Assessment of *in vitro* growth of apical stem sections and adventitious organogenesis to evaluate salinity tolerance in cultivated tomato

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Abstract

The possible use of *in vitro* shoot morphogenesis and shoot apex culture to evaluate salt tolerance in cultivated tomato (*Lycopersicon esculentum* Mill.) has been analyzed, using two cultivars with similar salt tolerance, Pera and Hellfrucht frühstamm (HF). The effect of salt on shoot regeneration was studied by culturing leaf explants on media supplemented with 0, 43, 86, 129 and 172 mM NaCl. The presence of NaCl in the regeneration media at 86 mM strongly inhibited shoot regeneration in the cultivar HF, but not in Pera. However, the substitution of NaCl by mannitol, maintaining the same water potential in the culture media, decreased the regeneration percentage in Pera but did not affect HF. Shoot apices of both cultivars were also subcultured at 6-week intervals, for 4 subcultures, at the same NaCl concentrations as used in the previous experiment, and the shoot growth, leaf and root number, rooted shoot and shoot necrosis were recorded at the end of each subculture. Root formation was the parameter most affected by salt in both cultivars, Pera being more sensitive than HF. The substitution of NaCl by mannitol significantly increased the percentage of rooted shoots in Pera after four subcultures, and slightly decreased this percentage in HF. Shoot necrosis was only observed in the last subculture at NaCl higher than 86 mM, the percentage of necrotic shoots being higher in Pera than in HF (75% and 45%, respectively). The lack of agreement between the results obtained with the *in vitro* tests, e.g., adventitious shoot formation and growth of apical stem sections, suggests that this approach may not be a reliable tool to evaluate salt tolerance in cultivated tomato.

Abbreviations: BA– benzyladenine; cvs – cultivars; HF – Hellfrucht frühstamm; IAA – indole acetic acid; LSD– least significant difference; MS–Murashige and Skoog.

Key words: adventitious organ formation, *in vitro* culture, *Lycopersicon esculentum*, mannitol, sodium chloride

Introduction

The excess of salt in the soil or in the irrigation water is one of the biggest problems in agriculture since almost all cultivated plants are sensitive to it. According to Epstein (1976), salinity is not only a problem in arid and semi-arid regions, but it also occurs in fertile and productive soils where overexplotation of water reservoirs, lack of rain, and use of large amounts of fertilizers have caused salt accumulation. Thus, selection of salt tolerant lines is one of the most important challenges in plant biology. One of the problems that appears when evaluating tolerance to a complex stress such as salinity is the labor intensive process required to screen thousands of plants and the lack of reliable salt stress markers (Cruz et al., 1990; Saranga et al., 1993; Cano et al.,

1996). These difficulties have been the cause that, in certain species such as tomato, few practical results have been obtained from traditional breeding programs. *In vitro* plant tissue culture has been proposed as an useful, quick and economical tool to evaluate salt tolerance. Although a lack of con cordance between growth of callus under salt stress and growth at the whole plant level has been observed in several species (Tal, 1984; McCoy, 1987), in others such as tomato, positive correlations have been found (Tal et al., 1978; Pérez-Alfocea et al., 1994; Cano et al., 1996). However, use of *in vitro* culture presents numerous disadvantages, such as somaclonal variation, culture medium and explant source effects (García-Reina et al., 1988) and, mainly, the lack of the whole plant integrity that exclude crucial mechanisms of salt resistance like ion exclusion. To avoid these problems, and as an alternative to the callus growth approach, several authors have evaluated the *in vitro* culture of shoot apices or buds under salinity conditions (Martínez et al., 1996; Cano et al., 1998). In both reports, rooting and root growth were the processes most affected by salt, and they were also positively correlated with the salt tolerance at the whole plant level, when wild and cultivated species were compared. The aim of the present work was to evaluate different *in vitro* tissue culture approaches to test salinity tolerance in tomato. Thus, shoot organogenesis from leaf explants and shoot apex growth in the presence of different salt concentrations was analyzed, using two tomato cultivars that displayed a similar salt tolerance.

Materials and methods

Plant material

Two tomato (*Lycopersicon esculentum* Mill.) cultivars, Pera and Hellfrucht frühstamm (HF), with similar salt tolerance (González-Fernández, 1996) were used. In order to establish a stock of plants growing *in vitro*, seeds were sterilized in a sodium hypochlorite solution (0.5%), with Tween 20 (2 drops/100 ml) for 15 min, and were rinsed 3 times with sterile water. *In vitro* germination was accomplished in test tubes with liquid medium, using a paper bridge support. Culture medium included the White's salts and vitamins (White, 1963), with Fe-EDTA at 0.3x the level used in the MS formulation (Murashige and Skoog, 1962), sucrose 20 g l^{-1} and inositol 100 mg l^{-1}. The pH was adjusted to 5.7 and the medium was autoclaved for 15 min at 121 ºC and 1.05 Kg cm^{-2}. Twenty-five ml were used in 25 x 150 mm test tubes. Two seeds were placed in each test tube and incubated in the dark until roots appeared. Afterwards, they were transferred to light (40 µmol m^{-2} s^{-1}, provided by Gro-lux lamps Sylvania, Germany), under a 16-h photoperiod, with a temperature regime of 25±1 ºC. When seedlings had reached 6–8 cm, microcuttings 2–3 cm in length were taken, grown and rooted in MS medium solidified with Bacto-agar (Difco) at 8 g l^{-1} to establish a stock of plants growing *in vitro*.

Culture medium and incubation conditions

The basal medium included the MS formulation (Murashige and Skoog, 1962), sucrose 3% (w/v), i-inositol 100 mg l^{-1}, the B5 vitamins (Gamborg et al., 1968) and Bacto-agar Difco, 0.8% (w/v). Growth regulators, sodium chloride and mannitol were incorporated into the basal medium, as indicated in each particular case. All experiments were carried out under light (40 µmol m^{-2} s^{-1}, 16 h). Culture conditions were the same as specified for *in vitro* grown seedlings.

Effect of salinity on in vitro growth of apical stem sections and shoot regeneration from leaf disks

The effects of sodium chloride (0, 43, 86, 129 and 172 mM) on growth of apical buds and shoot regeneration from leaf explants of both genotypes were studied. The first experiment was initiated using 1.5 cm long apical shoots obtained from an in vitro stock proliferating in basal medium. Apical shoots were cultured in test tubes containing 20 ml of the basal medium supplemented with different NaCl concentrations, through 4 subcultures of 6-week intervals. For subculturing,

the apical part of the shoot (1.5 cm long) was detached and cultured onto fresh medium. In the second experiment, the effect of NaCl treatment on shoot regeneration was evaluated. Leaf sections (5 x 5 mm) from in vitro rooted microcuttings were used as explants. Optimal culture conditions for shoot regeneration for both cultivars had been previously established (Sancho-Carrascosa, 1994), being 2 weeks at 17.8 mM BA/5.7 mM IAA and subsequent transfer to 1.3 mM BA, for cv. Pera; 4 weeks at 17.8 mM BA/5.7 mM IAA and subsequent transfer to 1.3 mMBA for cv. HF. After 12 weeks of culture, shoot regeneration percentages in the absence of salt were close to 90% in both cases. In both experiments, to determine if the differences on shoot apex growth or shoot regeneration capacity found between both cultivars were due to the toxic or osmotic effect of the NaCl in the medium, additional experiments were performed where mannitol at concentrations of 86, 172 and 256 mM was added to the medium to reach water potentials similar to those yielded by NaCl at 43, 86 and 172 mM, respectively. Data taken and statistical analysis The number of explants used in each treatment was in the range 15–20. In the experiments where development of apical shoots was evaluated, shoot length, number of roots and leaves, presence/absence of flower buds, percentage of necrotic shoots and shoot quality were recorded. In regeneration experiments from leaf disks, the amount of callus, number of shoots per regenerating explant, number of roots and the aspect of the cultures were evaluated after 6 and 12 weeks. Data were analyzed by One Way Anova and the LSD test was performed ($p \geq 0.05$) to detect differences among means.

Results

Effect of sodium chloride on shoot apex development

Shoot apices from both cultivars were grown for 4 subcultures at different sodium chloride concentrations. The results obtained after the first and fourth subculture are shown in Figures 1 and 2. Shoot length, number of leaves, rooted shoots and number of roots were reduced by salinity. Root development was the process most drastically affected. The main differences between cvs were found in this parameter. The percentage of rooted shoots was not decreased by salinity in the cv. HF after the first subculture (Figure 2), but Pera showed a drastic decrease in rooted shoots at 172 mM NaCl. After 4 subcultures, virtually no roots appeared at NaCl concentrations higher than 86 mM in both cvs, but the percentage of rooted apices from HF was higher than the observed in Pera. By contrast, the relative number of roots per shoot at the first subculture was slightly higher in Pera at NaCl concentrations up to 129 mM, although no differences were found after 4 subcultures (Figure 2). Shoot necrosis was only observed in the last subculture at NaCl concentrations higher than 86 mM. At the highest NaCl concentration, 172 mM, the percentage of necrotic shoots was lower in HF than in Pera (45% vs 75%).

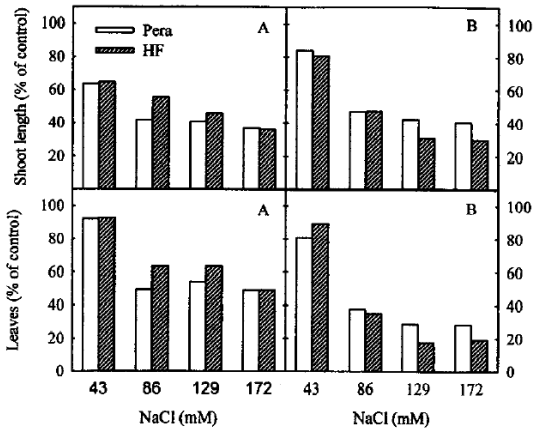

Figure 1. Effect of NaCl concentrations on shoot length and number of leaves of tomato shoot apices, cultivars Pera and HF, after one (*A*) and four (*B*) subcultures. Data are expressed as the percentage of control treatment without NaCl.

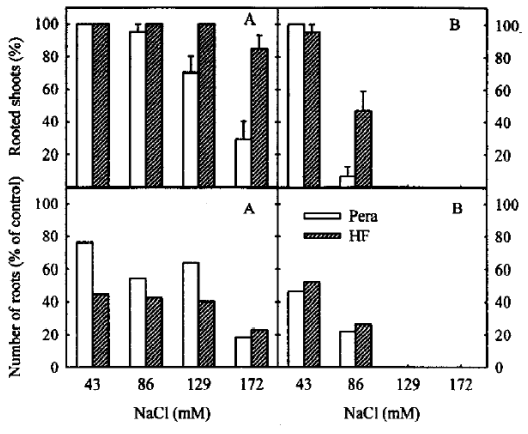

Figure 2. Effect of NaCl concentrations on the percentage of rooted shoots and number of roots of tomato apical stem sections, cultivars Pera and HF, after one (*A*) and four (*B*) subcultures. Root number is expressed as the percentage of control treatment without NaCl. Bars indicate the SE.

To determine if the lack of rooting was due to the osmotic effect of high NaCl concentrations, mannitol was added instead of NaCl to the medium at concentrations of 86 and 172 mM. These mannitol concentrations reduced the water potential of the MS medium to values similar to those obtained with NaCl at 43 and 86 mM, –0.57 and –0.75 MPa, respectively. In the first subculture, the response of apices cultured in mannitol was similar to the response obtained in NaCl at both water potentials (results not shown). However, after 4 subcultures in mannitol, the percentage of rooted shoots in cv. Pera at the lowest water potential was significantly higher than the percentage obtained in NaCl at the same water potential (Table 1). The relative root number was also slightly higher in mannitol than in NaCl. An opposite behavior was observed in HF, where the percentage of rooted shoots and the relative root numberwere slightly lower in mannitol than in NaCl at –0.75 MPa. Interestingly, 22.2% of the HF shoots developed flower buds after 3–4 subcultures at 86 mMNaCl. This phenomenon was not observed in HF apices when mannitol at 172 mM instead of NaCl was added to the medium. Flower bud inductionwas not observed in the case of cv. Pera in any salt or mannitol treatment.

Table 1. Percentage of rooted shoots and root number (% of control treatment) in apical stem sections of Pera and HF cultivars grown in MS supplemented with NaCl or mannitol at different water potentials of the medium (–0.55 and –0.75 MPa). Data were taken after four subcultures

	Water potential	Pera		HF	
	(MPa)	NaCl	Mannitol	NaCl	Mannitol
Rooted shoots (%)	–0.55	100	90	95	100
	–0.75	6.6	40	44.4	29.4
Root number (% of control)	–0.55	46.6	54.3	52.2	26.1
	–0.75	22.2	32.6	37.8	19.4

Effect of sodium chloride on shoot regeneration

The effect of salinity on shoot regeneration from leaf explants can be observed in Figure 3. The main differences between cvs was observed at moderate NaCl concentration, 86 mM, where the cv. Pera regenerated at a similar rate to the control, but the cv. HF yielded a 73% reduction in shoot regeneration. These differences disappeared at 129 mM of NaCl (30% of explants with shoots in both cvs). At higher NaCl levels, the explants were necrotic and died after few weeks of culture. The number of regenerated shoots per explant was also reduced by salinity, as observed by the regeneration rates. The reduction in shoot regeneration was higher in the cv. HF (4.5 vs. 2.1 in the control and 129 mM NaCl, respectively) than in Pera (4.2 vs. 3.4 in control and 129 mM NaCl, respectively). Mean values of length, weight and number of leaves of the shoots regenerated in the presence of NaCl were similar to those of shoots regenerated without salt, both in Pera and HF (results not shown). Shoot organogenesis in leaf explants on medium supplemented with mannitol at 86, 172 and 256 mM was tested. Figure 4 shows the regeneration rates of Pera and HF explants at different water potentials when mannitol was added to the medium. In general, the response of both cultivars to mannitol was opposite to that found in the presence of NaCl. In the case of HF explants, the regeneration rates were not affected by the decrease in the water potential in the medium, when mannitol was added to it. However, the

percentage of explants with shoots decreased significantly in Pera as the mannitol concentration increased, and even at –0.94 MPa (256 mM of mannitol), none of the explants was able to regenerate shoots.

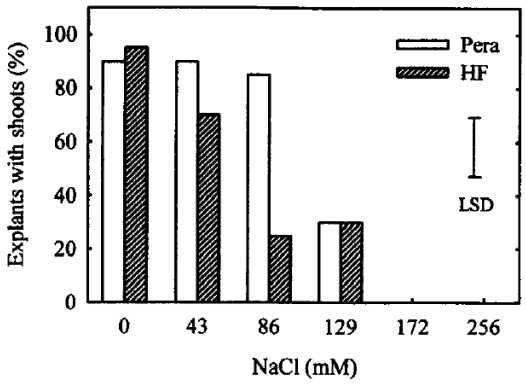

Figure 3. Effect of NaCl concentrations on shoot regeneration percentages of leaf disks explants of tomato cultivars Pera and HF, after 12 weeks of culture. Bar indicates the LSD at the 5% level.

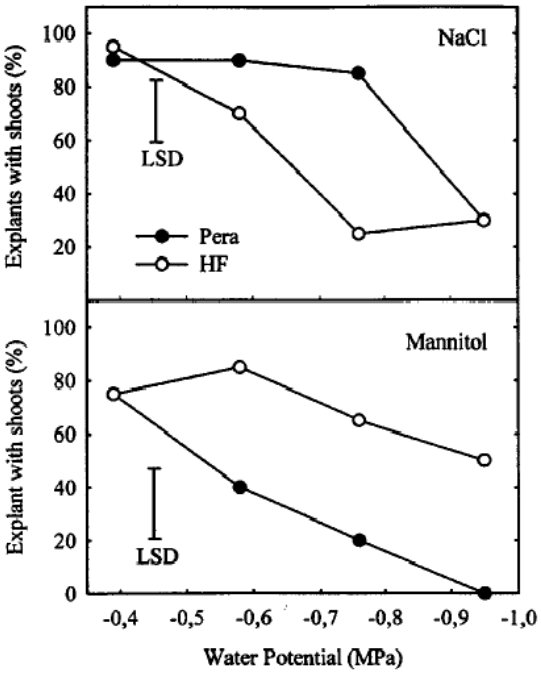

Figure 4. Shoot regeneration percentages of leaf disks of tomato cultivars Pera and HF after 12 weeks of culture at different water potentials produced by NaCl or mannitol added to the culture media. Bars represent the LSD at the 5% level.

Discussion

The two tomato cvs used in the present work displayed a similar salt sensitivity when evaluated at the whole plant level (González-Fernández, 1996). Both cvs showed a 90% reduction in fruit yield when the plants were watered with 30% sea water. In spite of these similarities, the response of the 2 cvs to the *in vitro* tests for salt tolerance evaluated in the present work, was completely different and contradictory. The results obtained with the shoot apex growth test showed that rooting was the parameter most affected by salt in both cvs, as previously observed by Morpurgo (1991) and Martínez et al. (1996) in potato and Cano et al. (1998) in tomato. Furthermore, the salt sensitivity of tomato apices increased with time in culture. The threshold NaCl concentration that significantly affected rooting varied among cultivars and subcultures. In the case of Pera, NaCl 172 mM significantly reduced rooting at the first subculture, while same effects were observed at 86 mM at the fourth subculture. By contrast, HF did not showed a reduction in rooting during the first subculture at any NaCl concentration but it decreased up to 50% after four subcultures at 86 mM NaCl. The decrease on rooting in Pera apical stem sections was mainly due to the toxic effect of Na and Cl ions since the substitution of salt for mannitol, maintaining the same water potential, only reduced the percentage of rooted shoots up to 40%, vs. the 6.6% obtained in the presence of salt at the fourth subculture. According to the results of this test, HF could be considered more salt tolerant than Pera. This last cv. was also used in a similar study by Cano et al. (1998). However, they found that no shoots developed roots after 32 days of culture at NaCl 105 mM. The better response obtained in our work could be related to the differences in the medium used to culture the apices. In their study, Cano et al. (1998) used MS medium at half strength and included IAA at 0.1 mg l□1, whereas we employed full strength MS without growth regulators. The effect of hormonal balance in the salt response of tomato callus has been previously reported (García-Reina et al., 1988).

On the other hand, and as observed for HF apical stem sections cultured in 86 mM NaCl, Liu and Li (1989) reported the *in vitro* formation of flower buds when culturing propagules of several tomato cvs at the same NaCl concentration. Sánchez-Díaz and Aguirreolea (1993) have pointed out the important role that water stress exerts on flower induction and differentiation processes. However, the absence of flowering of HF shoots cultured in mannitol, indicates an additional effect of sodium chloride on flower induction *in vitro* other than the osmotic one. The reason for the absence of flowering in Pera is unknown.

In relation to the regeneration experiment in the presence of salt, it is clear that Pera displayed a better response than HF, and the regeneration process of this cv. only decreased at NaCl concentrations higher than 86 mM. Regeneration capacity was completely inhibited in both cvs at NaCl higher than 129 mM, which is in accordance to the results of Kurtz (1981). Surprisingly, the response of both cvs to the osmotic stress induced by the presence of mannitol followed an opposite behavior and in contrast to the results obtained with the shoot apex culture experiment, HF was more affected by the toxic effect of NaCl than Pera in this experiment. This behavior has also been observed when growing leaf callus of both cultivars in the presence of salt (results not shown). The better response to high NaCl concentrations of Pera, at the cellular level, could be related to the use of Na or Cl ions as osmotic agents. In fact, Pérez-Alfocea et al. (1993) found that among 5 different tomato cvs, Pera accumulated more Na and Cl in the shoot than the other ones, a response typical of halophytic species. The decrease in the regeneration rates of Pera leaves in the presence of mannitol supports this hypothesis.

The lack of concordance between the results of the two *in vitro* tets of salt tolerance used in the present work are not unexpected, due to the complexity of this stress and the multiplicity of mechanisms that can be involved in salt tolerance. In this sense, Pérez-Alfocea et al. (1993) detected different salt stress responses among several tomato cvs, from an halophytic behavior, with Na and Cl accumulation in the shoot, to a glycophytic response, where translocation of Na and Cl to the shoot was restricted by the root. So, we think that this multiplicity of responses makes it difficult the use of *in vitro* culture tests to evaluate salinity tolerance in tomato.

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