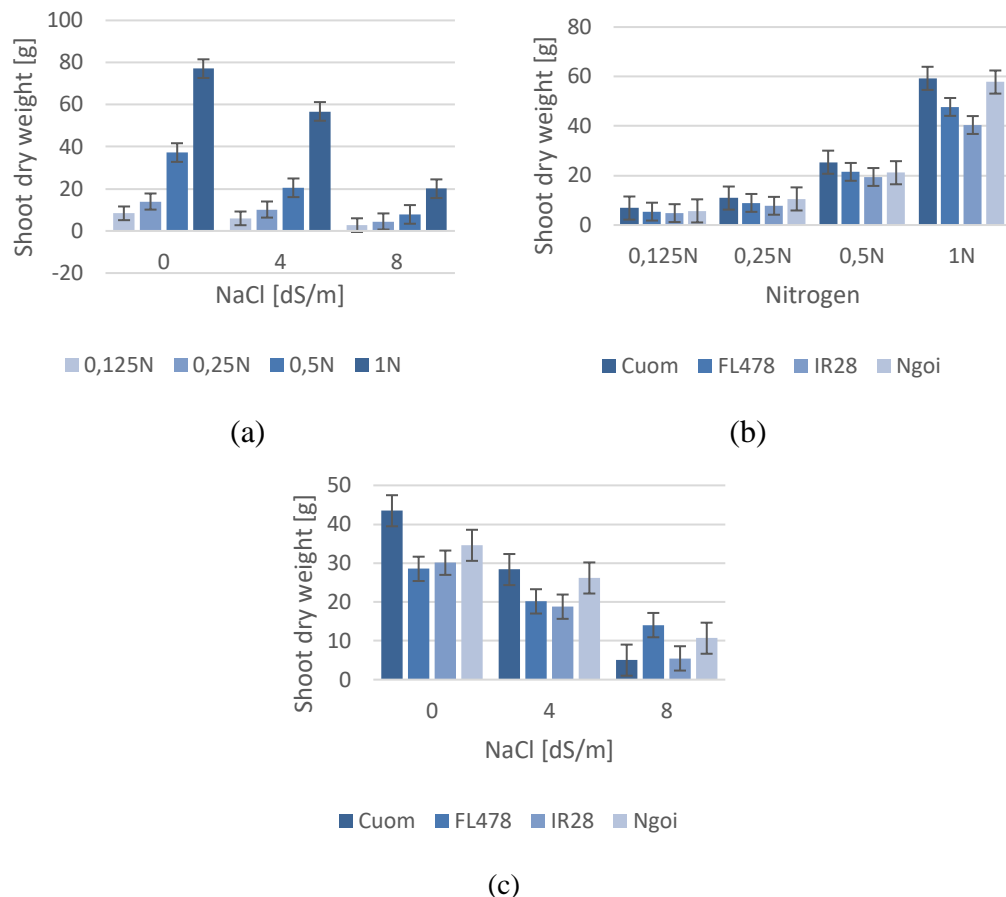


Figure 1: Shoot weight at heading stage: (a) in function of the salt concentration for the 4 N concentrations; (b) in function of the nitrogen concentration for the 4 genotypes; (c) in function of the salt concentration for the 4 genotypes. The complete GLM model was applied. Histogram: Least squares means. Error bar: 95% interval of confidence. Cultivar Cuom: trad. - tolerant; cultivar Ngoi: trad. - sensitive; cultivar FL478: improved - tolerant; cultivar IR28: improved - sensitive. 0,125N = 0.125g N; 0,25 = 0,25g N; 0,5N = 0,5g N; 1N = 1g N.

Agronomical NUE at Heading Stage (Vietnam Experiment)

The ANOVA (Table 4) of the complete model showed that the triple interaction parameter was not significant on the agronomical NUE, therefore the restrained model was calculated. The restrained model was considered suitable for analysis ($R^2 = 0.84$), but the results have to be taken cautiously as the number of repetitions was low. Almost all the parameters were significant on the agNUE. The interaction N-NaCl and genotype-N were not significant.

Genotype influence: The genotype had a significant influence on the agNUE (Table 5). Cuom and Ngoi had the highest agNUE, followed by FL478. IR28 had the lowest one (significantly different from the two highest).

Table 4: ANOVA models of the agNUE at heading stage

Type III ANOVA - Agronomical NUE							
Parameters	Gen	NaCl	N	N*NaCl	Gen*N	Gen*NaCl	Gen*N*NaCl
Pr > F	0.0006	<.0001	0.0013	0.1028	0.9694	0.0001	0.1984
Pr > F	0.0005	<.0001	0.0002	0.2100	0.8206	0.0002	

Table 5: LSMEANS of the agronomical NUE at heading stage

	LSMEANS - Agronomical NUE [kg kg ⁻¹]
Cuom	69.49 ± 6.95
FL478	64.49 ± 5.68
IR28	54.90 ± 5.68
Ngoi	72.18 ± 6.29

NaCl influence: The salt had a significant negative influence on the agNUE. The salinity levels results were significantly different from each other. The means rate of the medium salt level and strong salt level corresponded to 72% and 29% of the control, respectively.

N influence: The nitrogen had a significant effect on the agNUE. The N had a positive influence on the agNUE from 0.25N to 1N, but 0.125N was at the level of 0.5N. The mean rate of the standard N, 0.5N, 0.25N concentration corresponded to 131%, 105% and 84% of the 0.125 nitrogen one, respectively.

Interaction genotype-NaCl: The interaction had a significant effect on agNUE. The effects were difficult to characterize (Figure 2).

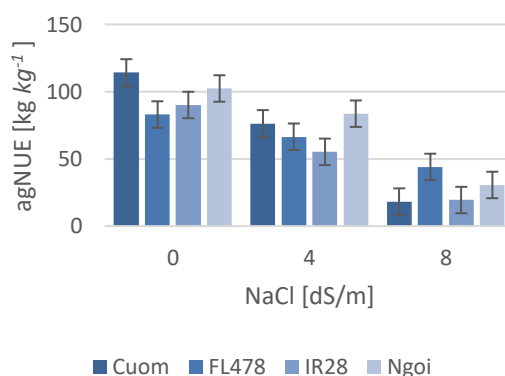


Figure 2: Agronomical NUE at heading stage in function of the salt concentration for the 4 genotypes. The complete GLM model was applied. Histogram: Least squares means. Error bar: 95% interval of confidence. Cultivar Cuom: trad. - tolerant; cultivar Ngoi: trad. - sensitive; cultivar FL478: improved - tolerant; cultivar IR28: improved - sensitive. 0,125N = 0.125g N; 0,25 = 0,25g N; 0,5N = 0,5g N; 1N = 1g N.

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Abstract Rice (*Oryza sativa* L.) is a major food supply in the world and many challenges for its cultivation sustainability have to be faced. One of these challenges consists in an efficient management of the nitrogen fertilization. Indeed, nitrogen is one of the most important elements for the plant growth and development. However, rice cropping conditions can be favorable to nitrogen losses leading to problems like eutrophication. Developing varieties reaching higher nitrogen use efficiency could represent a valuable improvement for the crop yield and economically, but also a sustainable improvement. Another challenge is to deal with the increasing risk of abiotic stress due to climate change, such as salinity which is more frequently present in Vietnamese deltas.

This study consisted in an analysis of the interactions between three fixed parameters: the nitrogen fertilization applied, the salinity level and the rice genotype. Two first experiments were conducted in hydroponics during the early vegetative phase with two nitrogen concentrations (standard N and 0.125N) and three increasing salt levels (no salt, moderate and strong salinity) for three genotypes Cuom: Vietnamese salt tolerant; Pokkali: salt tolerant reference; Ngoi: Vietnamese salt sensitive). The study was completed with an experiment in pots in Vietnam to validate the findings of the hydroponics analysis. This last experiment was conducted until the harvest but was focused on the active tillering.

Several conclusions can be highlighted with this work. The first one was the absence of salt sensitivity before the seedling emergence. The second conclusion showed that the N toxicity seemed to increase with the salinity. Finally, the salt tolerance had an influence on the Nitrogen Use Efficiency (NUE). A high salt-tolerance cultivar, such as Cuom, presented a high absorption (aNUE) and agronomical NUE (agNUE) coupled to a low physiological one (pNUE), while the opposite NUE levels were more related to salt-sensitive cultivar. The Vietnamese in-situ experiment did not invalidate the conclusions but can only confirm the negative influence of salt stress on rice and the basic nitrogen effects in absence of salt. Nitrogen Use Efficiency is a valuable indicator to be considered in future studies regarding the salinity tolerance of rice.