PRELIMINARY RESULTS ON *in vitro* SELECTION FOR TOLERANCE TO CHLORIDE EXCESS IN AVOCADO

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SUMMARY

The objectives of this study were to determine the viability of *in vitro* culture of mature avocado (*Persea americana* Mill.) embryos as a strategy to select rootstocks for their tolerance to excess chloride, as well as to evaluate genotypic differences between embryos of the Mexican (var. *drymifolia*) and West Indian (var. *americana*) avocado races. Mature embryos excised from seeds of the two avocado races were cultured in Murashige and Skoog medium supplemented with 2 mg·liter^{-1} of glycine, 10 mg·liter^{-1} of myo-inositol, 4 mg·liter^{-1} of thiamine-HCl, 30 g·liter^{-1} of sucrose and 7.5 g·liter^{-1} of agar. Furthermore salts of NaCl, $CaCl_2 \cdot 2H_2O$ and the mixture of NaCl + $CaCl_2 \cdot 2H_2O$ (ratio 1:1) in concentrations from 0.1 to 1.0 %, at 0.1 % increments, were added. Excised embryos were incubated at 27 ± 1 ºC for 21 d in darkness followed by 65 d of 18 h day and 6 h night. Percentage of germination and survival were greater for the West Indian race (24 and 11 %, respectively) than for the Mexican race (21 and 6 %, respectively). In general, water potential as well as osmotic potential were reduced as the concentration of salts increased, while the turgor potential increased. The results suggest that the avocado has some degree of osmotic adjustment.

ADDITIONAL KEY WORDS: *Persea americana* Mill., osmotic adjustment, salinity, tissue culture.

RESULTADOS PRELIMINARES SOBRE SELECCIÓN *in vitro* POR TOLERANCIA A EXCESO DE CLORUROS EN AGUACATE

RESUMEN

Los objetivos de este estudio fueron los de determinar si el cultivo *in vitro* de embriones maduros de aguacate (*Persea americana* Mill.) es una estrategia viable para seleccionar portainjertos tolerantes al exceso de cloruros así como el de conocer las diferencias genotípicas entre embriones de las razas Mexicana (var. *drymifolia*) y Antillana (var. *americana*). Se extrajeron embriones maduros de semillas de aguacate de las dos razas y fueron inoculados en el medio de cultivo de Murashige y Skoog adicionado con 2 mg·litro^{-1} de glicina, 10 mg·litro^{-1} de myo-inositol, 4 mg·litro^{-1} de tiamina-HCl, 30 g·litro^{-1} de sacarosa y 7.5 g·litro^{-1} de agar. Posteriormente se agregaron al medio sales de NaCl, $CaCl_2 \cdot 2H_2O$ y la mezcla de NaCl + $CaCl_2 \cdot 2H_2O$ (proporción 1:1) en concentraciones que fueron desde 0.1 a 1.0 % en intervalos de 0.1 %. Los embriones fueron cultivados a 27 ± 1 ºC durante 21 d en obscuridad y posteriormente durante 65 d con 18 h día y 6 h noche. El porcentaje de germinación y sobrevivencia resultaron mayores en los embriones de la raza Antillana (24 y 11 %, respectivamente) que en los de la raza Mexicana (21 y 6 %, respectivamente). En general, el potencial hídrico así como el potencial osmótico fueron reducidos conforme se incrementaron las concentraciones de sales, mientras que el potencial de turgencia se incrementó. Los resultados sugieren que el aguacate posee cierto grado de ajuste osmótico.

PALABRAS CLAVE ADICIONALES: *Persea americana* Mill., ajuste osmótico, salinidad, cultivo de tejidos.

INTRODUCTION

The sensitivity of avocado to salinity is well known. Avocado is not only sensitive to the total salt content in the soil but to specific ions like sodium (Na^+) and chloride (Cl^-). In Mexico, toxicity symptoms due to Cl^- excess in avocado are more common that those caused by Na^+ (Salazar-García *et al*., 1987; Salazar-García and Cortés-Flores, 1988). Leaf tip burn and marginal necrotic areas are the initial symptoms of Cl^- toxicity (Ayers *et al*., 1951; Solares-Morales *et al*., 1984; Salazar-García *et al*., 1987). Depending upon tree tolerance and orchard management, conti-

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nued exposure to high soil/water Cl^- may cause death of twigs and/or defoliation, resulting in low yields and eventually, death of the tree.

Usually, irrigation water is the main source of Cl^- for avocado orchards. However, toxicity caused by Cl^- in avocado is not exclusive of irrigated orchards. Use of low drainage soils and the improper use of chicken or hen manure and chemical fertilizers (like potassium chloride) have increased the problem of Cl^- toxicity in avocado. These conditions have become common in rainfed avocado orchards of the states of Michoacán and Nayarit (Salazar-García and Lazcano-Ferrat, 1999).

In Mexico, avocado rootstocks are typically obtained from seeds of unknown genetic origin. Due to cross-pollination in avocado, every seedling is genetically different to the mother tree and to other seedlings derived from seeds of the same tree. Consequently, the performance under field conditions of seed-propagated rootstocks is unpredictable.

Selection and evaluation of seedlings, to be asexually propagated as clonal rootstocks, has been a good strategy to reduce or even out several problems that affect yield (salinity, alternate bearing, drought, etc.) or cause tree death (avocado root rot). Aware of the potential to detect outstanding avocado genotypes, traditional breeding programs have been based on screening of seedling populations (Macías-González, 1981; Salazar-García *et al*., 1984). However, this procedure is expensive due to the time and space required. *In vitro* culture of avocado embryos may help breeders to screen large populations of seedlings with the goal of selecting promising genotypes in a shorter time (Llano-Agudelo *et al*., 1985). The objectives of this study were to establish the culture of mature avocado embryos as an option for selecting rootstocks for their tolerance to excess chlorides, and to evaluate the response of mature embryos of the Mexican and West Indian avocado races to several chloride concentrations *in vitro*.

MATERIALS AND METHODS

Mature embryos were obtained from fruit harvested from a single tree for each of the Mexican and West Indian avocado races. Trees were growing nearby the cities of Atlixco, Puebla and Santa María del Oro, Nayarit, México, respectively.

Seeds of both races were submerged in 70 % ethanol (v:v) for 1 min. The seeds were split and the embryo surface was sterilized for 15 min with 2 % sodium hypochloride (v:v) and rinsed in sterile double-distilled water. The embryos were excised with a small piece of cotyledon (Llano-Agudelo *et al*., 1995) and aseptically transferred to sterile culture tubes (25 x 200 mm) containing 10 ml of Murashige and Skoog (1962) (MS) medium supplemented with 2 $mg \cdot litro^{-1}$ of glycine, 10 $mg \cdot litro^{-1}$ of myo-inositol, 4 $mg \cdot litro^{-1}$ of thiamine-HCl, 30 $g \cdot litro^{-1}$ of sucrose, and 7.5 $g \cdot litro^{-1}$ of bacto agar. The MS medium was adjusted to pH 5.7 with 0.1 N NaOH or HCl. Culture tubes were capped with aluminum foil and sterilized by autoclaving at 120 ºC and 1.5 $kg \cdot cm^{-2}$ pressure for 15 min.

Nine hundred embryos were used in this research and a total of 60 treatments were evaluated. Treatments consisted of the combination of two avocado races (Mexican or West Indian) and 10 concentrations (0.1 to 1.0 %, at 0.1 increments) of NaCl, $CaCl_2 \cdot 2H_2O$, or a combination of NaCl plus $CaCl_2$ (ratio 1:1) added to the MS medium. Salinity treatments were started immediately after excision and transfer of embryos to the culture medium. There were 15 single embryo-replications per treatment.

Explants were incubated at 27±1 ºC in darkness for 21 d, followed by 65 d at the same temperature and 18-h day (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation from cool-white fluorescent lamps) and 6-h night. Embryo germination was considered the stage at which embryos showed a radicle or shoot growth ≥ 5 mm, which was approximately 50 days after beginning culture. Embryo survival was evaluated at the end of 65 days of culture.

Water potential ($\Psi\omega$) and osmotic potential ($\Psi\pi$) were determined 10 days after embryos germination for stem and root of four seedlings per treatment. A Peltier type termopar psychrometer (Wescor 5100) was used. Turgor potential ($\Psi\rho$) was estimated from the equation: $\Psi\omega = \Psi\pi + \Psi\rho$. These parameters were obtained only for treatments in which seedlings were alive and complete at the time of measurements.

There was a significant dead of explants or seedlings caused by the treatments applied. Thus, the standar error (SE) was calculated when enough data were available.

RESULTS

Germination of embryos

Germination of untreated control embryos was 70 and 80 % for the Mexican and West Indian races, respectively. A decrease in the germination of embryos was observed as salt concentrations increased.

Mexican race embryos exposed to NaCl at 0.1, 0.2, 0.3, 0.4 and 0.7 % had a germination of 50, 50, 60, 30 and 20 %, respectively. No germination was observed with other NaCl concentrations. Embryos treated with $CaCl_2$ at 0.1, 0.2, and 0.3 % had 50 % germination. $CaCl_2$ at 0.4 % resulted in 40 % germination. Embryos treated with other concentrations did not germinate. Germination of embryos treated with NaCl+$CaCl_2$ was lower than that obtained using individual salts. However, the salt mixture allowed embryo ger-

mination at higher concentrations, with the exception of the concentration of 0.4 % (Figure 1A).

Embryos of the West Indian race had higher germination rates in $CaCl_2$ than with other types of salts. Treatments with NaCl allowed germination only at levels of 0.1 to 0.5 %, being greater at 0.1 and 0.2 %. Germination in the mixture of NaCl+$CaCl_2$ was intermediate (Figure 1B).

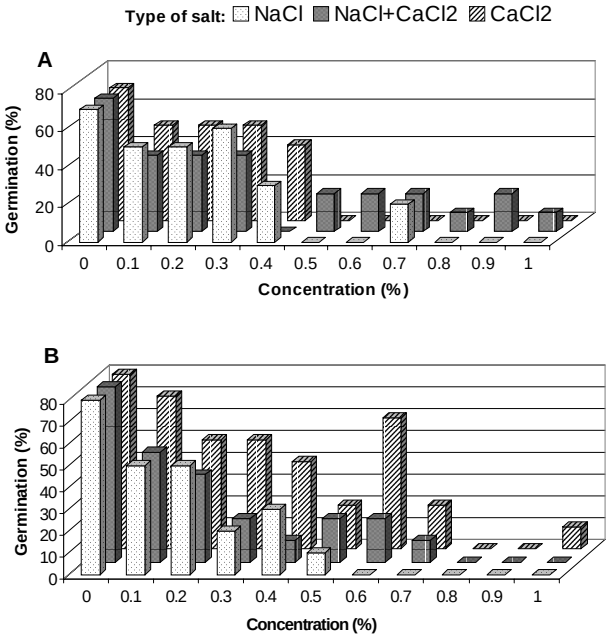

Figure 1. Germination of Mexican (A) and West Indian (B) race mature avocado embryos 50 days after being cultured in MS medium added with three types of salt.

Survival of seedlings

Sixty-five days after culture, average survival of Mexican race seedlings grown in different salt concentrations was 5.8 %. A clear-cut effect of the concentration or type of salt on seedling survival was not observed. However, a greater survival trend was noticed for seedlings treated with $CaCl_2$ (Figure 2A).

Seedlings originated from embryos of the West Indian race showed a greater average survival (10.6 %) than those of the Mexican race (5.8 %). Seedlings in $CaCl_2$ had the greatest survival, even at 1 %, whereas survival was strongly reduced by NaCl alone or mixed with $CaCl_2$ (Fi gure 2B).

Effect of salinity on plant water relations

Mexican race

Water relations of Mexican race seedlings were strongly influenced by the source of chlorides as well as the concentration of salts added to the medium. For seed-

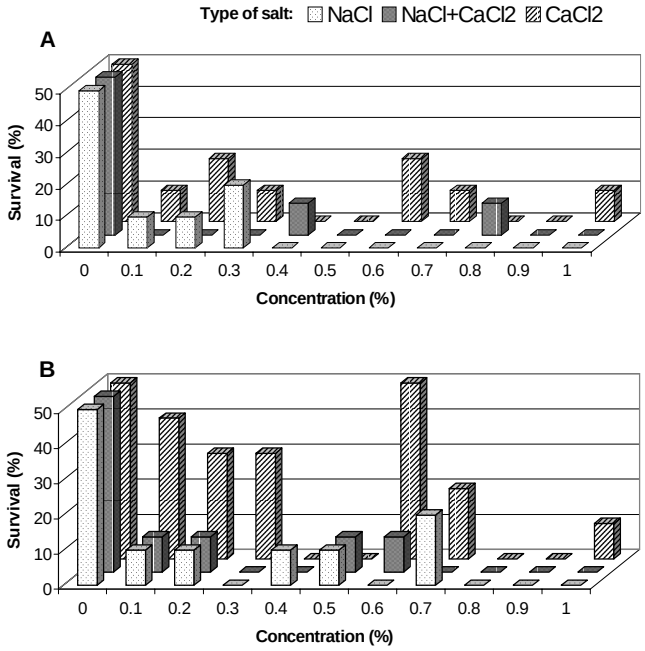

Figure 2. *In vitro* survival of Mexican (A) and West Indian (B) race avocado seedlings 65 days after culture in different salinity levels.

lings treated with NaCl, measurements were done only for concentrations of 0.1 and 0.3 %. Stem turgor potential increased with NaCl 0.1 % but it was not affected by 0.3 %. In the case of stem $\Psi\omega$ it was reduced only with 0.3 % NaCl. Both NaCl concentrations caused a reduction in stem $\Psi\pi$. Root determinations showed that $\Psi\rho$ was not affected by NaCl concentrations. However, NaCl 0.3 % caused an increase in both root $\Psi\omega$ and root $\Psi\pi$ as compared to the control and NaCl at 0.1 % (Figure 3).

$CaCl_2$ at 0.4 % caused a decrease in stem $\Psi\rho$, but this effect disappeared at 0.8 %. Neither the stem $\Psi\omega$ or $\Psi\pi$ resulted affected by the $CaCl_2$ concentrations evaluated. Root $\Psi\omega$ and $\Psi\pi$ decreased as $CaCl_2$ concentrations increased, but they were accompanied by an increase in root $\Psi\rho$, which at 0.8 % was notoriously greater than that of the control (Figure 3).

A greater number of Mexican race seedlings were available for treatments with the mixture of NaCl+$CaCl_2$. A drop of the stem $\Psi\rho$ was observed with the lowest salt mixture concentrations (0.1 and 0.2 %); however, values of stem $\Psi\rho$ were above the control for treatments with NaCl+$CaCl_2$ at 0.7 and 1.0 % (Figure 3). It was worthy of note that at these concentrations the lowest values of stem $\Psi\omega$ and $\Psi\pi$ were observed (-1.1 to -1.5 and 1.8 to 2.1 MPa, respectively). As observed for the treatments with NaCl alone, the increase of the stem $\Psi\rho$ was associated to a notorious decrease (more negative) of the stem $\Psi\pi$. Roots of seedlings grown in the salt mixture had a similar response to the stem. However, there was an obvious decrease on both root t $\Psi\omega$ and root $\Psi\pi$ with NaCl+$CaCl_2$ at 0.1 and 0.2 % (Figure 3).

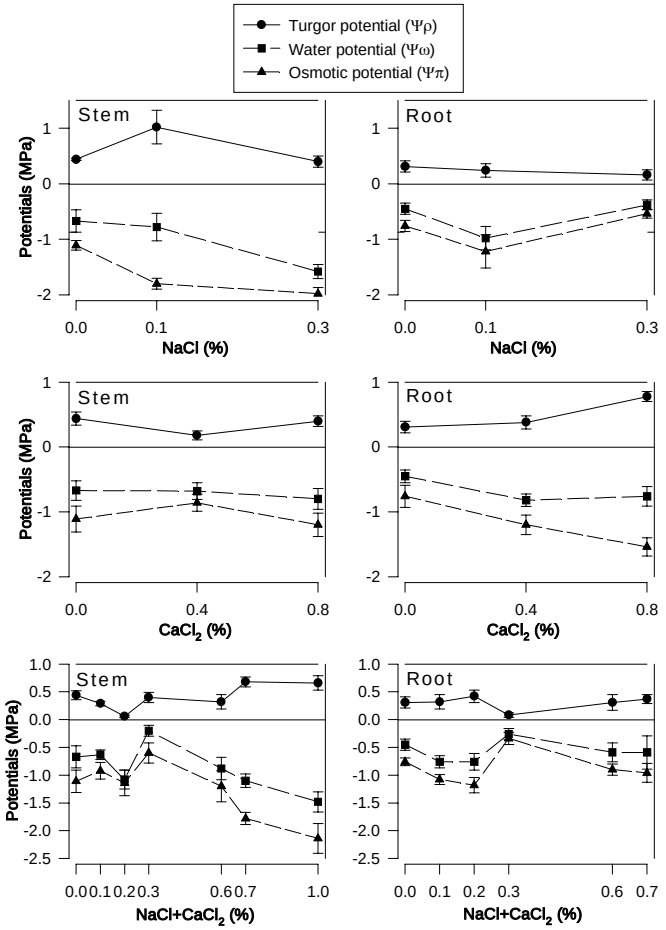

Figure 3. Stem and root water relations (±SE) of Mexican race avocado seedlings 10 days after germination *in vitro* with different salinity treatments.

West Indian race

West Indian seedlings cultured in MS medium added with NaCl at 0.2 and 0.4 % showed a marked increase in the stem Ψρ. This was accompanied by a parallel reduction (more negative) of the stem Ψπ. However, with NaCl 0.5 %, all water relations were similar to the control (Figure 4). Root response was not as clear as for the stem; however, in most cases, a change in the root Ψρ was accompanied by an opposing change. Higher NaCl concentrations (0.5 and 0.7 %) were characterized by an important accumulation of solutes (Ψπ) in the root (Figure 4).

Fluctuations of the stem water relations were less pronounced when the salt added to the MS medium was $CaCl_2$. This statement was true for those concentrations no greater than 0.7 %, as a steep fall of all stem potentials occurred with $CaCl_2$ 1 % (Figure 4). A similar response was observed for the root; although in this case the Ψρ remained high, even with $CaCl_2$ at 1 % (Figure 4).

Mixture of salts added to the MS medium had no effect on stem water relations, even at the highest concentration where data were available (NaCl+$CaCl_2$ at 0.6 %) (Figure 4). Roots were more sensitive to the concentrations based on NaCl+$CaCl_2$. An acute decrease in both root Ψω and root Ψπ was observed as salt concentrations went above 0.1 %. However, a corresponding increase on root Ψρ was detected for these concentrations (Figure 4).

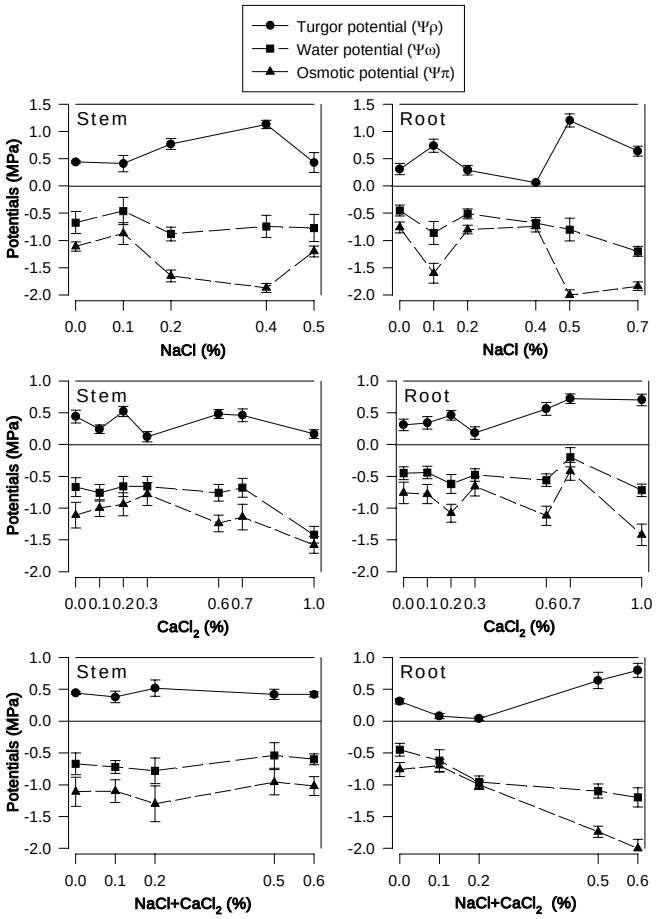

Figure 4. Stem and root water relations (±SE) of West Indian race avocado seedlings 10 days after germination *in vitro* with different salinity treatments

DISCUSSION

In general, embryo germination and seedling survival for both Mexican and West Indian races were lower in treatments with NaCl than with NaCl+$CaCl_2$ Inhibition of germination and delay of seedling growth was clearly noticed. This response may have been influenced more by the presence of Na^+ than by Cl^- (Waisel, 1972). There also exists the possibility that high NaCl levels caused an osmotic potential that prevented the complete hydration of the embryo, reducing its germination (Roundy *et al*., 1985). This may explain a greater germination and survival of embryos of both races at higher $CaCl_2$ concentrations.

Use of the mixture of salts seems to be a promising salinity treatment for *in vitro* selection of avocado seedlings with tolerance to excess chloride. When $CaCl_2$ plus NaCl were added to the MS medium a higher germination of embryos of the Mexican race was observed, compared to

the use of NaCl alone. Addition of $CaCl_2$ to the culture medium could be important to maintain an adequate ratio Na^+/Ca^{2+}. It is well documented that Ca^{2+} has a protective effect against the possible damages by excess Na^+ by regulating ion transport and maintaining the integrity of the cellular membrane (Nieman and Willis, 1971; Epstein, 1961, 1972; Kurth *et al*., 1986; Hoffman and Bisson, 1988). Presence of Ca^{2+} may reduce the damage caused by excess Na^+ during embryo germination and growth by displacing Na^+ at the cellular membrane, as it has been reported for several gramineous species (Cramer *et al*., 1985). Similar findings have been reported for avocado seedlings and young trees irrigated with saline water containing NaCl plus $CaCl_2$. The combination of salts was helpful to reduce the injury caused by Na^+ (Haas, 1950; Salazar-García *et al*., 1984).

A wide variation in the response of embryos and seedlings to salinity was detected between and within explants of Mexican and West Indian races. Individuals of the West Indian race exhibited a higher tolerance to salinity. Similar reports for young and mature plants showed a greater tolerance of West Indian to salinity (Kadman, 1963), especially Cl^-. However, some individuals of the Mexican race have also shown a high degree of tolerance to progressive soil salinity (up to 2,800 mg·liter^{-1} NaCl+$CaCl_2$) (Salazar-García *et al*., 1984).

An interesting response of seedlings of the Mexican race treated with NaCl+$CaCl_2$ was the reduction of stem $\Psi\omega$ and stem $\Psi\rho$ (more negative) while the Yr increased (more positive). A similar response was obtained for roots of seedlings of the West Indian race. Our data suggest that a different adaptive mechanism may be present in avocado as water relations were affected by the organ analyzed as well as the botanical race.

We have shown that avocado seedlings cultured in vitro in a saline medium have the capacity of adjusting osmotically by reducing their $\Psi\rho$ in greater proportion than their $\Psi\omega$. To our knowledge, this is the first report documenting the physiological response of avocado embryos to excess chlorides *in vitro*. Our results provide evidence that under salinity conditions, the avocado possesses the capacity to adjust osmotically by accumulating solutes. The results of this study are encouraging because the genetic variation showed by avocado embryos and seedlings opens the possibility of using this technique to select new salinity tolerant avocado rootstocks. However, field data on the behavior of rootstocks selected with this technique should be obtained. A relationship between the salinity levels tested in this study and those present under field conditions remains to be determined.

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