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Long Term Effects of Sodium Chloride Salinity on Growing *in Vitro*, Proline and Phenolic Compound Content of Jojoba Explants

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Abstract

Jojoba (*Simmondsia chinensis* L.) explants were cultured *in vitro* under four levels of sodium chloride salinity (0, 0.33, 0.66 and 0.99% w/v) during the proliferation stage. Explant growth was seriously affected by salinity treatments. The mean shoot number and the total mean shoot length per explant were significantly reduced under high salt concentration. Fresh and dry weight of explants were increased till the medium level of salinity and decreased at the high one. Proline and phenolic compound contents of explants were determined in relation to salinity treatments. At the high salt level, proline content was increased while the total phenol and o-diphenol content was higher in explants deriving from control treatment. Ferulic acid and 4-coumaric acid contents decreased at the high salt levels. There were no significant differences between treatments, during most of the time at the proliferation stage, concerning vanillic acid and protocatechuic acid content.

Zusammenfassung

Langfristige Wirkungen des Salzgehalts auf die *in vitro* Entwicklung, den Gehalt an Prolin und phenolischen Verbindungen von Jojoba-Explantaten. Jojoba- (*Simmondsia chinensis* L.) Explantate wurden während der *in vitro* Proliferationsphase bei vier verschiedenen Natriumchlorid-Konzentrationen (0, 0.33, 0.66 and 0.99% w/v) kultiviert. Das Explantatwachstum wurde durch den Salzgehalt deutlich beeinflußt. Die durchschnittliche Sprosszahl und die durchschnittliche Sprossgesamtlänge pro Explantat war bei dem hohen Salzgehalt statistisch nachweisbar reduziert. Das Frisch- und Trockengewicht der Explante nahm mit steigendem Salzgehalt bis zur mittleren Stufe zu und mit weiterer Zunahme (hoher Salzgehalt) wieder ab. Auch der Gehalt an Prolin und phenolischen Verbindungen in den Explanten war durch den Salzgehalt bestimmt. Beim hohen Salzgehalt war der Prolingehalt erhöht, während der Gesamtphenol- und der o-Diphenol-Gehalt in der Kontrollbehandlung am höchsten war. Der Gehalt an Ferula- und 4-Cumarsäure nahm bei der hohen Salzkonzentration ab. Hinsichtlich Vanillin- und Protokatechinsäure war während der Proliferationsphase zumeist kein signifikanter Unterschied zwischen den Behandlungen nachweisbar.

Key words. Jojoba – *in vitro* – phenolic compounds – proline – salinity

Introduction

Jojoba (*Simmondsia chinensis*) is an industrial crop with increasing interest, especially in arid and semi-arid regions, as it is considered to tolerate fairly high levels of salinity and water stress (BOTTI et al. 1998). For this crop to be an economically profitable alternative for arid and semi-arid zones, it is necessary to select plants of high productivity under stress conditions.

Salt stress affects many physiological and biochemical processes in plants, resulting in the alteration of some metabolic pathways (FRECHILLA et al. 2001). The nature of salt tolerance is complex and not well understood, but may be attained through salt compartmentation in the cell and within the plant (KHAN et al. 2000). In response to salt stress, many plants synthesize and accumulate osmotically active, low molecular weight compounds such as proline, polyols, carbohydrates, amino acids and organic acids (FRECHILLA et al. 2001; GIRIJA et al. 2002).

Proline through its function as osmoprotectant makes an environment compatible with macromolecular structure and function (GIRIJA et al. 2002) Moreover proline accumulation may reduce stress-induced cellular acidification and proline itself may act as a substrate for respiration, which might provide energy, needed for recovery from stress (AZIZ et al. 1999).

Phenolic compounds are a large group of secondary metabolites, which can play a role in virtually any interaction a plant can have with its environment (WATERMAN and MOLE 1994). These compounds have been implicated to stress resistance against biotic and abiotic factors (BERGMANN et al. 1994, COHEN et al. 1994, WATERMAN and MOLE 1994). On the other hand, there are not so many research data about their role against abiotic ones, such as drought or salt stress, except for their unquestionable role against UV radiation (WATERMAN and MOLE 1994).

Since screening germplasm in saline fields could be time and money consuming, a promising, efficient and simple alternative could be to screen organs or propagules *in vitro* (MILLS and BENZIONI 1992). There is evidence that *in vitro* nodal segments of jojoba respond to

salinity in a similar way as the whole plant, so that this technique could be used for preselection and evaluation for salt tolerance (MILLS and BENZIONI 1992).

The aim of the present study was to evaluate a jojoba clone under four levels of sodium chloride salinity *in vitro*, using single node explants and reveal the role of proline and some phenolic compounds to the adaptation of the species to salt stress.

Materials and Methods

In vitro culture and salt treatments

Jojoba explants originated from an one year culture, maintained by successive subcultures in a basal medium described by ROUSSOS et al. (1999), supplemented with 4 mg l^{-1} BA. At least fifty single node and shoot tip explants were used per treatment. After excision, explants were grown in 25 x 150 mm culture tubes filled with 10 ml of basal medium, supplemented with the above mentioned concentration of BA for invigoration. Cultures were incubated at 25 ± 1 °C with a 16-h photoperiod provided by cool white fluorescent lamps (38 mol m^{-2} s^{-1}). At the end of the third week, the explants were transferred to 250 ml Erlenmeyer flasks, containing 100 ml of basal medium, supplemented with the same BA concentration plus four concentrations of sodium chloride, e.g. 0 (Control), 0.33 % (w/w) (Low), 0.66 % (w/w) (Medium) and 0.99 % (w/w) (High). The explants remained at this stage for three months. At the end of every month ten flasks from each treatment were withdrawn in groups of five (two groups per treatment) and lyophilized.

At the end of the third month the number and length of shoots, of twenty separate flasks, were recorded along with their fresh and dry weights, in order to evaluate jojoba's growth under salt stress conditions.

Proline determination

The method of proline determination was essentially as described by BATES et al. (1975). Each sample was assayed twice.

Phenolic compounds extraction and determination

Phenolic compounds were extracted as described by ROUSSOS and PONTIKIS (2001) with some modifications. Briefly, 100 mg of lyophylized tissue were extracted with 10 ml of 60 % methanol in water in an ultra sound bath for 30 min. Right after extraction the mixture was centrifuged at 4000 g for 6 min and 300 µl of the supernatant was used for total phenols and o-diphenols determination. Methanol was evaporated from the rest of the supernatant under a stream of nitrogen, in a water bath at 45 °C and the aqueous solution was used for phenolic compound purification.

The purification was accomplished using 500 mg, 3 ml bed volume, C-18 non-endcapped solid phase extraction columns (IST international). The columns were pre-equilibrated with 3 ml absolute methanol acidified with 1 % acetic acid, followed by 3 ml water acidified with 1 % acetic acid. The samples were loaded to the columns and phenolic compounds were eluted by 3 ml of 60 % methanol and 1 % acetic acid in water. The final eluate was brought to 5 ml with the same solvent and filtered through a 0.22 µm pore nylon syringe filter before HPLC analysis. Four phenolic compounds were identified and quantified, e.g. protocatechuic acid (PA), vanillic acid (VA), ferulic acid (FA) and 4-coumaric acid (4CA). Analysis was performed with a Hewlett-Packard 1050 HPLC instrument equipped with a UV-Vis detector on a Lichrospher RP-18 column (4.6 x 250 mm), at 28 °C while phenolic compounds were detected at 280 nm. The solvent system for the HPLC analysis consisted of 15 % acetonitrile, 2 % methanol and 1 % formic acid in water. The phenolic compounds were eluted from the column with a timetable flow rate as followed: from 0–5 min flow rate at 0.2 ml min^{-1}, at 5.5–15 min flow rate at 0.5 ml min^{-1}, at 15.5–22 min flow rate at 1 ml min^{-1} and at 23 min and thereafter flow rate was at 1.8 ml min^{-1}. Each sample was assayed twice.

Total phenol and o-diphenol content

The total phenol and o-diphenol content was determined according to ROUSSOS and PONTIKIS (2001).

Statistical analysis

Experiments with jojoba explants were arranged in randomized complete block design with five explants per treatment and two replications, while there were four replications for the growth parameters measurements, at the end of the third month. The data of the biochemical analyses were analyzed as a two-way Anova, with one factor being time and the other the salt treatment. For the figures SEs were calculated from the residual variances. The data of growth parameter measurements and fresh and dry weights were analyzed for significance by analysis of variance with mean separation by Duncan's multiple range test. Statistical analysis was performed using Statgraphic plus, version 2.1 for Windows.

Results

Effect of salinity treatments on jojoba explant growing at the end of the proliferation stage.

Salinity treatments had a serious effect on the growing of jojoba explant (Fig. 1). As it can be concluded by Table 1, the mean shoot number was significantly reduced by the addition of NaCl to the medium. The higher the concentration of the salt in the medium, the lower was the number of shoots per explant, reaching almost four shoots per explant when 0.99 % (w/v) NaCl was added to the medium. On the other hand, the length of shoots did not experience such a negative influence. On the contrary, the addition of the salt to the medium induced longer shoots, reaching a height of 28.5 mm at the highest concentration of salt. By the mean total shoot length (mean shoot number x mean shoot length) it can be concluded, that there was not any significant difference between the control and the two lower salt concentrations, while the highest one reduced the mean total shoot length per flask significantly.

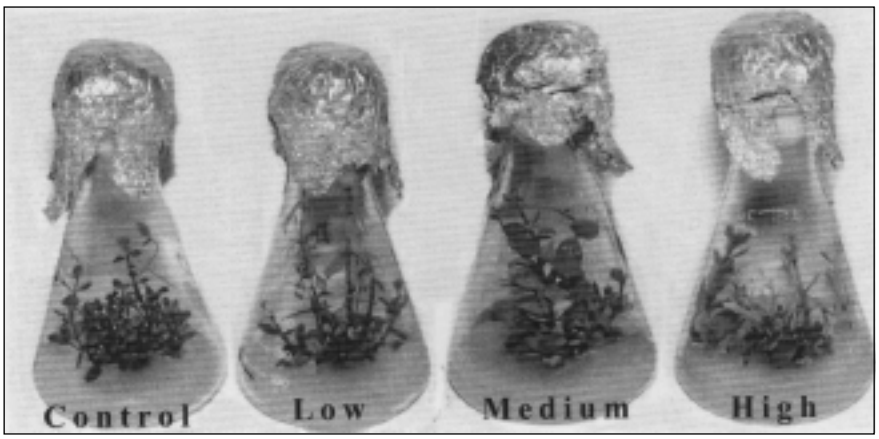


Fig. 1. Jojoba explant growing *in vitro* under four levels of salinity during the proliferation stage (3 months).

Table 1. Effect of various concentrations of sodium chloride on jojoba explant growing during the proliferation stage *in vitro* (three months).

Salinity treatments	Mean shoot number	Mean shoot length (mm)	Mean total shoot length (mm)	Fresh weight (g)	Dry weight (g)	Fresh/Dry weight
Control	16.0 ± 0.2[1] c[2]	17.0 ± 1.0 a	272.2 ± 15.4 b	4.9 ± 0.1 a	3.7 ± 0.1 b	1.32 ± 0.12 a
Low	11.1 ± 0.3 b	22.9 ± 0.4 b	253.7 ± 2.3 b	7.0 ± 1.9 ab	3.9 ± 0.9 b	1.75 ± 0.18 ab
Medium	10.4 ± 1.3 b	25.6 ± 2.2 bc	272.4 ± 57.0 b	9.8 ± 1.1 b	4.5 ± 0.2 b	2.17 ± 0.15 c
High	4.2 ± 0.1 a	28.5 ± 0.5 c	119.6 ± 2.0 a	3.4 ± 0.3 a	1.8 ± 0.04 a	1.9 ± 0.11 bc

[1] Mean ± standard error.
[2] Means within the same column followed by the same letter are not significantly different at p=0.05 according to the Duncan's multiple range test.

Table 2. Correlation coefficients (r^2) between the various salinity levels and the growth parameters, the phenolic compounds and the proline content of jojoba explants.

Parameters	Salinity level	Parameters	Salinity level
Shoot length	0.90[1] ***	Total phenols	ns
Shoot number	–0.94 ***	o-diphenols	ns
Total shoot length	–0.65 *	VA	ns
Fresh weight	ns	PA	ns
Dry weight	ns	FA	ns
Fresh/dry weight	ns	4CA	ns
		Proline	0.75 *

[1] Correlation coefficients, *, $p<0.05$; ***, $p<0.001$; ns, not significant; VA, vannillic acid; PA, protocatechuic acid; FA, ferulic acid; 4CA, 4-coumaric acid.

The highest fresh weight per explant was achieved by the medium concentration of salt. The other three treatments resulted in significantly lower fresh weight per explant. On the other hand, there was not any significant difference between the three first salt levels considering the dry weight of the explants, while only the high one did it result in significantly lower dry weight per explant.

A succulence index can be achieved by dividing fresh weight by dry weight measurements (BENZIONI et al. 1992). The higher this index the more succulent the explants. Explants deriving from the control treatment exhibited the lower succulence index, while those from the two higher salt concentrations exhibited the highest succulence indexes.

As can be concluded by Table 2, there was a significant high positive correlation between salt level and jojoba explant length, while the opposite stood (high negative correlation) for the mean shoot number and the mean total shoot length.

Effect of salinity treatment on the proline content of the explants.

Salinity treatments and time of culture influenced the proline content of the explants significantly (Table 3). Explants growing on medium supplemented with the two low salt concentrations (0 and 0.33 % w/v) exhibited almost the same low proline content during the entire culture period (Fig. 2). Explants growing on medium supplemented with the highest salt concentration had the highest proline content during the entire culture period.

Proline concentration seemed to be decreasing through time of culture, with the most significant decrease taking place on explants of the two higher salt treatments. Furthermore, proline exhibited a significant positive correlation with salinity level (Table 2).

Effect of salinity treatments on the phenol content of explants.

Salinity treatment affected the total phenol content, the total o-diphenol content and the concentrations of both ferulic and 4-coumaric acid significantly (Table 3),

Table 3. Probabilities of effects of salt level and time of explant sampling, as a factorial experiment, on proline and phenolic compounds concentration of jojoba explants during the *in vitro* culture.

Variables	Source of variation		
	SL	*Time*	*SL*Time*
Proline	**<0.001**	**<0.05**	>0.05
Total phenols	**<0.05**	**<0.001**	**<0.01**
o-Diphenols	**<0.01**	**<0.001**	>0.05
o-Diphenol/Total Phenols	>0.05	**<0.001**	>0.05
Vannillic acid	>0.05	>0.05	>0.05
Protocatechuic acid	>0.05	>0.05	>0.05
Ferulic acid	**<0.001**	**<0.01**	**<0.05**
4-coumaric acid	**<0.05**	>0.05	>0.05

SL, salt level; Time, time of explant collection; *, interaction.

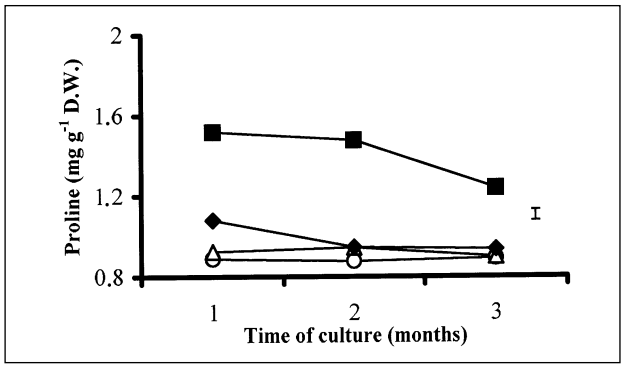


Fig. 2. Fluctuations of jojoba explant proline content under four levels of salinity during the proliferation stage. –○–: control; –△–: low; –◆–: medium; –■–: high salt treatment. The vertical bar is the SE.

while there was not any significant correlation between salinity levels and phenolic compound content (Table 2). The time of culture had a significant effect on total phenol and o-diphenol content, on the ratio of o-diphenol/total phenol content and the concentration of ferulic acid. There were significant interactions between salinity treatment and time of culture only concerning the total phenol content and ferulic acid concentration of jojoba explants.

During most of the time, explants growing on medium supplemented with the two higher salt concentrations, exhibited the lower total phenol content. This was significantly lower than that achieved by the two low salt levels, especially during the second and third month (Fig. 3a). The total phenol content seemed to be decreasing through time of culture, except for the case of the two low salt concentrations, where the significant increase during the second month was followed by a similar decrease during the third one.

Explants growing on salt-free basal medium exhibited the highest o-diphenol content during the entire culture period, but especially during the last two months (Fig. 3b). Generally, explants from the other three treatments did not differ significantly, concerning their o-diphenol content. The o-diphenol content of the explants seemed to be increasing significantly through time, at all treatments.

The ratio o-diphenol/total phenol content could be a useful indicator of a possible alteration of the phenolic compound metabolism of the explants. It seemed that the participation of o-diphenols to the total phenol content of jojoba explants increased significantly throughout the culture period (Fig. 3c).

Explants growing on medium supplemented with salt, exhibited high level of VA, during the third month, higher than those growing on salt-free medium (Fig. 4a). During the first month only the exlpants from the low concentration of salt did they present high level of VA, while at the second month all treatments resulted in the same VA values.

Explant PA content seemed to be decreasing during the second month and increasing during the third one

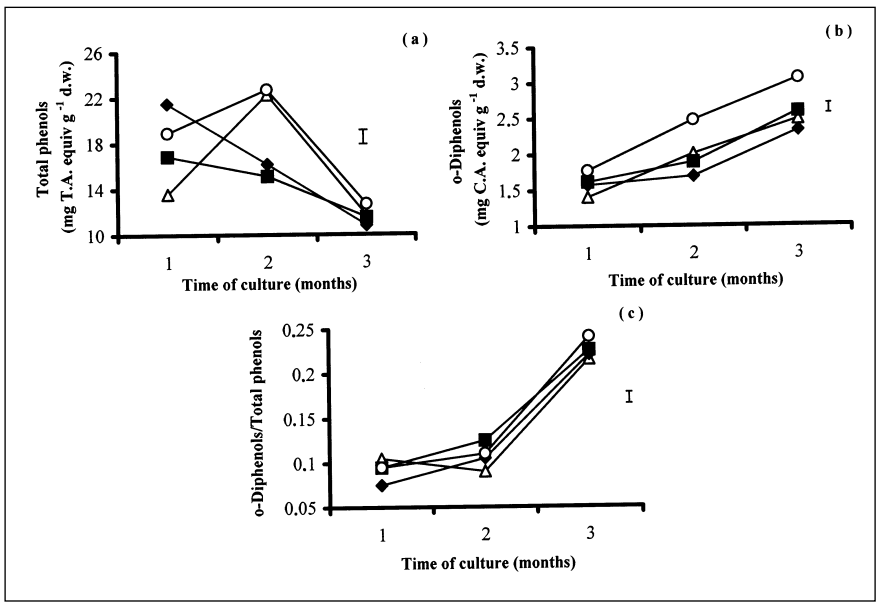


Fig. 3. Fluctuations of jojoba explant total phenol and o-diphenol content as well as their ratio, under four levels of salinity during the proliferation stage. –○–: control; –△–: low; –◆–: medium; –■–: high salt treatment. The vertical bar is the SE.

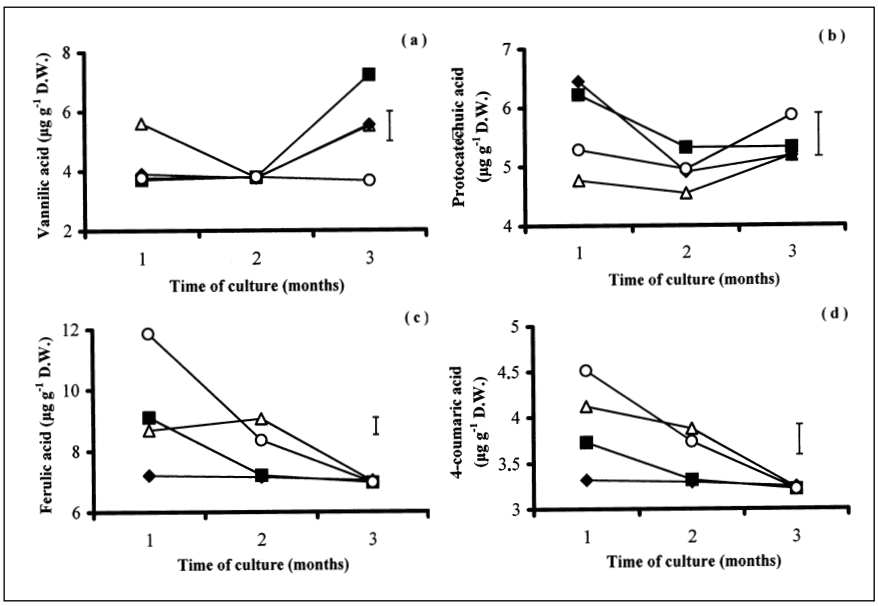


Fig. 4. Fluctuations of jojoba explant phenolic acid content under four levels of salinity during the proliferation stage. –○–: control; –△–: low; –◆–: medium; –■–: high salt treatment. The vertical bar is the SE.

(Fig. 4b). Explants, deriving from medium supplemented with the two higher levels of salt, exhibited during the first month the higher PA level, while later on, during the culture period there were not any differences between treatments.

On the other hand, explants growing on salt-free medium exhibited the highest FA and 4CA content during the first month (Fig. 4c and d)., while explants deriving from medium supplemented with the two higher salt concentrations had low levels of both phenolic acids, during the whole period of culture. It seemed that explants growing on medium supplemented with the two lower salt concentrations exhibited higher levels of both FA and 4CA during the first two months. It is also noteworthy that at all treatments the content of these two phenolic acids was decreasing through time of culture.

Discussion

Metabolic stress caused by sodium chloride results in reduced plant growth and changes in the metabolic pathways of many primary and secondary metabolites. Intracellular mechanisms such as compartmentation of ions and solutes as well as osmotic adjustment in salt tolerant species may allow continuance of growth and survival of the plant in the presence of NaCl (GIRIJA et al. 2002).

Jojoba is considered to be salt tolerant and therefore one would expect a minor decrease of its growth under saline conditions. Nevertheless, the number of shoots was drastically decreased under the high salt level, which is in accordance with the findings of MILLS and BENZIONI (1992), working with nodal segments of jojoba *in vitro*. One thing noteworthy is the reaction of explants, regarding shoot elongation, under saline conditions. It seemed at first that shoot elongation was favoured by the presence of sodium chloride in the medium. The same results report also MILLS and BENZIONI (1992) regarding concentrations of salt up to almost 1 %. When a similar experiment was conducted, with young plants of jojoba growing in containers, the plant height was neither reduced, up to a specific salt level, nor enhanced (BENZIONI et al. 1992).

The mean total shoot length of jojoba explants growing *in vitro* under saline conditions seemed to be decreasing as salt level increased, presenting a high correlation coefficient with the NaCl concentration used. This could be an indication of the fact that when a small number of shoots are growing on a medium, these shoots will probably elongate more than in the case of many shoots growing on the same one, independently of the stress factor present. There is in fact a strong competition between shoots, regarding nutrient and organic compounds uptake, which is clearly shown by the significant negative correlation between the number of shoots and their length ($R=-0.79$, $p<0.01$).

The fresh weight of explants increased significantly by the low and medium salt concentration in relation both to that of control and the high one. Only the highest salt level affected the dry weight of explants significantly. The majority of research reports a reduction of fresh and dry weight of plants growing under saline conditions (SULTANA et al. 1999; ROMERO-ARANDA et al. 2001). Nevertheless, there are also reports on fresh and dry weight increase, up to a level of salt concentration (GUPTA et al. 1995, KHAN et al. 2000), over which a drastic decline follows, as in this case. Explants growing on medium supplemented with salt exhibited higher succulence index, measured as fresh/dry weight ratio. It is known that salinity limits growth and production of plants primarily due to osmotic stress and ion imbalances (FRECHILLA et al. 2001; ROMERO-ARANDA et al. 2001). Since water availability in *in vitro* culture does not constitute a problem (MILLS and BENZIONI 1992) it would be obvious that explants should not experience high osmotic stress. On the other hand, it seemed as if jojoba explants overcame salinity stress by increasing water uptake from the medium, in order to dilute high salt intracellular concentrations. This is being referred as "salt dilution by succulence" and is one way of plant adaptive reaction to salt stress (HAGENMEYER 1996). Similar increases in succulence of jojoba leaves, in response to salinity treatments, have been reported by BENZIONI et al. (1992) and by YER-

MANOS et al. (1967). In the present experiment, succulence was obvious by the major leaf thickness of explants growing at high salinity.

Proline accumulated in jojoba explants only when explants were exposed to the highest level of salt concentration. Proline is known to be accumulated during salt stress and it is considered to play a role in the plant tolerance mechanism (HARE and CRESS 1997; AZIZ et al. 1999; SULTANA et al. 1999; GHOULAM et al. 2002). It seems to be a late adaptive response of plants under saline conditions (AZIZ et al. 1999), but in our case proline content decreased with time, which could mean that plants had already started the adaptive response reaction by the first month. It must be noticed here that the short term (during the first week after salinity treatment) changes could be very different to those observed in a long term experiment.

Phenolic compounds are a large group of secondary metabolites, which have a role in plant resistance against biotic but also abiotic stress factors (RIVERO et al. 2001, WATERMAN and MOLE 1994). In the present experiment the total phenol and o-diphenol content seemed to be higher in the explants growing on salt-free medium or on low salt concentration. The o-diphenol content was increased through time like the ratio o-diphenol/total phenol content. It is well known that o-diphenols play a substantial role in the plant resistance against stress factors (BERGMANN et al. 1994). The lower o-diphenol content of explants growing on medium supplemented with salt, could indicate a reduction of these compounds through enzymatic oxidation. Oxidative stress is known to occur in plants under saline stress (DIONISIO-SESE and TOBITA 1998; RIOS-GONZALEZ et al. 2002) and o-diphenols could act as relieving agents, as it is known to be strong antioxidative compounds (YURTTAS et al. 2000). Furthermore, phenolics may inhibit the production of reactive oxygen species, act as radical scavengers or radical-chain breakers thus extinguishing strongly oxidative free radicals such as the hydroxyl radical (GRASSMANN et al. 2002).

Ferulic acid and 4-coumaric acid content were significantly affected by salinity treatment. Their concentration was higher in explants growing on salt-free medium or on medium supplemented with the low salt concentration, possibly due to lower oxidation rates.

It seemed that salt stress has affected phenolic compound metabolism, either by reducing phenylalanine-ammonia lyase activity, the key enzyme in the phenolic compound biosynthesis, as has been reported by DUNN et al. (1998) or by inducing their oxidation.

Jojoba is reported to be a salt tolerant species with differences among the varieties regarding to this characteristic (BENZIONI et al. 1992). All growth parameters were affected by salinity treatments, indicating that jojoba can withstand saline conditions up to some level of salt concentration and of salinity durance, over which major metabolic changes take place and as a consequence reduce growth and proliferation. Although phenolic compounds are a vast group of secondary metabolites, they have not been extensively investigated over their role in salt resistance. Even if it seemed that o-diphenols and some phenolic acids could play a major role in regulating oxidative reactions under salt stress, still further research is needed to evaluate their role parallel to oxidative enzyme activities.

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