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Effect of exogenously produced proline on callogene cultures of *citrus aurantium*

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Abstract
To verify the role of certain biochemical substances such as proline in regulating the biochemical activity of citrus fruits, calli of *citrus aurantium* were grown under Murashig and tucker (MT) culture media containing 3, 6, 8, 9 $g.l^{-1}$ of NaCl and 0.5, 25, 50 mg/l of proline. Results showed that addition of exogenous proline to the culture medium neutralized the inhibitory effects of NaCl and increased the induction and growth rate of the calli, particularly at high NaCl levels. These results suggest the potential role of proline in protecting these fruit species, such as *Citrus aurantium*, under abiotic stress.

Keywords: citrus rootstock, callogenesis, NaCl, exogenous proline, *citrus aurantium*

1. Introduction
In citrus fruit, *Citrus aurantium* calli was used by several researchers to produce salinity tolerant seedlings [2, 8]. In addition, plants subjected to salt stress are known by the accumulation of many high concentration organic compounds, such as proline. However, some studies have shown that addition of exogenous proline to saline stressed calli culture media increases salinity tolerance in tomatoes [8]. There are no *in vitro* studies on the effect of application of exogenous proline on the salinity tolerance of citrus rootstocks. We propose an analysis of the metabolic behavior at the cellular level and of the calli reaction of local *Citrus aurantium* put in a culture medium enriched with exogenous proline.

2. Materials and methods
2.1 Experimental conditions
Calli were obtained from the in vitro culture of the excised embryos of local disinfected seeds of Citrus aurantium. The concentration of 2,4-D and BAP used was 1/0.5 $mg.l^{-1}$ for the maintenance of the calli.

2.2 Interaction between NaCl and exogenous proline on the fresh weight of calli
The primary calli of Citrus aurantium, obtained after induction, were then divided into fragments of initial mass of 100 mg of fresh material (FM), and incubated in the MT medium with three doses of NaCl (0.3, 6.8 g/l). Their growth was studied according to the proline addition to the culture medium (0, 5, 25, and 50 mg/l). The calli PF was determined after 3, 6 and 9 weeks.

2.3 Interaction between NaCl and exogenous proline on endogenous proline
After nine weeks, the endogenous proline concentration was determined using the modified ninhydrin method [4]. The reaction of stressed calli with NaCl in a medium enriched or not with exogenous proline was examined through the kineticsof free proline accumulation in the calli. The contents were expressed in µg of proline per g of FM after reading the optical density (D.O.) at 515 nm.

2.4 Statistical analysis
The results were analyzed by ANOVA, and the values were compared by LSD (Fisher's Minimal Significant Difference Test).

3. Results and discussion
Results showed that addition of exogenous proline to the culture medium neutralized the inhibitory effects of the salinity. This addition made it possible to increase the endogenous concentration of proline and soluble sugars and to promote the growth of the calli (Fig. 1, 2). Furthermore, the behavior of stressed *Citrus aurantium* calli in a medium supplemented with exogenous proline showed a variability in the accumulation of free proline according to the NaCl dose. Many studies reported that proline accumulates in plant cells under adverse conditions [18], which reflects the character of resistance to stress [11]. In sensitive plants, however, the presence of this amino acid is lessened [6, 3]. According to Feitosa *et al.* (2001)[10] and Meloni *et al.* (2004) [15], the role attributed to proline in plant responses to stress is sometimes controversial; for [17], its accumulation contributes to the development of this resistance by maintaining cell turgescence in many species, created by the osmotic adjustment for which proline is responsible. The mechanism of proline accumulation makes it possible to think of the presence of plant resistance sites to the stress. Our results showed that addition of proline to the control culture medium increased greatly the fresh weight of calli (Fig. 1). this increase in FP is due to the fact that proline is a source of nitrogen which is a

respiratory substrate that generates ATP and increases the process of cell division, as well as its osmotic role [1, 12]. Figure 1 showed also that there was a significant interaction between NaCl and proline. The inhibitory effect of NaCl on the fresh weight of calli was reduced by the addition of proline, in particular at 5 mg/l. Indeed, addition of 5 mg/l of proline in saline culture media caused a significant accumulation of free proline in the *Citrus aurantium* calli. On the other hand, at a dose of 25 and 50 mg/l of the exogenous proline, it was found that accumulation of the endogenous proline decreased in the calli maintained on these media. This slowing of the free proline accumulation in these calli, presupposes an inhibition of its precursor [19] or a rapid activity of proline dehydrogenase, implicated in the degradation of the amino acid [16], probably related to contribution of proline to this concentration threshold (50 mg) creating an antagonistic effect on salinity as suggested by Heyser *et al.* (1989a) [13] and confirmed by Belkhoudja *et al.* (2007) [5].

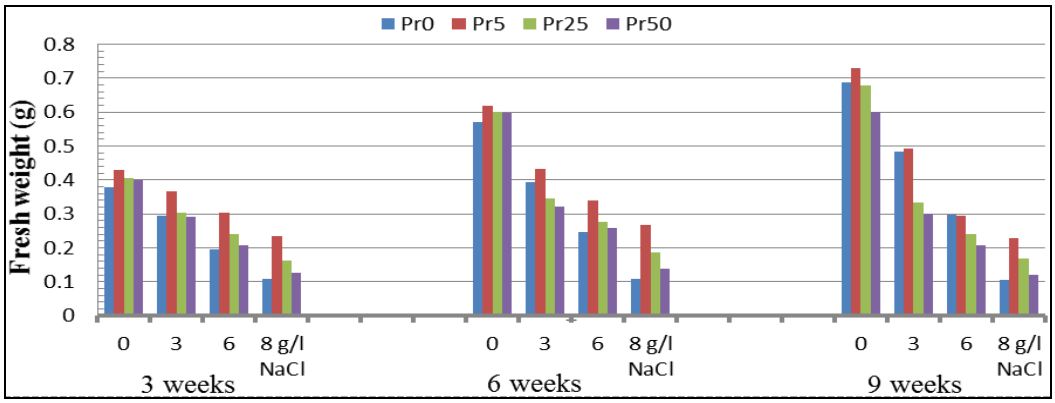

Fig 1: Effect of NaCl (g/l), proline (mg/l) and their interaction on FP (g) of Citrus aurantium calli after 3, 6 and 9 weeks of culture. Pr: proline.

Figure 2 showed the effect of NaCl, proline and their interaction on proline levels in calli. After three weeks of stress, free proline analyzed in calli varied according to saline treatment. In presence of 5 $g.l^{-1}$of NaCl, free proline was accumulated significantly in the calli, increasing substantially with the exogenous proline concentration of the culture medium. Exogenous proline resulted in a significant increase in the concentration of endogenous proline, it was probably due to the absorption of proline by calli tissues. Similar results have been found on other calli cultures such as date palm [19]. Therefore, the accumulation of proline under the effect of NaCl at 3 g/l became more important after addition of exogenous proline, this was due to the synergy between proline and NaCl. From 6 g/l of NaCl, the dose of endogenous proline increased significantly even at 8 g/l of NaCl, which proves that the cells are alive despite the stoppage of growth proved by the decrease of the FP.

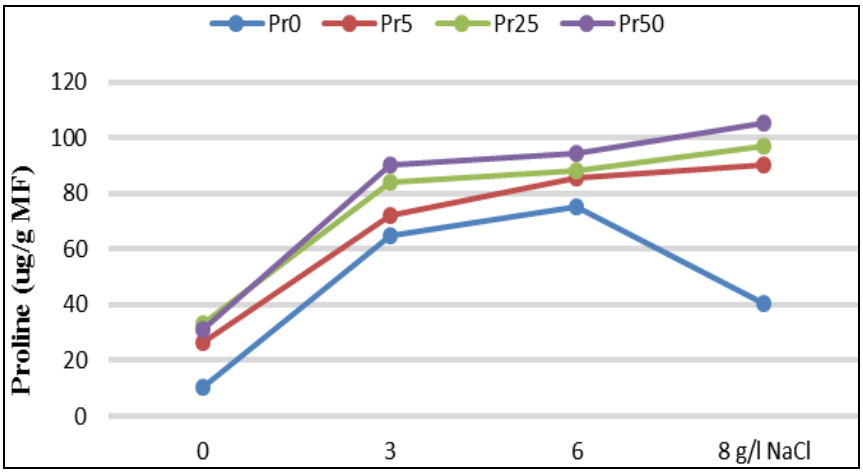

Fig 2: Free proline levels (µg/gPF) of 3-week-old Citrus aurantium calli and stressed with NaCl in an enriched MT medium at 0, 5, 25 and 50 mg/l exogenous proline.Pr: proline.

Generally, the role attributed to proline in plant responses to stress, remains controversial; for Qian *et al.* (2001) [17], its accumulation contributes to the acquisition of this resistance through the osmotic adjustment of which the proline is responsible. It could also intervene in the regulation of pH [7] or a reduced carbon and nitrogen reserve used by the plant after the stress period [14].

4. Conclusion

Results showed that proline addition to the medium containing

sodium chloride counteracted the NaCl inhibitory effect and increased induction and growth rate of calli, particularly at high NaCl levels. After three weeks of stress, the free proline analyzed in calli varied according to saline treatment. In presence of 3 $g.l^{-1}$of NaCl, the free proline accumulated significantly in calli, increasing with the exogenous proline concentration of the culture medium. Our results showed that callogene cultures were able to grow actively in saline and proline-rich environments. Similarly, calli growth was inversely correlated with salt concentration with its complete inhibition at 8 $g.l^{-1}$of NaCl. The addition of proline to the culture medium stimulated the growth of the cultures, even at 8 $g.l^{-1}$of NaCl. These results suggest the potential role of proline in protecting these fruit species, such as *Citrus aurantium*, under abiotic stress.

Conflict of interest

The authors declare that there is no conflict of interest.

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