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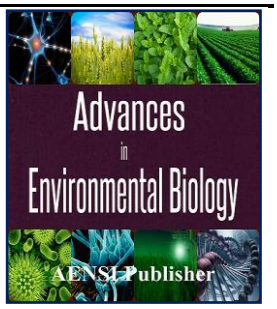

Optimization of Somatic Organogenesis *in Vitro* of *Solanum Tuberosum* in Salt Stress Condition

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ABSTRACT

The induction of somaclonal variant resistant at salt stress *in vitro* requires a selection of resistant cell lines. For better resistance to abiotic stress, the Induction of stem and leaf callus of *Solanum tuberosum* L., at Spunta and Kondor variety was initiated in MS medium with 0.5 mg.l^{-1} ANA, and 0.5 mg.l^{-1} 2.4-D. Their exposure to different concentrations of sodium chloride (from1 to 14 g.l^{-1}), showed the direct effect of this stress on the texture, growth and somatic callus organogenesis. Determination of salt tolerance stress callus performed until the second generation of transplanting. The application of concentration superior to 5 g.l^{-1} of NaCl induces a non-organogenesis texture (friable callus, smooth and necrotic), it's the lethal concentrations. Microscopic calluses observations, followed by statistical analysis, have outlined the tolerance threshold of 4 g.l^{-1} in the two explants. In terms of tolerance, there is no difference between stem callus and leaf callus, but only at the level of growth, which is more favorable in stem callus. These are proving more of somatic organogenesis in the presence of salt stress. The measure of stress tolerance index shows the dominance of meristem callus on leaf callus in terms of tolerance .The Induction of somatic meristems is optimized for G4 generation in stem calluses and G3 in leaf calluses; and disappear from G6 in leaf calluses to G7 in stem calluses. The variety of factors has a significant effect on the intensity of organogenic callus.

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INTRODUCTION

Potato is a strategic culture in Maghreb. With the intensification, this culture is affected by many diseases, and soil salinity. In Algeria Over 80% of land is affected by salinization problem, due to irrigation with brackish water, or by excessifs integrations of Fertiliser [15]. In western Algeria, salinity has reached a high enough level to affect agricultural yields [6]. This Salt excess results of increasing of soil solution's osmotic pressure and, thereafter, create a major limiting factor of production [8; 3]. Thus, tolerance of crops is limited, given the complexity of involved mechanisms in tolerance to salt plants [11].

This type of stress affects the yield of many crops such as potatoes. Plant's exposure to salt stress causes the plant's physiological disorders, resulting in the reduction of tubers and caliber's number. Its tolerance is determined by F.A.O [5] at 1.28 g.l^{-1} of NaCl [3]. The introduction of tolerant plants to salinity is one of the most recommended technics for valuing affected soils by these phenomena [17]. Biotechnology provides an easy access to innovative genetic variation to support breeding. Tissue culture is the most potent part of biotechnology and is mainly employed in *Solanum* germplasm maintenance, besides the production of

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somaclonal variants and the development of transgenic plants [2]. *In vitro* selection lines can be applied in that event of improving tolerance to NaCl by callogenesis. In this context, studies were done to induce calluses tolerant potato by Akossiwoa Quashie *et al.* [1], and durum wheat by Koutoua *et al.* [13]. The various hormonal concentrations/combinations and culturing duration were the main factors contributing the somaclonal genetic variability in micropropagated potato callus [7 ; 16]. Nevertheless, somaclonal variation is undesirable in some of the tissue culture based techniques such as *in vitro* propagation and genetic transformation where genetic stability of regenerable culture is required. But, somaclonal variation also could provide a natural source of variability. Furthermore, a particular importance should be reserved at the choice of explant *in vitro*. This allows for better prevention applications and a relative gain in time and space for selecting tolerant species. The delimitation of *in vitro* callus of tolerance depends on several parameters, such as their ability to regenerate according of explant to the choice [7 ; 16].

Therefore, the present study assess tolerance at salt stress depending on the choice of the explant (stems and leaves), and evaluate the effect of the junction of the salt stress and transplanting Generations factor on induction of somatic meristems tolerant at salt stress.

MATERIALS AND METHODS

Callus's induction:

The explants of 4 to 5 mm of stems and leaves *in vitro S.tuberosum* ; Spunta and Kondor variety ; have been used for exhibiting callus induction. The selected culture medium is MSC [7], composed of MS (Murashige and Skoog), enriched by vitamins of Morel and Wetmor, added with 0.5 $mg.l^{-1}$ of BAP (6-benzyl aminopurine), and 0.5 $mg.l^{-1}$ of 2.4-D (2,4-dichlorophenoxyacetic acid). The explants were incubated at 25±1°C and 12 hours light intensity of 15000 Lux [7].

Salinity Test:

Induced callus are subject to salt stress by subculturing, at the rate of 30 calluses per test, on the MSR codified culture medium: MSC medium supplemented with the concentrations of NaCl: T0 (0 $g.l^{-1}$) to T14 (14 $g.l^{-1}$). Tolerant callus selected after two subcultures in saline, are oriented in somatic organogenesis.

Induction and growth of tolerant meristems:

According to Ghomari *et al.* [7], MSC environment is an inducer middle of somatic meristems. Therefore, the MSR environment is used, also, for somatic organogenesis tolerant callus.

Evaluation of salt stress tolerance:

Binocular microscope observations are made, followed by the evaluation of callus growth rate and somatic organogenesis. The degree of salt tolerance is estimated by applying a Principal Component Analysis (PCA) with the STATISTICA software version 6, of the two generations of callus in saline. A measure of the Index tolerance to salt stress callus (ITS) is established by applying the rule :

$$\frac{\text{Middeweights callus witnesses X Middeweights of stress tolerant callus}}{\text{(middleweight of all calluses)}}$$

3. Results:

Effect of salinity on the callus's growth:

The use of the MSC culture medium promoted callogenesis explants of both varieties with a compact texture, and globular greenish. The integration of NaCl to the culture medium at increasing concentrations favored the relative change in the texture of a smooth brownish and friable callus. For the first generation (G1), the T1 to T5 rate had submitted a similar texture to the check (Figure 1), with a reduction in the massive volume nearly 6% in Spunta and 10% in Kondor. From T6 this effect was accentuated by 40% in Spunta and 48% in Kondor. Again, the texture of the callus changes as a friable callus, smooth and necrotic (lethal concentrations). At T8, a significant drop was triggered massive volume, reaching nearly 80% in both varieties. Transplanting tolerant calluses in generation G2 did not influence the change of texture.

a: Organogenic callus texture (compact, globular greenish); b: non organogenesis hypertrophic callus (friable and white smooth).

Statistical analysis was made by PCR of the callus growth according to their sensitivity to salt stress and this for the two calluses generations (G1 and G2). The results of the G1 (Figure 2A) indicates the presence of a significant correlation between some individuals, classifying them into four groups:

- Group 1: (callus T0, T1, T2 and T3) of very low sensitivity to salt stress, being in the negative part of the axis 2. The callus T1, T2 and T3 were well developed compared to T0 ;
- Group 2: (callus T4, T5, T6 and T7) low sensitivity to stress with its location in the positive part of the axis 2. A high correlation of T6 and T7 individuals was observed.

- Group 3: (callus T8 to T12) increasing sensitivity to the salinity of these individuals, with a high correlation between them. Calluses are low-growing, with necrosis.
- Group 4: (callus T13 and T14) high sensitivity to salt stress, characterized by an absence of callus's growth.

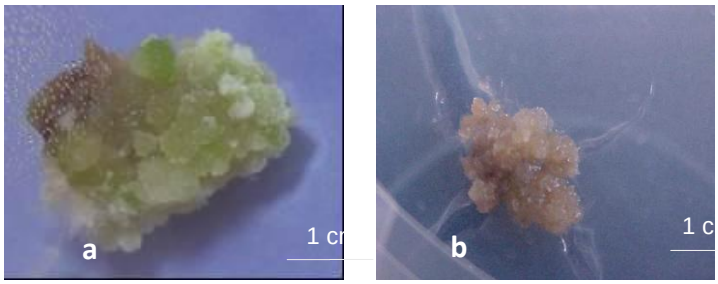
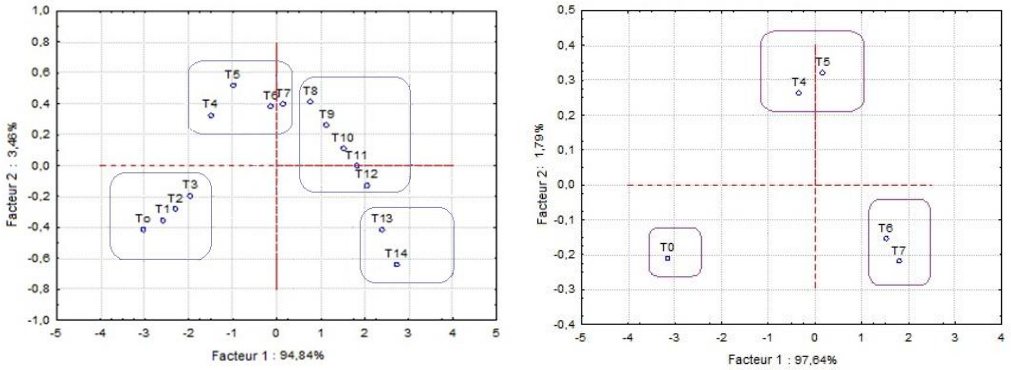


Fig. 1: Callus cauline Structure 0 mg.l^{-1} of NaCl (a) and the leaf callus 8 mg.l^{-1} of NaCl (b) of the Kondor variety, after 25 days of growth.



A B

Fig. 2: Screening of the callus's sensitivity G1 (A) and G2 (B) to salt stress on the factorial axis of the PCA.

A: Generation 1 of calluses exposed to salt stress in four groups (from T0 to T3: Individuals high tolerance of T4 to T7: Maximum tolerance for individuals, both groups of T8 to T14: non-tolerant individuals)

B: Generation 2 of calluses G1 exposed to salt stress in three groups (T0: Individuals witnesses; T4 and T5: individual's tolerance; T6 and T7. Low tolerance of these two individuals' results of the threshold tolerance's studies is determined between T4 and T5.

Therefore, tolerant calluses in group 2 of G1 are transplanted. The results in Figure 2B, show a correlation of individuals to form three groups:
- Group 1 check (callus of T0);
- Group 2 with tolerant calluses (T4 and T5 callus) representing the tolerance to salt stress of both varieties;
- Group 3 (callus T6 and T7) with higher sensitivity to salt stress than group 2.

The calculation of the tolerance index to salinity (Table 1) (ITS) callus G2 allows to define the tolerance for both explants of the two varieties.

Tolerance T4 is noticed among Kondor stem callus and Spunta leaf callus. But at T5, tolerance was observed in Spunta calluses stem and Kondor leaf callus. The junction of these results with the observations prompt us to define the tolerance for T4 (4 g.l^{-1}) in both varieties.

Table 1: Measurement of the salt stress tolerance index (STI) on the calluses of both varieties.

salinity test	Kondor Stem Callus	Kondor Leaf Callus	Spunta Stem Callus	Spunta Leaf Callus
T4	2.32	1.94	1.77	1.78
T5	1.65	1.89	1.72	1.55
T6	1.14	1.15	0.99	1.11
T7	1.03	1.00	0.95	0.92

The high value of SIT of stem callus shows that is the calluses with more tolerance than leaf callus. Thus, the Kondor variety has a higher tolerance than the Spunta variety.

Tolerant meristem induction:

According to microscopic observations of T0 callus in regeneration phase (Figure 3), the new formation of somatic meristem is more favorable in callus stem than leaf callus. This was expressed in 76 % of callus stem against 66 % in Kondor leaf callus; and in the same order of succession, about 33 % and 14 % in Spunta. A good evolution of cell clusters was observed, thus forming the primary meristem (3-a) after 60 days in Kondor and 65 days in Spunta. The first primordial leaves develop in 10 days after, leading to the formation of secondary somatic axillary meristem (Figure 3).

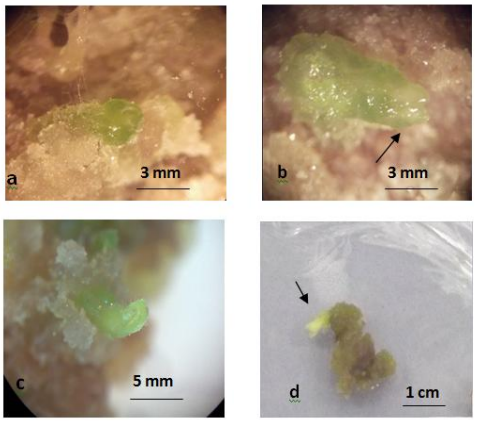


Fig. 3: Neoformation phases of Somatic meristem in stem callus of Spunta variety a: primary axillary meristem to callus in 65 days of growth; b: formation of the first leaf primordial after 67 days of growth; c: secondary axillary meristem after 75 days of growth; d: somatic bud meristem after 83 days of growth.

In the presence of salt stress tolerant Kondor callus variety are revealed to be more organogenic than Spunta variety. T4 and T5 concentrations presented an inhibition of somatic organogenesis callus in G1 and G2 generations. Only from G3 where the meristematic neoformation is observable in both varieties. At the G4 generation, the number of meristems decreased by over 30% in leaf callus and increased to almost 70 % in callus stem. This organogenic neoformation disappear from G6 in leaf callus and from G7 to stem calluses.

4. Discussion:

In this study, the results obtained in both varieties showed a low sensitivity to salt stress tests for T1, T2 and T3. Although the statistical study by CPA of the first callus generation has indicated a wide tolerance for NaCl concentrations ranging from 4 $mg.l^{-1}$ to 7 $mg.l^{-1}$, a considerable decrease of massif exhibiting callus is recorded from T6. Changing the texture of callus expresses the non-tolerance callus to the high concentrations of NaCl. Hussein *et al.* [10] explain that in several plant species, damages caused by salt stress commonly manifested by a sequence of morphological and physiological changes. The high salt concentrations cause cellular and ionic imbalance and plants toxicity; which can affect some vital metabolic processes [4; 14], such as reducing growth, and the necrosis of sensitive calluses.

Transplanting of T4 to T7 callus, tolerant in generation G1, elucidated the results in G2. Microscopic observations, added to statistical analysis confirmed that the organogenic calluses present a T4 threshold tolerance. The calculation of stress tolerance index varieties Spunta and Kondor *in vitro* allowed to say that Kondor callus, although less developed, are more tolerant. Stem calluses of both varieties; come with a greater tolerance than the leaf calluses. Koutoua *et al.* [13] comment that the leaf callus in the normal state, are richer than the stem callus K^+ and that the presence of NaCl in the medium culture, lowers the levels of K^+ of the two types of callus, especially in concentration of 6 $g.l^{-1}$. They also explain that the levels of Ca_2^+ leaf calluses are higher than those of stem calluses. Therefore, we can say that the leaf callus should be difficult to regulate cell osmotic pressure, which promotes greater sensitivity.

According to the observations, the induction of somatic organogenesis tolerant callus is more favorable in stem callus than leaf callus. Thilaga *et al.* [18] affirms that the segments of internodes have better capacity for regeneration on a saline environment at leaf fragments. Increasing concentrations of NaCl had a reducing effect, even though with inhibitor regeneration. Indeed, in 5 $g.l^{-1}$, organogenesis was strongly affected by abortion bud and shoot formation of a very reduced growth. The addition of NaCl to the culture media decreased the osmotic potential of the media inducing salinity stress that adversely affected the plants growth of potato cultivars [12].

The number of transplant tolerant callus has strongly influenced the meristem induction. Similar observations were reported by Anwar *et al.* [2] on Desiree variety using the two types of explants. In our study, this is consistent with the work of It was until the third generation that regeneration has been triggered, to be

more favorable in the fourth generation. This could be explained by the cells adaptability in saline medium, allowing thereafter their organogenesis. According to Hanana *et al*. [9], the problem of salinity is multiple : in addition to salt stress, ion toxicity (Na^+ and Cl^-), plants have difficulty absorbing water from medium because of its elevated osmotic pressure ; which leads to water stress and thus complicates and impairs their physiological state in an exponential way. Consequently, cells try to adjust their water potential by ion homeostasis regulation via vacuolar compartmentation and/or extrusion out of the cell of the toxic ions. this requires an adjustment period to stress to continue the physiological actions. El Yacoubi and Rochdi [4] explain that tolerance callus is linked with the regulatory capacity and ionic separation. Under stress, vacuolar captivity of salt ions is counterbalanced by the synthesis and accumulation of organic solutes (proline and soluble sugars). This promotes thereafter, the osmotic adjustment between the vacuolar compartment and the cytosol of cells tolerant callus.

To assess optimum *in vitro* salt stress tolerance, it is not enough to achieve a good callogenesis, but to achieve optimal meristematic induction under stress conditions. Therefore, the T4 concentration is considered optimum *in vitro S.tuberosum* tolerance to salt stress.

5. Conclusion:

The *Solanum tuberosum* L., determination of salt stress tolerance callus only performed until the second generation of transplanting. Cell physiology changes with the persistence of this type of stress. The concentration 4 $g.l^{-1}$ proves the tolerance level of calluses *S.tuberosum*. Sensitivity to salt stress is expressed by the decrease in cell growth, which results in the reduction of exhibiting callus mass. For lethal concentrations, callus texture changes in callus and friable necrotic smooth. In terms of tolerance, there is no difference between stem callus and leaf callus, but only at the level of growth, which is more favorable in stem calluses. These are proving more capable of somatic organogenesis in absence as in the presence of salt stress.

The addition of NaCl to the culture medium has hampered the induction of somatic meristems, which was triggered only from the generation G3. This induction is very related to the number of planting, with an optimum G4 registered under stress in stem calluses, and G2 in the absence of stress. This new organogenic formation vanishes from G6 in leaf callus and from G7 in stem calluses. The variety of factors has a significant effect on the intensity of organogenic callus. In this context the variety Kondor is more organogenesis *in vitro* in saline environment than Spunta.

However, the production of potato plants, tolerant to salt stress from selected cell lines, can have a significant economic interest, especially for coastal areas and semi-arid of North African regions.

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