RESULTS OF IN VITRO ANDROGENESIS UNDER INCREASING SALINITY CONDITIONS FOR THREE MOROCCAN SPRING BARLEY VARIETIES ( Hordeum vulgare L.)

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INTRODUCTION

In arid and semi-arid regions, high soil salinity is one of the many abiotic stresses limiting the agricultural yields of wheat, rice, maize or barley. Soil salinity is the result of either irregular and insufficient rainfall, or strong evaporation of water. The drier the climate, the more saline is the soil and higher the osmotic stress (Hillel et al., 2008), the two traits showing to be linked, it is then difficult for the roots to extract water from the soil, which results in weaker growth (De-Jong-van Lier et al., 2009). Hence, in cereals salt has a depressive effect on germination rates, biological growth and grain production (Bennaceur et al., 2001). Compared to wheat and rice, barley is considered to be the most salt tolerant; being rich in fibers, vitamins and hormones it is used for both animal and human nutrition and turns out to be one of the main cereals. Therefore, it appears interesting to develop a complementary research to use doubled haploid methods to obtain quickly improved salt tolerance new lines.

In vitro regeneration is largely demonstrated to be a source of variability for either somatic (Sibi, 1976) or gametophytic tissues (El-Goumi, 2015). In barley biometrical analyses of doubled haploid lines obtained through in vitro gynogenesis and androgenesis, show the last path to be source of wide variability (San Noeum and Ahmadi, 1982) and that is why in barley anthers culture was chosen in this work.

In this species, after embryonic and plantlet stage, doubled haploid lines can be generated (Esteves, 2014) without any colchicine or other antimitotic treatments (Kahrizi and Mohammadi, 2009), as in barley spontaneous doubling is reaching the results at the same level, permitting to exclude the hypothesis of accidental lethal action. In this experiment, after the successive androgenesis steps, the doubled haploid barley regenerated on medium containing progressively increased NaCl concentrations, will constitute the first stage of a rapid creation of new barley lines for which, afterward, the tolerance to salt and drought will be analyzed, as well as agronomic traits.

Furthermore, during this first step, as the responses of the material might well be related to the genotype, three varieties entered the program to be compared during the in vitro phase, and because of the number of seeds necessary to repeat the experiments, three successive generations were made, thus by the way, the genetic homogeneity of each variety could be tested through the comparisons of the results at the same level, permitting to exclude the hypothesis of accidental residual heterozygosity.

MATERIAL AND METHODS

Technical processes

The plant material consisted of three spring barley fixed varieties (2n = 14), Asni (As) and Tamelalt (Tm) with two rows, and Arig (Ar) with six rows, provided by INRA-Settat. This material was stated as RS0 (before selfing), RS1, after one round of natural self-fertilization and RS2, after two rounds. To simplify the global name of each case, for example, Tamelalt before selfing, will be written as "Tm RS0", and considered as a "category", giving for the three varieties a total of nine categories.

All the donor plants were grown in 20 cm diameter pots, containing seven grains per pot, and cultivated in the glasshouse. The spikes harvested at microspore uninucleated stage, were covered by filter paper soaked with water, wrapped in aluminum foil, and kept in refrigerator at 4°C for 14 days cold pretreatment (El-Goumi et al., 2017; Hentour et al., 2016; Kahrizi et al., 2011; Powell, 1988) to induce androgenesis and increase embryos yields.
These spikes were sterilized by using 95% ethanol. Glumes and husks were removed, and the anthers were extracted with sterile tweezers and placed on one side (El-Gouni, 2015; El-Gouni et al., 2017) at a density of one spike per 55 mm diameter Petri dishes containing induction FHG (Hunter, 1988) medium supplemented by 2 mg.L\(^{-1}\) of naphthaleneacetic acid (ANA) at pH 5.7. Every medium was sterilized by autoclaving at 120°C, 1 bar, for 20 minutes. The inoculated dishes were sealed with Parafilm and incubated at 25°C for 4 to 8 weeks, in the dark chamber. Progressively, a total of 11,343 anthers followed this process enabling the development of 2,273 calluses or embryos, i.e. formations. When ranging from 1 to 2 mm, the formations (El-Gouni, 2015) were gradually transferred in 90 mm Petri dishes containing regeneration FHG medium at pH 5.8, supplemented with 35 g.L\(^{-1}\) of sucrose, and 0.4 mg.L\(^{-1}\) of benzyl amino purin (BAP).

Simultaneously with control cultures on salt free regeneration medium, according to Figure 1, increasing concentrations of NaCl were progressively added to this medium, up to the sub-lethal concentration. After one to two weeks, they generated either green or albino haploid plantlets. The green ones were transferred to FHG rooting medium containing 20 g.L\(^{-1}\) of sucrose. Once rooted, the plants were transplanted into 7 cm diameter pots containing sand and potting soil (2/3, 1/3) to ensure development and tillering, thus the progeny.

![Figure 1: Experimental design](Image)

**Legend:** After the induction phase, the calluses or embryos (formations) were placed on regeneration medium. During the whole experiment, control dishes consisted of cultures on salt free medium. In parallel, the formations were placed on the same regeneration medium containing at the beginning 2.5g.L\(^{-1}\) of NaCl. Every two weeks transfer was made on new fresh medium supplemented by the increasing salt concentrations of 5, 7.5, 10, 11, 12 and 13 g.L\(^{-1}\); up to 10 g.L\(^{-1}\) a gap of 2.5 g.L\(^{-1}\) was used, beyond 10 g.L\(^{-1}\) of NaCl a 1 g.L\(^{-1}\) increment was applied.

### Statistical analyses

The following parameters were calculated:

- Induction rate (I%) = (number of responding anthers/number of cultured anthers) \(\times\) 100
- Regeneration rate out of embryos (R%) = (total number of regenerated plants / number of cultured embryos) \(\times\) 100
- Albino rate out of total plants (A %) = (number of albino plants / total number of regenerated plants) \(\times\) 100

Several analyses were made:

- The induction rates were ranked according to Duncan’s multiple range tests (p<0.05) (Duncan, 1955) with SPSS software (Table 1).
- The ratios in percent of the total regenerated plants and of the albino ones were listed in Table 2, while the numbers of green or albino plants were presented in Table 3.
- To examine the combined effects of the genotypes and salt stress on regeneration or albinism, the data were statistically analyzed by \(\chi^2\) test, using SPSS software (Table 4).

A p value <0.05 is considered as statistically significant.

### RESULTS

#### Induction phase

The induction rates comparisons of the 2,243 formations issued from the 11,343 anthers for the three genotypes are presented in the Table 1.

| Genotype | 1% group 1 | 1% group 2 | 1% group 3 |
|----------|------------|------------|------------|
| Ar RS1   | 1.26       |            |            |
| Ar RS0   | 1.42       |            |            |
| Tm RS0   | 2.05       |            |            |
| Tm RS1   | 2.37       |            |            |
| Ar RS2   | 3.73       | 3.73       |            |
| Tm RS2   | 4.15       | 4.15       |            |
| As RS0   | 9.29       | 9.29       |            |
| As RS2   | 13.97      |            | 14.07      |
| As RS1   |            |            | 14.07      |

**Significance (p)**: 0.377 0.060 0.106

**Symbols:**

- **Genotypes:** Ar: Arig, As: Asni, Tm: Tamelalt
- **Categories:** RS0: before selfing, RS1: 1st generation from selfing, RS2: 2nd generation from selfing
- **I%**: Induction rate of formations, the values are presented in % of anthers cultivated

**Legend:** The three groups 1, 2 and 3 can be distinguished by the ability to induce formations. In a same group the values do not differ significantly.

After Duncan analysis three groups were observed. The recorded rates of formations expressed by Arig were ranging from 1.26% for Ar RS1, to 3.73% for Ar RS2, and those expressed by the three Tamelalt generations from 2.05% for Tm RS0 to 4.15% for Tm RS2, even if this last generation value is slightly higher, all of them being not statistically different belong to the group 1.

Asn exhibited significantly higher rates of induction, with 9.29% for As RS0, 13.97% for As RS2 and 14.07% for As RS1, the three generations being linked statistically and constituting the group 3.

The group 2, including Ar RS1 (3.73%), Tm RS2 (4.15%) and As RS0 (9.20%), showed statistical links with the groups 1 and 3 that differ from each other statistically.

#### Regeneration phase

As shown in the experimental design (Figure 1) the 2,273 formations obtained were progressively transferred from the induction medium to the salt free or to a saline regeneration medium. In both cases, either green plants or albino were regenerated, and the total regeneration or albinos rates were evaluated and listed in Table 2.
Table 2 Regeneration and albino rates obtained for each category, developed on control or salt type regeneration medium

| NaCl (g.L⁻¹) | Ar RS0 | Ar RS1 | Ar RS2 | As RS0 | As RS1 | As RS2 | Tm RS0 | Tm RS1 | Tm RS2 |
|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|             | R%   | A%   | R%   | A%   | R%   | A%   | R%   | A%   | R%   |
| 0           | 29.6 | 37.5 | 8.7  | 100  | 100  | 100  | 8.4  | 100  | 100  |
| 2.5         | 3.7  | 100  | 0    | 0    | 0    | 4    | 100  | 0    | 0    |
| 5           | 3.7  | 100  | 0    | 0    | 0    | 0.8  | 100  | 5.8  | 94.1 |
| 7.5         | 3.1  | 100  | 0    | 0    | 0    | 0.7  | 100  | 0.5  | 94.1 |
| 10          | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    |
| 11          | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    |
| 12          | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    |
| 13          | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    |

Symbols:
Genotypes: Ar: Arig; As: Asni; Tm: Tamelalt
Categories: RS0: before selfing, RS1: after one round of selfing and RS2: after two rounds of selfing
R%: Regeneration rate out of the embryos, A%: Albino ratio out of the regenerated plants

Legend: The outcomes are reported for each category. The total regenerants and the albinos ratios are given in percent, out of formations numbers, after culture on salt free or on the increasing salt concentrations medium.

In control conditions, the regeneration maximum rates were of 29.6% for Ar RS0, 12.4% for As RS1 and 15.1% for Tm RS1. But despite this efficiency, there was a high level of regenerated albino, often reaching 100%. Only Ar RS0 with 37.5% of albinism and Tm RS1 with 50% gave green plants.

After culture on saline medium, the higher regeneration values were expressed at 5 g.L⁻¹ salt stress, for As RS1 and As RS2, with the rates of 5.8% and 4%, respectively, and for which albinism appeared at a level of 94.1% for As RS1 and 92.3% for RS2.

Most of the regenerants showed to be albinos, however regeneration occurred in several salt concentrations values, up to 12 g.L⁻¹ as for As RS1 with a rate of 0.2%.

Thus, whatever the control or salt conditions were, the total numbers of either albinos or green plants have been listed in Table 3, showing a summarized view of the results.

Table 3 Number of green and albinos plants regenerated under control and salt stress conditions, according to the genotype

| Genotypes | Salt free Green | Albinos | Sum | Salt stress Green | Albinos | Sum | Total numbers |
|-----------|----------------|--------|-----|------------------|--------|-----|---------------|
| Arig      | 5              | 8      | 13  | 0                | 3      | 3   | 16            |
| Asni      | 0              | 69     | 69  | 2                | 40     | 42  | 111           |
| Tamelalt  | 13             | 22     | 35  | 0                | 8      | 8   | 43            |
| Total numbers | 18    | 99     | 117 | 2                | 51     | 53  | 170           |

Legend: Numbers of regenerants inventoried for the salt free path and also for the cumulated results from all concentrations for the salt stress experiments. Among the total number of 170 regenerants, there were 150 albino and 20 green plants.

Based on Table 3, the control path gave a total number of 117 regenerated plants for the 3 varieties, comprising 99 albino and 18 green plants. The salt stress path generated 53 plants, out of which 51 were albino, while 2 green plants were obtained for Asni, on the 5 g.L⁻¹ of salt medium.

Table 4 Statistical analysis of genotype and salinity effect on regeneration and albinism by χ² test

| Effect | Genotype/NaCl | NaCl | NaCl/genotype |
|--------|---------------|------|---------------|
| Parameters | Control | NaCl | Arig | Asni | Tamelalt |
| Reg χ² Test | 79.520 | 48.988 | 44.000 | 305.452 | 219.750 |
| ddl | 2 | 12 | 1 | 1 | 1 |
| p-value | 0.0004*** | 0.213 | 0.142 | 0.049* | <0.000*** |
| Alb χ² Test | 6.000 | 1.143 | 4.000 | 5.000 | 2.000 |
| ddl | 2 | 12 | 1 | 1 | 1 |
| p-value | <0.0001*** | 0.565 | 0.261 | 0.287 | 0.157 |

Symbols:
Reg: Regeneration, Alb: Albinism
Genotype/NaCl: genotype response to salt stress (All genotypes cumulated)
NaCl/genotype: salinity effect on each genotype (All NaCl concentrations cumulated)
p-value: significance, *: significant, ***: highly significant

The χ² test showed a genotype effect most marked for the control without addition of salt, for regeneration and albinism. A statistically significant difference was noted between the control and salt stress (p<0.05) showing the adverse effect of salt.

For salt stress medium (cumulated results from all concentrations), Tamelalt and Asni varieties indicate significant results compared to Arig with p-values of <0.0001 and 0.049 respectively. Furthermore, the χ² test showed that the interaction between genotype and salt stress (NaCl) indicates non-significant results, i.e. the same negative effect, on regeneration and albinism, with p-values of 0.213 and 0.565 respectively.

DISCUSSION

In this study, the genetic material was composed of the three varieties, Asni, Tamelalt and Arig, the level of homozygosity of which has been verified by INRA-Settal. Each variety was multiplied by two successive self-fertilizations in order to provide sufficient number of anthers and permitting at the same time to test the possible effect of accidental heterozygosity. During the first phase, the experiments started with barley androgenesis on a salt free induction medium, as to initiate divisions in the microspores. Additionally, it should be noted that in order to maximize the reactivity, all the anthers have to be plated on only one side on the in vitro medium, as a previous work (Fakiri, 1995) showed that the anther wall acts as a filter, allowing higher androgenic
division rates. The calluses or embryos, i.e., formations, developed were counted for each generation and variety. The data presented in Table 1, showed firstly that induction rates, relative to the number of cultivated anthers, exhibited values that do not differ statistically, for the three generations of each genotype, ranging from 1.2% to 3.73% for Asri, 2.05% to 4.15% for Tamelalt and from 9.29% to 14.07% for Asni. These results confirming the homogeneity of the successive generations by selfing; in other terms, at that step, genetic stability or homozygosity of the three varieties was expressed, avoiding any possible effect of accidental heterozygosity. Moreover, while Arig and Tamelalt varieties showed a tendency for a decrease with the lowest values, ranging from 1.26% to 4.15%, Asni exhibited a separate position with the significantly highest rates, mentioned above, and showing the special behavior of this genotype.

The next regeneration phase concerning salt stress applications differed from the other works cited in the literature; indeed, most of them were made on somatic tissue cultures, with differentiation and high efficiency of embryo production, for durum wheat (Aktbar et al., 2012) selecting single-gene traits. The progressive introduction of salt, proposed here, up to sub-lethal levels might well produce several other kinds of tolerances, implying multiple hereditary traits, differing from monogenic ones easy to be reverted (Sibi and Fakiri, 2000). So, here the stressing NaCl agent was introduced upon each transfer, according to increasing concentrations, in the regenerating medium, versus control, i.e., salt free culture.

Concerning the albinos plants observed here, it should be emphasized that in spring barley, albinism is the essential problem for this species facing androgenesis (Caredda et al., 2004; Castillo et al., 2000; Hunter, 1998). Because the high frequency of albinos developed on either control or salt stress medium (150 out of the 170 totally regenerative plants), and often reaching 100%, for the three varieties, every albino or green plants were counted and the data reported as ratios values in Table 2, or as numbers of regenerative plants in Table 3. Thus for the control path, only Arig (Ar RS0) with 37.5% of albinism, and Tamelalt (Tm RS1) with 50%, gave respectively 5 and 13 green plants, while every Asni regenerated plants were albino.

After in vitro culture on salt stress medium, all the regeneration rates appeared to be lower than those of the control path. Meanwhile, green plants results seem to depend on the limitation expressed by albinism. Then, with rates of 100% of albinos, among the 11 plants generated by Arig and Tamelalt, on until 10 g L⁻¹ of salt in the medium, none were green. Furthermore, in spite of the regenerative plants obtained with Asni on salt concentrations values, up to 12 g L⁻¹, the only efficient green plants were obtained on 5 g L⁻¹ of salt medium, for As RS1 and As RS2, with rates of 5% and 4%, respectively, among which albinos rates were a little lower than 100%, thus resulting in one chlorophyllian plant for each case. However, even if high sodium chloride concentrations showed an adverse effect on regeneration, it should be pointed out that in previous work, a fertile green regenerated plant of Tamelalt has been obtained with 15 g L⁻¹ salt concentration in the medium (Sibi and Fakiri, 2000) suggesting that high concentrations values can be successfully applied.

After the three varieties did follow the successive androgenesis steps, the genotypic effects were clearly revealed at the different levels of the experiment, this being consistent with many other works (Bennaceur, 2000; El-Goumi, 2015; El-Haddourey et al., 2000; Kährizi et al., 2011) and remaining among the important and critical phases in the development of plastids during microspore embryogenesis in barley. Protoplasma, 208(1–4), 248–256. https://doi.org/10.1007/BF01279096

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These experiments show promising results for further utilization of androgenesis and gynogenesis, combined with salt stress, in order to provide improved elite lines (germplasm) for barley breeding in Morocco.

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