

Full Length Research Paper

Salinity stress tissue-regenerated *Rosa chinensis* Jacq. improves water and proline content

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Rosa chinensis Jacq. is used worldwide in ornamental and medicine. The goal of this study was to establish a convenient media for plant regeneration and embryogenesis from nodal segments excised from greenhouse-grown plants and cultured on MS (200 mM NaCl-free/not) medium. Salinity stress could cause embryo death, plantlet morphological change and drastic necrosis during rooting and pot soil transplantation. The most effective embryo development (Dv), proliferation (PI) and differentiation (Df) media under salt stress were respectively supplemented with [2.00 mg/L zeatin, 0.30 mg/L α -naphthleneacetic acid (NAA), 1.50 mg/L gibberellic acid (GA₃)]; [5.00 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.50 mg/L zeatin, 1.50 mg/L GA₃] and [2.00 mg/L zeatin, 1.50 mg/L GA₃, 3.00 mg/L abscisic acid (ABA)]. These media provided an average of 100±0.00, 88.35±0.68 and 51.59±1.78% respectively as relatively high percentage of well developed embryos. Analysis of macromolecules nitrogen (N), phosphorus (P) and potassium (K) of 200 mM NaCl stress *in vitro* (SSIV) were significantly higher compare to control and 200 mM NaCl-free recovered *in vitro* (RIV) plantlets. Chlorophyll in contrary was significantly higher in control and RIV compare to SSIV plantlets. Interestingly, Water and proline remained significantly higher in RIV (371.42±0.88 μ g/g and 69.24±0.65% respectively) compare to control (368.79±0.58 μ g/g and 54.67±1.31% respectively) and thus, China rose was improved of physiological activity.

Key words: Embryogenesis, plant regeneration, *Rosa chinensis* Jacq., transplantation, salinity stress.

INTRODUCTION

Rosa chinensis Jacq., commonly known as China rose, is a member of genus *Rosa* native to China. It is an economically important plant used in ornamental, medicine and also, its industrial products are worldwide distributed. In the past decades, research in plant tissue culture focused on different points. Cell and tissue culture have been used to study various physiological and

biochemical processes affected by induced stress (Reid and Stephen, 1991).

Rose plants have been investigated for short term tissue regeneration, including growth regulators, genetic transformation, disease resistance, temperature, humidity and engineering for stress resistance rose (James et al., 1986; Matthews et al., 1991; Reid and Stephen, 1991; Liping and Manzhu, 2002; Emma and Simeon, 2008). The study of the influence of growth regulators and element have been dwelling in the control, maintenance and improvement of plant physiology and metabolism (Zohreh and Morteza, 2005; Kumar et al., 2009; Das, 2010).

Progresses were made in plants in general as well as in roses in particular to physiological stress resistance (Cid et al., 1984; Alexandra et al., 2005; Yehoshua et al., 2009). Studies focused on rose tissue engineering, including cut rose response to low temperature (John and Shimon, 1984), mass cloning of rose via somatic

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Abbreviations: ABA, Abscisic acid; 6-BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; Df, differentiation; Dv, development; GA₃, gibberellic acid; IAA, indole-3-acetic acid; K, potassium; NAA, α -naphthleneacetic acid; N, nitrogen; P, phosphorus; PI, proliferation; RIV, 200 mM NaCl-free recovered *in vitro*; SE, standard error; SSIV, 200 mM NaCl stress *in vitro*.

embryogenesis by manipulating growth regulators and culture conditions (Das, 2010), and the physiological responses to water availability of Rosa 'Eurored' plants. Improvements in the study involving rose plant response to salinity in *Rosa hybrida* cv. and cut rose 'Frisco' for effects of sodium (Na⁺) on one hand and a decreasing nutrient emission from closed nutrient systems on the other hand, through high irrigation rates in the presence of accumulated Na and Cl (Lorenzo et al., 2000; Baas and van der Berg, 2004).

The specific needs of China rose for industrial raw material, wide culture and growth in a salt stress environment justify continued research for enhanced varieties. To the best of our knowledge, no report has yet mentioned the study of China rose tissue engineering in response to salinity stress and therefore, is still without positive salt stress response. Explants were cultured in 200 mM NaCl- containing tissue culture medium, associated with different embryogenic media designed orthogonally. Plantlets with weak adaptation to whole plants were induced to recover in a NaCl-free medium. The goal of this work was to investigate the influence of salinity stress on China rose tissue regeneration, physical changes and physiological response.

MATERIALS AND METHODS

Plant material

R. Chinensis Jacq. (China rose) was grown from surface disinfected internodes fragments obtained from fresh cultured plants in the green house. The plants in the green house were cultured by "taking of cutting", giving 2 to 3 fresh shoots from which nodal fragments were harvested and used as explants. These explants were surface-sterilized with 50% (w/v) sodium hypochlorite for 10 min under gentle agitation and washed three times with sterile deionized water before inoculation into a callus induction medium.

Culture media and conditions

The basal media consisted of MS (Murashige and Skoog, 1962) with 200 mM NaCl. Salinity stress was alleviated at the stage of plantlet and rooting development. Embryogenic (Dv, PI and Df) media were supplemented with different concentrations of growth regulators and arranged according to Taguchi methods for orthogonal experiment design (Roy, 1990). All cultures were maintained in a growth chamber at 25 ± 1 °C in the dark or under a 16 h photoperiod, using a total irradiance of 125 to 150 μmol m⁻² s⁻¹ provided by cool-white fluorescent light.

(i) Callus growth media were supplemented with 2.00 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1.50 mg/L zeatin, pH 5.7. Cultures in Petri dishes were sealed with parafilm and incubate in the dark for 4 to 5 weeks;

(ii) Well developed calli were successively transferred to the orthogonally experimentally designed embryogenic development, proliferation and differentiation media according to the following groups of three parameters and 3 levels respectively [(A = zeatin mg/L: A₁ = 1.00; A₂ = 1.50; A₃ = 2.00), (B = α-naphthleneacetic acid (NAA) mg/L: B₁ = 0.10; B₂ = 0.25; B₃ = 0.30) and (C = gibberellic

acid (GA₃) mg/L: C₁ = 0.50; C₂ = 1.00; C₃ = 1.50)]; [(A = 2,4-D mg/L: A₁ = 3.00; A₂ = 5.00; A₃ = 7.00), (B = Zeatin mg/L: B₁ = 0.50; B₂ = 1.00; B₃ = 1.50) and (C = GA₃ mg/L: C₁ = 0.50; C₂ = 1.00; C₃ = 1.50)]; [(A = Zeatin mg/L: A₁ = 1.00; A₂ = 1.50; A₃ = 2.00), (B = GA₃ mg/L: B₁ = 0.50; B₂ = 1.00; B₃ = 1.50) and (C = abscisic acid (ABA) mg/L: C₁ = 1.00; C₂ = 2.00; C₃ = 3.00)]. Cultures were accomplished in 6, 4 to 5 and 3 weeks, pH 5.7, 5.5 and 5.5 under light conditions respectively for embryo Dv, PI and Df; (iii) Fully germinated embryos were transferred to plantlet medium based on 1/2 MS (200 mM NaCl-free/not) salts supplemented with 3.0 mg/L 6-benzylaminopurine (6-BA), 0.3 mg/L indole-3-acetic acid (IAA), pH 5.8 and cultured for 4 to 5 weeks in the presence of light. Plantlets bearing three or more leaves were transferred to rooting medium based on 1/2 MS (200 mM NaCl-free/not) salts supplemented with 4 mg/L IAA, pH 5.6, and then transplanted into mix vermiculite pots soil (1/1).

Embryogenesis rates were expressed as the percentage of well developed embryos to the induced callus. Some physical aspects of embryos were noted: dead and well developed cells. Plant regeneration could confirm the efficiency of the culture medium.

Total chlorophyll and proline determination

The amount of total chlorophyll as well as proline was determined on young fresh leaves of SSIV plantlets and RIV plants before transplantation. Chlorophyll fluorescence was measured in triplicate as described in Min et al. (2009).

Proline content was measured according to Monreal et al. (2007) with some modifications. Each sample was harvested, soaked into liquid nitrogen and stored at -80 °C until use. For experimental condition, leaf samples were washed, divided into small pieces with an electric blender, mixed thoroughly, distributed in several portions (30 g) and stored at -20 °C. Proline was quantified by the acid-ninhydrin procedure. Leaf samples of 0.5 g were ground with 3% sulphosalicylic acid (10 ml) and clarified by centrifugation. 2 ml of the supernatant was mixed with an equal volume of acid-ninhydrin and acetic acid, the mixture was oven incubated at 100 °C for 1 h, and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 ml toluene and absorbance was read at 517 nm, using toluene as a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis. Each sample was measured in triplicate.

Measurement of Potassium (K), Nitrogen (N) and Phosphorus (P)

K was measured as described (Zhang et al., 2009). For N and P analysis, leave samples were dried in the oven at 105 °C for 15 min and then at 65 °C for 3 days. Dried material was first milled to a fine powder and 1 ± 0.001 g samples were then digested in a hydrogen peroxide-sulphuric acid digestion mixture as by the Kjeldahl procedure. Standard colorimetric assays were used to determine N and P (Anderson and Ingram, 1993). All N and P measurements gave the total elemental N and P (organic plus inorganic) present in the plant tissue. The macroelements (K, N, and P) were determined on young fresh leaves of SSIV and RIV plantlets preparing for transplantation.

Measurement of soil water, salt and pH values

The soil of our experimental farm was divided into 6 sections as represented in Table 1. 30 g portions of fine mix surface and subsoil were sampled from each site, and the mass (m) loss on

Table 1. Embryogenesis from different calli excised from 200 mM NaCl-stress nodal stem segments cultured in the absence of light on MS medium supplemented with 2.00 mg/L 2,4-D and 1.50 mg/L zeatin.

Experiment number	Dv (%)	PI (%)	Df (%)
1	60.06±0.04	1.77±0.09	20.32±0.27
2	70.23±0.20	50.00±0.00	40.42±0.39
3	87.34±0.15	75.17±0.15	65.03±0.03
4	72.67±0.06	87.52±0.03	50.27±0.02
5	80.11±0.11	100.00±0.00	60.31±0.27
6	70.21±0.21	18.73±0.02	20.85±0.09
7	90.21±0.30	100.00±0.00	60.8±0.16
8	54.55±0.03	12.60±0.24	25.25±0.21
9	100.00±0.00	23.06±0.02	50.13±0.11

Embryos Dv, PI and Df were cultured on a stressed MS media supplemented with different concentrations of plant growth regulators, after 6, 4 to 5 and 3 weeks of culture, respectively. Data are given as mean ± standard error (SE) of development in each medium composition of each experiment.

oven heating at 50°C for a week was determined according to the formula:

$$m_{\text{water}} = m_{\text{wet}} - m_{\text{dry}}$$

Salt content was determined by taking a fine mix portion of surface and subsoil samples from each site. Large aggregates were crushed and removed with any gravel or particle so that the study will have a fine mixture to test. The soil was oven-dried at 40°C for a week, and weighed. The dry samples were placed in a jar with addition of distilled water to a ratio of 1:5, and then the jar was shaken periodically for over one hour. The mixture was stand undisturbed for half an hour or a little longer until the suspension was clear enough. The suspension was then filtrated and the salt content could be read in the FP6410-flame photometer, referring to the manual. Thus, values were calculated according to the following formula:

$$\text{Soil Na}^+(\text{mg/g DW}) = \text{tested filtrate Na}^+ \times \text{water volume} / \text{soil dry weight (mg DW)} \times \text{dilution factor.}$$

Purified Na⁺ was used for standardization

For pH determination, 5 g of soil was weighed in test tubes, with an addition of 25 ml of distilled water and was shaken for about 2 to 3 min. The soil was allowed to settle for 2 min, followed by the suspension filtration before pH measurement using the PHS-3C Precision pH Meter. Every rough measurement ensured a 1:5 soil dilution.

Statistical analysis

All data were subjected to analysis using the SAS software. The experiments were repeated at least three times, using groups of 25 explants. The rates of embryos Dv, PI and Df from induced callus were recorded after 6, 4 to 5 and 3 weeks respectively. Variances in orthogonal experiment were analyzed at a 5% probability level. Regenerated plant physiological parameters were also determined and a two-tailed tests statistical procedure was used to compare the null and alternative hypothesis that: H: $\mu = \mu_0$ and H1: $\mu \neq \mu_0$.

RESULTS

The effects of NaCl on tissue regeneration capacity were

investigated using the nodal stem segment as explant isolated from greenhouse-grown plants. Stress-induced calli from explants were subcultured on 9 different media composition for embryo Dv, PI and Df. A substantial amount of data were obtained by evaluating the percentage of developed, proliferated and differentiated embryo irrespective of the medium composition and generating a convenient media (Table1) for a better NaCl stress embryogenesis. Physical characteristics of tissue regeneration under stress condition (Figures 1, 2 and 3) and physiological analysis of control, SSIV and RIV plantlets (Figures 4 and 5) were obtained on tissue regenerated from the convenient media for embryogenesis and NaCl-free plant recovery.

Efficiency of medium composition on embryo development, proliferation and differentiation under NaCl stress conditions

Upon transfer of well developed callus from precultured explants on MS medium supplemented with 2.00 mg/L 2,4-D and 1.50 mg/L zeatin to nine embryogenic tissue media all under salinity stress (200 mM NaCl) conditions irrespective of the culture media, embryo development ranged between 60.06±0.04 and 100.00±0.00%. Developed embryos were transferred to a proliferation medium relatively to its serial experiment number, and proliferated embryos ranged between 1.77±0.09 and 100.00±0.00%. In the same way, proliferated embryos were transferred to a differentiation medium, and differentiated embryos ranged between 20.32±0.27 and 65.03±0.03%. It was clear that the differences between percentages of embryos developmental stage were significantly influenced by the variation of some parameters.

However, analysis of the orthogonal experimental results provided important information on the influence of each element variation on the culture medium (Table 1). The Dv medium was significantly influenced by zeatin

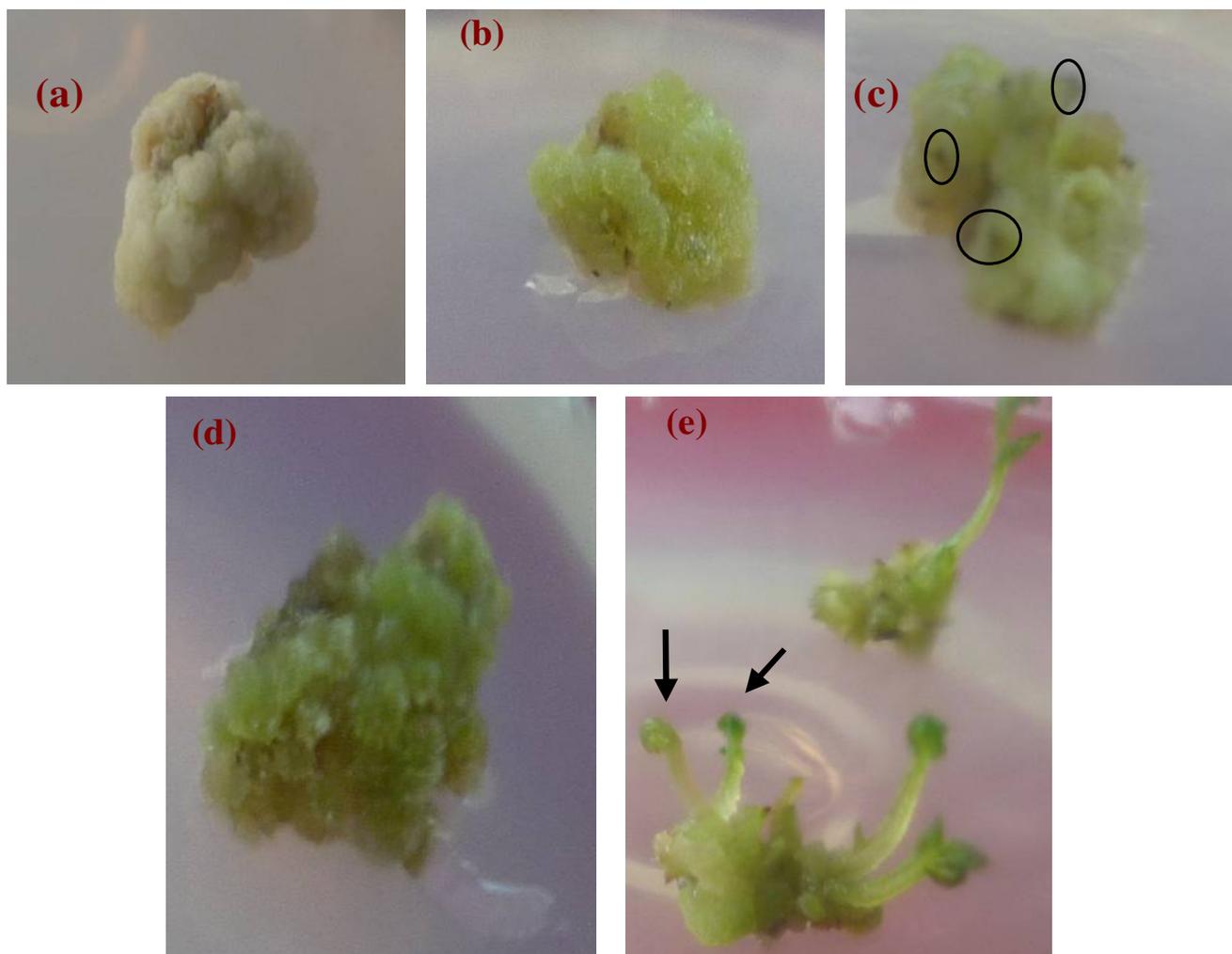


Figure 1. *In vitro* regeneration, callogenesis and embryogenesis of *R. chinensis* Jacq. in the most efficient medium with 200 mM NaCl. (a) Callus induced from intermodal segment cultured on MS medium supplemented with 2.00 mg/L 2, 4-D, 1.50 mg/L zeatin, pH 5.7, after 4 to 5 weeks in darkness. (b) Embryo formed from induced callus cultured in MS medium supplemented with 2.00 mg/L zeatin, 0.30 mg/L NAA and 1.50 mg/L GA₃, pH 5.7, after 6 weeks in the presence of light. (c) Embryo proliferated in MS medium supplemented with 5.00 mg/L 2, 4-D, 0.50 mg/L zeatin, 1.50 mg/L GA₃, pH 5.5, after 5 weeks in the presence of light. Circles represent the initiation of death to unadapted embryo. (d) Differentiated embryo in MS medium supplemented with 2.00 mg/L zeatin, 1.50 mg/L GA₃, 3.00 mg/L ABA, pH 5.5, after 5 weeks in the presence of light. (e) Shoots developed from differentiated embryo (also called here SSIV: 200mM NaCl Stress In Vitro plantlets) in 1/2 MS medium supplemented with 3.0 mg/L 6-BA, 0.3 mg/L IAA, pH 5.8, after 5 weeks in the presence of light. Arrows represent thick bend shape of induced plantlets.

and GA₃ concentrations. Their variation could highly affect embryo development under salt stress conditions, and analysis of variance suggested 2.00 mg/L zeatin, 0.30 mg/L NAA and 1.50 mg/L GA₃ producing 100±0.00% developed embryo under 200 mM salt stress. The PI medium was not significantly influenced by GA₃ variation under salt stress conditions and analysis of variance suggested 5.00 mg/L 2,4-D, 0.50 mg/L zeatin and 1.50 mg/L GA₃, producing 88.35±0.68% proliferated embryo. The Df medium was significantly influenced by the variation of ABA concentration under salt stress conditions and analysis of variance suggested 2.00 mg/L zeatin, 1.50 mg/L GA₃ and 3.00 mg/L ABA, producing

51.59±1.78% differentiated embryos (Table 2). The most productive results were achieved when the Dv, PI and Df media contained a combination of (2.00 mg/L zeatin, 0.30 mg/L NAA, 1.50 mg/L GA₃); (5.00 mg/L 2,4-D, 0.50 mg/L zeatin, 1.50 mg/L GA₃) and (2.00 mg/L zeatin, 1.50 mg/L GA₃, 3.00 mg/L ABA) respectively as suggested in Figure 1.

Physical characteristics of tissue development and Influence of NaCl stress on Shoots regeneration

Shoot production via the development of preexisting



Figure 2. Plantlets recovery and regeneration to a whole plant on a 200 mM NaCl-free medium. (a) Shoots developed from differentiated embryo in the presence of NaCl (also called here SSIV: 200 mM NaCl stress *in vitro* plantlets) were transferred to NaCl-free 1/2 MS medium supplemented with 3.0 mg/L 6-BA, 0.3 mg/L IAA, and pH 5.8. Plantlets recovery was observed after 4 weeks of culture under light conditions. (b) Recovered plants induced to rooting (also called here RIV: 200mM NaCl-free recovered *in vitro* plantlets) in 1/2 MS supplemented with 4 mg/L IAA, pH 5.6. (c) Regenerated plants transferred into mix vermiculite pots soil (1/1) and cultured in greenhouse.

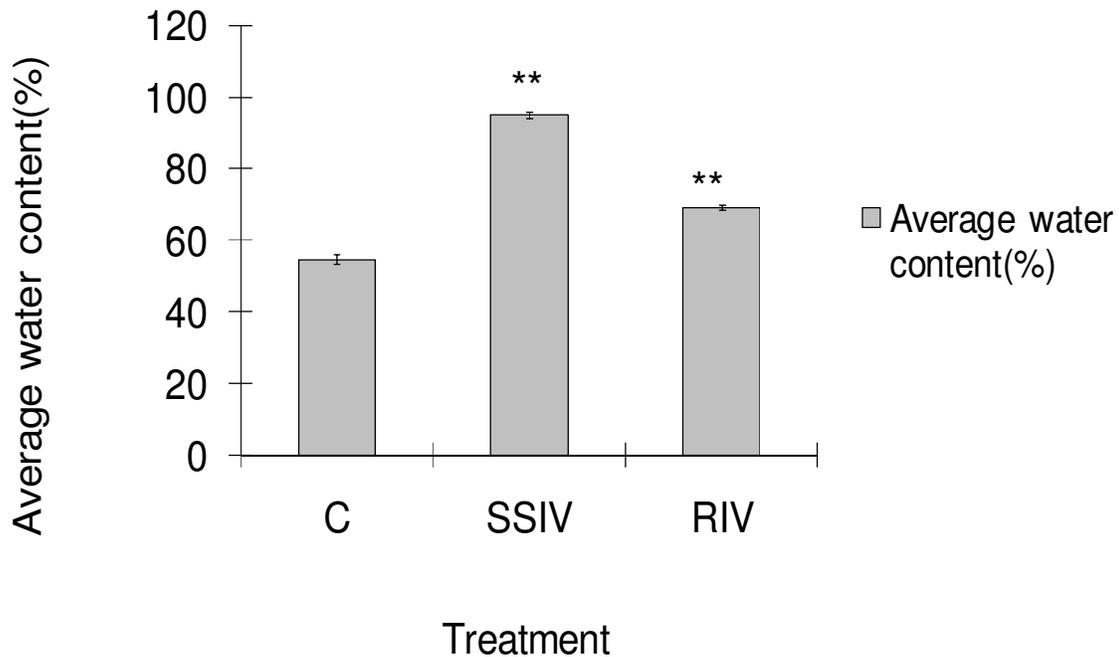


Figure 3. Average water content of fresh leaf in different developmental stage. C = Control plants from greenhouse cut culture; SSIV = 200 mM NaCl stress *in vitro* plantlets; RIV = 200 mM NaCl-free Recovered *in vitro* plantlets; ** = values are significantly different from control plants at the 5% probability level. Each value is the mean of three replicons ± SE.

meristems was observed from nodal stem segments (1 to 4 shoots/explant) induced for callusgenesis and embryogenesis in culture media each containing 200 mM

NaCl. A differentiated calli was incised to embryogenic tissue medium and yellowish turned green after 3 weeks of culture as somatic embryos was formed. These

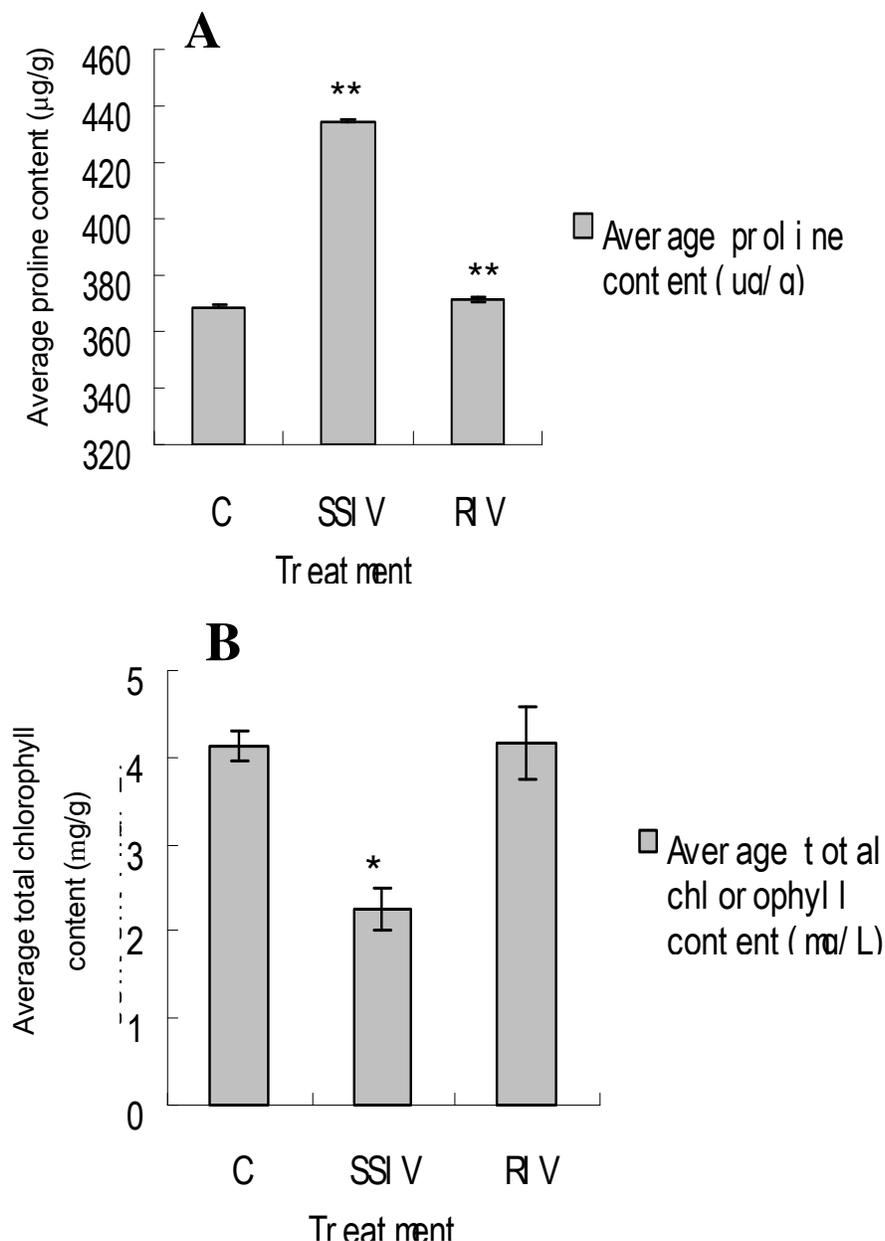


Figure 4. Proline and total chlorophyll content of fresh leaf in: C = Control plants from greenhouse cut culture; SSIV = 200 mM NaCl stress *in vitro* plantlets; RIV = 200 mM NaCl-free recovered *in vitro* plantlets. A, B are respectively proline and total chlorophyll accumulation. *; ** = values are respectively not significantly and significantly different from control plantlets at the 5.00% probability level. Each value is the mean of three replicons \pm SE.

embryos proliferated, differentiated and germinated efficiently in the appropriate medium described above, providing shoots and plantlets.

Non adapted embryo died, turning into dark, while adapted cells grew normally in a 200 mM NaCl containing culture medium. Although callus development, embryo formation, proliferation and differentiation could in certain growth regulator concentrations be processed, shoot induction and plantlet development were highly

influenced with only very few (1 to 4 shoots) improper shoots development induced and mostly thick-bent leaf shape in salinity stress conditions (Figure 1).

Rose development of plantlets from undifferentiated cells in tissue culture was successfully regenerated in a stressed tissue culture medium (200 mM of NaCl) (Figure 1). Leaves in plantlet medium presented a thick and bent shape with high cellular water content (Figure 3). Under salt stress conditions, plantlets were unable to develop

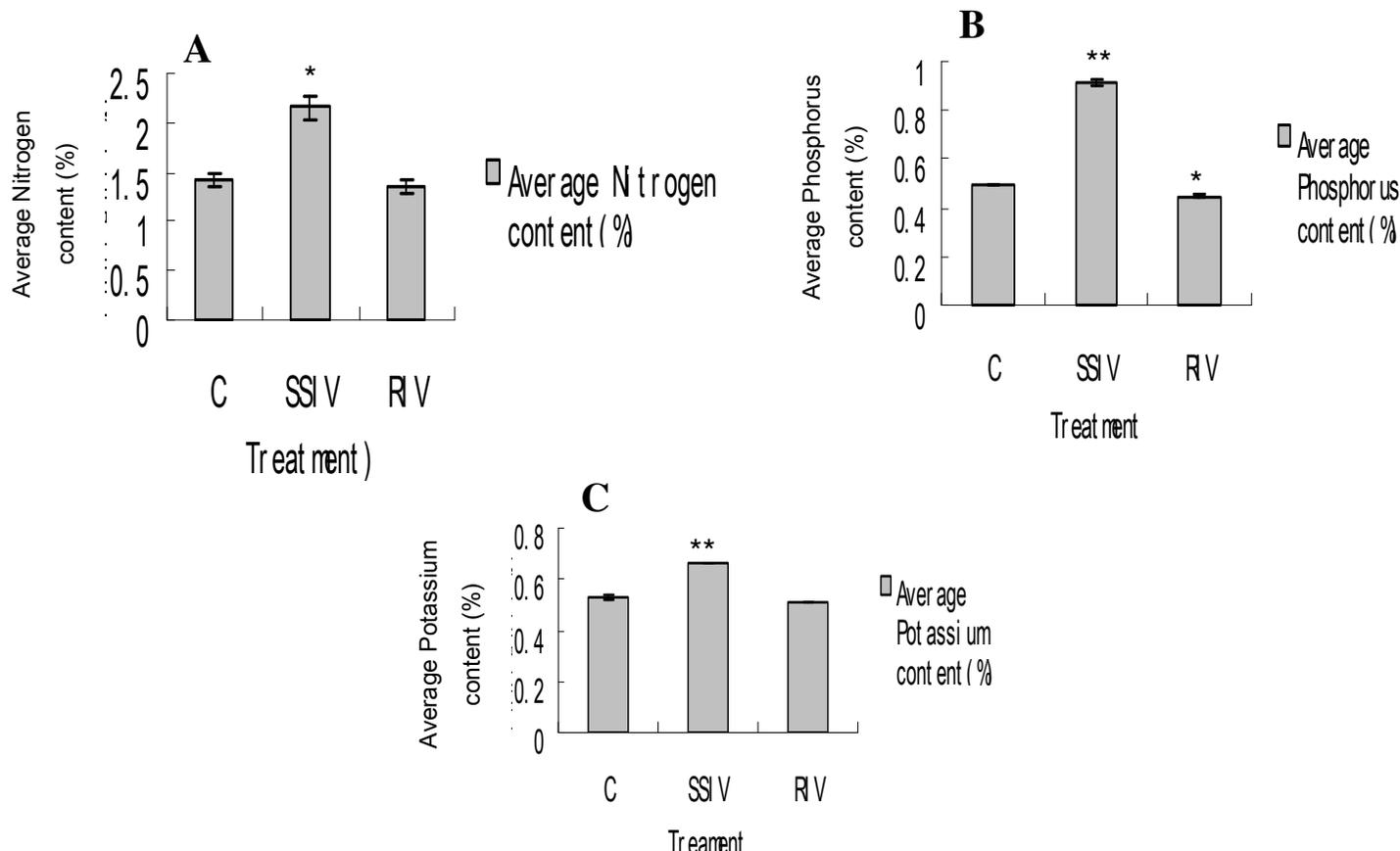


Figure 5. Macroelement N, P and K content of dry leaf in: C = Control plants from greenhouse cut culture; SSIV = 200 mM NaCl stress *in vitro* plantlets; RIV = 200 mM NaCl-free Recovered *in vitro* plantlets. A, B and C are respectively N, P and K percentage of leaves dry weight. *, ** = values are respectively not significantly and significantly different from control plantlets at the 5.00% probability level. Each value is the mean of three replicons ± SE.

Table 2. 200 mM NaCl-stress embryogenesis on MS media supplemented with the most productive combination of growth regulators and percentage of developed embryos at the stage of: embryo Dv, tissue PI and Df after 6, 5 and 3 weeks of culture respectively.

Medium	Dv	PI	Df
Most productive combination of growth regulators	Zeatin: 2.00 mg/L, NAA: 0.30 mg/L, GA ₃ : 1.50 mg/L	2,4-D: 5.00 mg/L, zeatin: 0.50 mg/L, GA ₃ : 1.50 mg/L	zeatin: 2.00 mg/L, GA ₃ : 1.50 mg/L, ABA: 3.00 mg/L
Percentage of well developed embryo (%) ^a	100±0.00	88.35±0.68	51.59±1.78

^aData are given as mean ± SE of development in each medium composition of each recommended experiment.

and induced to death after transplantation despite abundant absorption and distribution of water; this could not compensate the amount of water loosed by transpiration and induced a low survival rate (nearly zero percent). The soil of our experimental farm growing wild type was analyzed and proved to be in convenient conditions for the growth of plants (Table 3) from which explants were sampled. Plantlet development on 1/2 MS salts supplemented with 3.0 mg/L 6-BA and 0.3 mg/L IAA

was found to be highly influenced by the addition of NaCl to the medium. Therefore, shoots developing in a salt stress medium were excised and induced to develop in a medium without salt stress. Plantlets gradually recovered, turning to normal development and leaves observed to rapidly loose their water by transpiration. Plantlets were then transferred to a rooting medium based on 1/2 MS 200 mM NaCl-free supplemented with 4 mg/L IAA, and further transplanted to pot soil mix vermiculite and perlite

Table 3. Soil water, salt content and pH value of our experimental field. The field was divided into six sections, water, salt content and pH value were analyzed in each section and from which the average values were obtained.

	North1	North2	North3	South1	South2	South3	Average ^a
Water content	9.009 g	9.007 g	9.010 g	8.998 g	9.011 g	9.006 g	9.006±0.004
pH	8.77	8.78	8.70	8.67	8.73	8.69	8.72±0.04
salt content (‰)	10.0	6.1	6.8	5.9	7.6	7.2	7.2±1.48

^aAverage of water, salt content and pH value are given as mean ± SE.

(1/1) and cultured (Figure 2) in the greenhouse as normal plants.

Physiological analysis of control, SSIV and RIV plants

To assess the impact of stress-associated changes in plants physiology, total chlorophyll, proline, macroelements P, N and K were analyzed on plants that had received the same treatment schemes. Plantlets fresh leaves were all sampled from control (cut cultured plants from which explants for tissue culture were obtained), SSIV, RIV, and analyzed in triplicates for their macroelements (K, N, and P), as well as chlorophyll and proline content. Cultures were conducted under a temperature of 18 to 26°C, and 12 h / d light intensity of 125 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Proline and total chlorophyll analysis

Plantlets induced from tissue culture under salt stress conditions were found with a non significant (5% probability level) reduced amount of total chlorophyll (Chlorophyll a + b) in leaves of SSIV and RIV compare to control and recovered plantlets (Figure 4B). These results indicated that, the photosynthetic capacities of plantlets remained higher during salt treatment in control plantlets than those of SSIV and RIV.

In contrary, plantlets induced from tissue culture under salt stress conditions were found with increased proline content in fresh leaves of SSIV and RIV compare to control (Figure 4A). Salt induced proline accumulation was significantly higher at a 5% probability level in SSIV and RIV compare to control.

Macroelements P, N and K analysis

Plantlets induced from tissue culture under salt stress conditions were found with increased amount of P, N and K in fresh leaves compare to control and recovered plantlets. P and K contents were significantly different in SSIV, whereas N content was different but not significantly in SSIV, all compare to their respective

control plantlets (Figures 5A, B and C) at a 5% probability level.

DISCUSSION

This study describes the effect of growth regulators and NaCl stress on embryogenesis and shoot regeneration capacity using nodal stem segments excised from greenhouse grown *R. chinensis* Jacq. on one hand, and the determination of plants physiological activity on the other hand. Plant regeneration was obtained through the development of preexisting meristems to callus under NaCl stress, followed by the study of growth regulators and NaCl stress on embryogenesis, then the study of NaCl effect on shoot regeneration. These processes were studied using MS or 1/2 MS basal media.

Plant growth regulators have been studied in plants and tissue culture and found to negatively or positively affect tissue development when added to a certain concentration. Several studies described the appropriate media for better rose plant regeneration (Zohreh and Morteza, 2005; Kumar et al., 2009; Das, 2010). We used the orthogonal experimental design at the Dv, PI and Df stage to describe a possible protocol of salt stress tissue regeneration of *R. chinensis* Jacq. with 200 mM NaCl as induced stress. When we compared the different growth media irrespective of embryogenesis and regeneration media (Table 1, Figures 1 and 2), it was clear that those media containing high concentrations of zeatin (2.00 mg/L), NAA (0.30 mg/L) and GA₃ (1.50 mg/L) were the most effective in embryos development. In the same way, high concentrations of zeatin (2.00 mg/L), NAA (1.50 mg/L) and ABA (3.00 mg/L) were the most effective in embryo differentiation. The media containing relatively high concentration of 2,4-D (5.00 mg/L), low concentration of zeatin (0.50 mg/L) and high concentration of GA₃ (1.50 mg/L) were the most effective in embryo proliferation. These plants growth regulator concentrations were efficient in the maintenance of embryogenesis under salt stress conditions. High zeatin and GA₃ concentrations were previously reported by Fusun et al. (2003) who demonstrated that their high content in leaves of wheat (*P. acutifolius*) induced plant resistance to salt. This indicates that the absorption of plant regulators by plant tissue may provides salt resistance and that,

despite high zeatin and GA₃, high ABA and NAA could also provide a resistance effect to NaCl stress. A combination with low concentration of zeatin is required for a most productive embryo differentiation. Effective embryogenesis may be due to the regulation of growth regulators and metabolism in cellular compartment. Although plantlets were improperly developed with thick bent leaf shape under stress condition, they could recover well in plantlet medium until rooting without any salt concentration.

We report that *R. chinensis* Jacq. was successfully regenerated in a convenient tissue culture media under salt stress conditions. Meanwhile, plantlets improperly developed with thick bent leaves shape, unable to survive under salinity stress, presenting drastic death after inoculation into plantlets or rooting medium. After transplantation to salinity stress medium, its adaptation, including the maintenance of high cellular water content conferring resistance to plantlet was influenced by salt stress. Transplants loosed cellular water content by transpiration and changes in leaves shape from thick to bend was view as the possible main reason of high death rate of plantlets. The comparison of two different shoot regeneration media irrespective of the growth regulator compositions revealed that plantlet were sensitive to salt stress, and no addition of NaCl were the most prolific treatment for shoot regeneration and potted plantlets survival. Changes in leaves shape could provide easily survival plants in salt-free medium with high absorption and water transpiration (Figure 3). These results were consistent with the findings of Sinclair and Hoffmann (2003) who reported that leaf size, shape and maximum internode length were tested to be sensitive to salinity stress, and have been potential tools for monitoring salinity in vineyards. Those traits variability, including high water absorption and plant transpiration may considerably affect plant response to stress, although it is difficult to separate genetic and environmental factors.

We also evaluated the effect of NaCl on plant physiological activity. All regenerated plantlets were confirmed of physiological function by total chlorophyll and proline analysis. Furthermore, macromolecules N, P and K content were also determined. NaCl stimulated response to resistant plantlets by decreasing total chlorophyll concentration in SSIV plantlets to 2.25±0.24 mg/L. This concentration was increased after alleviation of salt stress in RIV plantlets to 4.18±0.42 mg/L. In contrary, Proline, as well as K, N and P were increased in SSIV plantlets and decreased (except for proline) in RIV plants. These chlorophyll content, proline and important macroelements variability play a key role in the plant physiology. Mark et al. (2009) described K and P availability to be integrated at the whole plant level into physiological and metabolic adaptations. The higher level of proline in SSIV (434.75±0.44 µg/g) and RIV (371.42±0.88 µg/g) could be attributed to the resistance effect of salt stress and to the ability of the recovered plantlet to produce resistance effect respectively.

Significant changes observed in total chlorophyll, N, K and P were absolute for plantlets recovery and transplants survival. This in accordance to previous studies which demonstrated that carbohydrates are supplied mainly through the process of photosynthesis whose rates are usually lower in plants exposed to salinity and especially to NaCl (Ashraf and Harris, 2004; Parida and Das, 2005).

Conclusion

It was evident that after embryogenesis on convenient media under stress condition, alleviation of NaCl stress could provide better adaptation and recovery to plantlets. Meanwhile, although proline was reduced in recovered plantlets (371.42±0.88 µg/g), this value was still significantly higher compare to control (368.79±0.58 µg/g). Also, water retention due to relief of salinity stress was still significantly higher in RIV (69.24±0.65%) plantlets compare to control (54.67±1.31%). The plantlet ability of water retention, proline synthesis and availability could unravel the regulation of metabolism in its new environment. The enhanced regeneration protocol described in this report is expected to contribute to the efforts of biotechnological improvement of Chinese rose, since the establishment of its regeneration from salt stress might improve the plant physiological activity.

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