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In Vitro Flowering of Miniature Roses (*Rosa*×*hybrida* L. 'Red Imp') in Response to Salt Stress

S. Cha-um and C. Kirdmanee
(National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand)

Summary

Miniature rose plantlets in the flowering stage were photoautotrophically grown on MS (Murashige and Skoog 1962) medium and subsequently exposed to 0 (control), 25, 50 or 100 mM NaCl for 14 d. The Na^+ in the leaf tissues of salt stressed plantlets was enriched, resulting in proline accumulation and the degradation of total chlorophyll. Pigment degradation, chlorophyll a fluorescence diminution and net photosynthetic rate (P_n) reduction in miniature rose plantlets exposed to 100 mM NaCl were maximized when compared to the control, especially P_n reduction (77.96 % of control), leading to growth reduction. In contrast, the proline content in the salt stressed plantlets was enriched, indicating salt stress. In plants cultivated *in vitro*, the flowering percentage and the number of flowers per plantlet declined significantly under both 50 and 100 mM NaCl salt stress. The flowering stage of miniature rose plantlets was susceptible to salt stress, the reproductive developmental process being suppressed, which may be an effective indicator for salt tolerance screening in rose genotypes.

Key words. chlorophyll a fluorescence – flower initiation – growth reduction – net-photosynthetic rate – pigment degradation

Introduction

Salt-affected soil is a severe environmental problem, limiting plant growth and development, leading to loss of productivity, especially in horticultural crop species (Shannon and Grieve 1999; Hasegawa et al. 2000). Salt affected soils are identified by excessive levels of water-soluble salts, including sodium chloride (NaCl), sodium sulfate (Na_2SO_4), calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$), among others (Tanji 2002). In Thailand, inland salt-affected soil is located in the North-Eastern region and amounts to 3 million ha (40 % of the agricultural area), and is defined as having underground rock salt (Yuvaniyama et al. 1996). Moreover, shrimp production using sea water which is then released untreated into the natural fresh water, is a major problem in the central area of Thailand, where it has led to an increase in the area affected by inland soil salinity (>22,000 ha) (Miller et al. 1999; Flaherty et al. 2000). In saline soil, NaCl is a major salt contaminant. It is a small molecule, which when oxidized by water forms sodium ions (Na^+) and chloride ions (Cl^-), which are easily absorbed by the root cells of higher plants and transferred to the whole plant through the xylem uploading channel (Maathuis and Amtmann 1999; Tester and Davenport 2003). These toxic ions cause ionic and osmotic stress at the cellular level of higher plants, especially in susceptible species (Mansour and Salama 2004; Chinnusamy et al. 2005). Glycophyte species are salt susceptible and exhibit wilting, chlorosis, necrosis, burning, senescence, slow growth and loss of productivity (Lutts et al. 1999; Shannon and Grieve 1999). The reproductive stage in plant growth and development has been reported as sensitive to salt stress in many plant species, including roses (Safi et al. 2005; Reezi et al. 2009). The treatment of salt water is a promising way of reducing salt contamination of irrigation for horticultural crops, including roses, carnations (Safi et al. 2005) and melons (Miranda et al. 2008).

Roses are an important horticulture crop, with a high value in the international trade both as cut flowers and in pot culture. *In vitro* flowering of roses has been reported on in the cultivars 'Parade' (Wang et al. 2002) and 'First Prize' (Vu et al. 2006) but the investigation into *in vitro* flowering of roses in response to salt stress has not been reported on previously. The aim of this research was to investigate the physiological and biochemical changes in miniature roses in response to salt stress during *in vitro* flowering.

Materials and Methods

Plant materials and treatments

Shoots of a miniature rose (*Rosa*×*hybrida* L. cv. 'Red Imp') were propagated on MS medium (Murashige and Skoog 1962) containing 8.88 μM benzyl adenine (BA), 3 % sucrose and 0.25 % Phytagel® for 6 weeks. Multiple shoots were elongated on MS medium without plant growth regulators for 2 weeks. Then, single shoots (1.5±0.2 cm in

height) were excised and roots were induced on MS medium supplemented with 2.46 μM indole butyric acid (IBA) for 2 weeks. Plantlets were cultured *in vitro*, under conditions of 25±2 °C ambient temperature, 60±5 % relative humidity (RH) and 60±5 μmol m^{-2} s^{-1} photosynthetic proton flux density (PPFD) provided by fluorescent lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) with a 16 h d^{-1} photoperiod. Rose plantlets were transferred to MS sugar-free liquid medium (photoautotrophic conditions) using vermiculite as supporting material for 7 d. The amount of air exchange in the glass vessels was adjusted to 2.32 μmol CO_2 h^{-1} by punching a hole (Ø1 cm) in the plastic cap and covering the hole with a microporous filter (0.20 μm pore size; Nihon Millipore Ltd., Tokyo, Japan). The open capped vessels containing photoautotrophic seedlings were transferred aseptically to culture chamber boxes (Carry Box Model P-850, size 26×36×19 cm, Japan) maintained at 65±5 % RH in 1.5 L NaCl-saturated solution. The amount of air exchange in the culture chambers was increased to 5.1±0.3 μmol CO_2 h^{-1} by perforating the side of the plastic chambers with 32 holes and covering each hole with gas-permeable microporous polypropylene film (0.22 μm pore size). These chambers were incubated in an EYELA plant growth incubator at a temperature of 28±2 °C/25±2 °C (12 h photoperiod/12 h dark-period), 500±100 μmol mol^{-1} CO_2 concentration, 60±5 % RH, and 120±5 μmol m^{-2} s^{-1} PPFD by fluorescent lamps, for 14 d (Cha-um et al. 2009). The salt concentration in the culture medium was adjusted to 0 (control), 25, 50 or 100 mM NaCl for 14 d. Sodium ions (Na^+), potassium ions (K^+), photosynthetic pigments, proline content, chlorophyll a fluorescence, net-photosynthetic rate (P_n), growth characters, flower initiation and flower numbers were measured.

Data collection

One-hundred milligrams of leaf tissue was ground in liquid nitrogen. Sodium and potassium ions in the crude leaves were extracted by the acidic method (HNO_3 and $HClO_4$) and assayed according to Dionisio-Sese and Tobita (1998) using an atomic absorption spectrophotometer (AA, Model M6, Thermo Elemental, MA, USA).

Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC), were analysed following the methods of Shabala et al. (1998) and the total carotenoids (C_{x+c}) concentration was assayed according to Lichtenthaler (1987). One hundred milligrams of leaf material was collected and placed in a 25 mL glass vial, along with 10 mL 95.5 % acetone, and blended using a homogeniser. The glass vials were sealed with parafilm to prevent evaporation, and then stored at 4 °C for 48 h. Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer at 662 nm and 644 nm wavelengths. The C_{x+c} concentration was also measured by spectrophotometer at 470 nm. A solution of 95.5 % acetone was used as a blank.

Proline in the leaf tissues was extracted and analysed according to the method of Bates et al. (1973). Fifty-milligrams of fresh material was ground with liquid nitrogen in a mortar and the homogenate powder mixed with 1 mL aqueous sulfosalicylic acid (3 % w/v) and filtered through filter paper (Whatman #1, England). The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M H_3PO_4) and incubated at 95 °C for 1 h. The reaction was terminated by placing the container in an ice bath. The reaction mixture was mixed vigorously with 2 mL toluene. After cooling to 25 °C, the chromophore was measured by spectrophotometer DR/4000 at 520 nm using L-proline as a standard.

Chlorophyll a fluorescence emission from the adaxial surface on the second-fully leaf of the was measured using a fluorescence monitoring system in the pulse amplitude modulation mode, as previously described by Loggini et al. (1999). A leaf, adapted to dark conditions for 30 min using leaf-clips, was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (<0.5 μmol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (>6.8 μmol m^{-2} s^{-1} PAR) and calculated using FMS software for Windows®. The variable fluorescence yield (F_v) was calculated by the equation of F_m–F_0. The ratio of variable to maximum fluorescence (F_v/F_m) was calculated as maximum quantum yield of PSII photochemistry. The photon yield of PSII (Φ_{PSII}) in the light was calculated by $\Phi_{PSII} = (F_m' - F)/F_m'$ after 45 s of illumination, when steady state was achieved. In addition, photochemical quenching (qP) and non-photochemical quenching (NPQ) were calculated according to Maxwell and Johnson (2000).

Net photosynthetic rate (P_n) was calculated by comparing the different concentrations of CO_2 inside (C_{in}) and outside (C_{out}) the glass vessel containing the rose plantlets. The CO_2 concentrations at steady state were measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The P_n of *in vitro* cultivated seedlings was calculated according to the method of Fujiwara et al. (1987).

In vitro flowering percentage, number of flowers per plant, shoot height (SH), fresh weight (FW), dry weight (DW) and leaf area (LA) of rose plantlets were recorded. Rose plantlets were dried at 80 °C in a hot-air oven for 2 d, and then incubated in desiccators before the measurement of dry weight. The leaf area of rose plantlets was measured using a leaf area meter DT-scan.

Experiment design and statistical analysis

The experiment was arranged as a completely randomized design (CRD) with eight replicates (n=8). The mean values were compared by least significant difference (LSD) and analyzed by SPSS software (SPSS for Windows, SPSS Inc., Chicago, USA). Relationships between Na^+ and proline, Na^+ and TC, Chl_a and F_v/F_m, TC and Φ_{PSII}, Φ_{PSII} and P_n, P_n and plant dry weight were analysed using Pearson's correlation coefficient.

Results

Sodium ions (Na^+) in the salt stressed leaves of miniature rose plantlets accumulated depending on exogenous NaCl application in the culture media, while the level of potassium ions (K^+) decreased, causing an increase in the Na:K ratio (Table 1). Na^+ is a major toxic ion and an increase in the levels in salt stressed leaves led to proline accumulation as an organic osmolyte ($r^2 = 0.99$) (Fig. 1A), in turn damaging the chlorophyll pigment ($r^2 = 0.99$)

Table 1. Sodium (Na^+), potassium ions (K^+) and sodium/potassium ratio (Na : K) of miniature rose plantlets under salt stress for 14 d.

NaCl (mM)	Na^+	K^+	Na : K
	(mg g^{-1} DW)		
0 (Control)	0.76 d	36.44 a	0.02 c
25	5.77 c	28.29 b	0.20 c
50	14.35 b	18.77 c	0.77 b
100	31.26 a	12.61 c	2.48 a
r^2	0.991	0.933	0.946

Different letters in each column show significant difference at $P \leq 0.01$ by Least Significant Difference (LSD).
r^2: Regression coefficiency

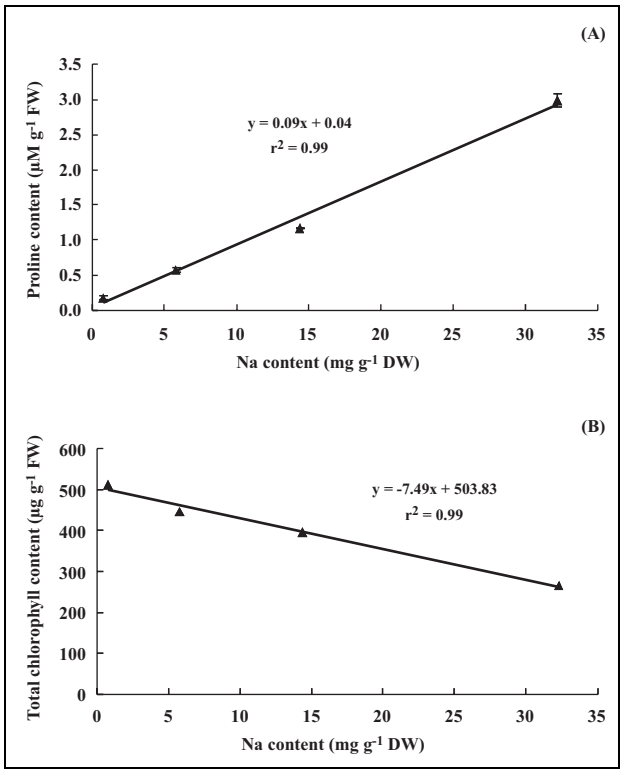


Fig. 1. Relationships between sodium ions and proline content (A), sodium ions and total chlorophyll content (B) in the leaf tissues of miniature rose plantlets under salt stress for 14 d. Error bars represent ± SE.

(Fig. 1B). Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) content in the leaf tissues of salt stressed plantlets declined significantly, relating to the NaCl salt concentration in the culture medium (Table 2). In 100 mM NaCl conditions, those pigments were degraded by 41.70, 54.42, 48.09 and 61.31 %, respectively. On the other hand, proline content in the leaf tissues was enriched by 3.17, 6.51 and 16.62 times in plants exposed to 25, 50 and 100 mM NaCl stress, respectively (Table 2). Proline accumulation in the salt stressed plantlets may play a role as an osmolyte in the plant tissues as protection against salt stress, in terms of osmotic adjustment and antioxidant defence systems. In addition, the function of photosynthetic pigments was evaluated, as represented by chlorophyll a fluorescence parameters, i.e., maximum quantum yield of PSII (F_v/F_m), quantum efficiency of PSII (Φ_{PSII}), photochemical quenching (qP) and non-photochemical quenching (NPQ). Positive correlations between Chl_a and F_v/F_m ($r^2 = 0.99$), TC and Φ_{PSII} ($r^2 = 0.95$) were demonstrated, as shown in Fig. 2A–B. F_v/F_m, Φ_{PSII}, qP in response to 100 mM NaCl stress, were significantly diminished and were lower than the control by 30.28, 41.60 and 31.64 %, respectively. The exception was NPQ, which was enhanced (Table 3). The diminution of Φ_{PSII} in salt stressed leaves was positively related to net photosynthetic rate (P_n) reduction ($r^2 = 0.89$) (Fig. 2C), leading to reduced plant dry weight ($r^2 = 0.79$) (Fig. 2D). P_n, or the CO_2 assimilation rate, in salt stressed plantlets decreased progressively, especially in 100 mM NaCl (77.96 % of the control). Shoot height, fresh weight, dry weight and leaf area of salt stressed plantlets dropped significantly, especially in 100 mM NaCl by 46.62, 57.02, 63.99 and 59.50 %, respectively when compared with the control (Table 4). The reproductive developmental process of *in vitro* miniature roses was sensitive to salt stress, especially

Table 2. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC), total carotenoids (C_{x+c}) and proline content of miniature rose plantlets under salt stress for 14 d.

NaCl (mM)	Chl_a	Chl_b	TC	C_{x+c}	Proline
	(μg g^{-1} FW)				(μmol g^{-1} FW)
0 (Control)	254.6 a	256.6 a	511.2 a	83.5 a	0.18 d
25	245.5 a	201.3 b	446.8 b	75.0 b	0.57 c
50	232.5 b	161.3 c	393.8 c	62.6 c	1.17 b
100	148.4 c	117.0 d	265.4 d	32.3 d	2.99 a
r^2	0.905	0.950	0.999	0.968	0.971

Different letters in each column show significant difference at $P \leq 0.01$ by Least Significant Difference (LSD).
r^2: Regression coefficiency

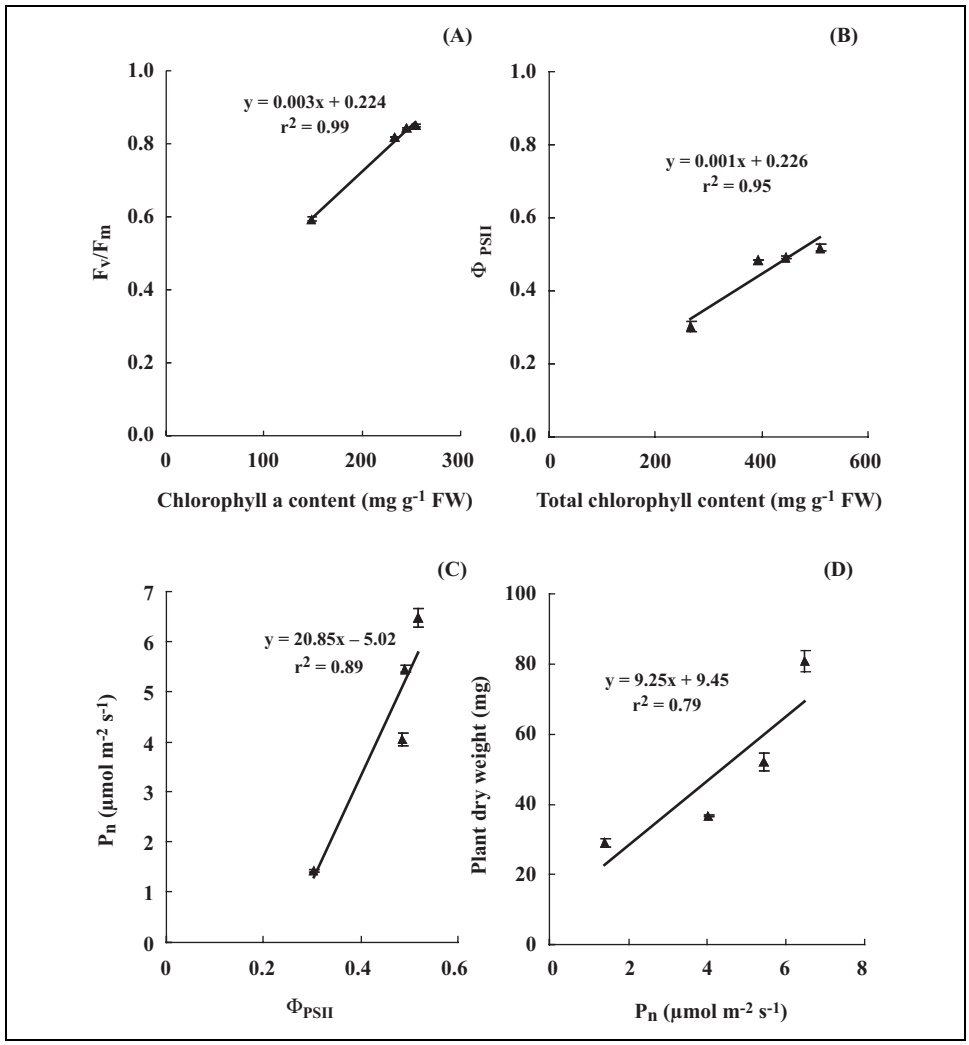


Fig. 2. Relationships between chlorophyll a content and maximum quantum yield of PSII (F_v/F_m) (A), total chlorophyll content and photon yield of PSII (Φ_{PSII}) (B), Φ_{PSII} and net photosynthetic rate (P_n) (C), and P_n and plant dry weight (D) of miniature rose plantlets under salt stress for 14 d. Error bars represent ± SE.

Table 3. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}), quantum efficiency of PSII (qP), non-photochemical quenching (NPQ) and net-photosynthetic rate (P_n), of miniature rose plantlets under salt stress for 14 d.

NaCl (mM)	F_v/F_m	Φ_{PSII}	qP	NPQ	P_n (μmol CO_2 m^{-2} s^{-1})
0 (Control)	0.852 a	0.518 a	0.642 a	0.127 d	6.47 a
25	0.842 a	0.491 a	0.620 a	0.242 c	5.45 b
50	0.818 b	0.485 a	0.479 b	0.366 b	4.04 c
100	0.594 c	0.303 b	0.439 c	0.444 a	1.43 d
r^2	0.858	0.873	0.863	0.925	0.998

Different letters in each column show significant difference at $P \leq 0.01$ by Least Significant Difference (LSD).
r^2: Regression coefficiency

in response to 50 and 100 mM NaCl. The flowering initiation percentage of *in vitro* miniature roses was reduced to 50 and 25 % when subjected to 50 and 100 mM NaCl, respectively (Fig. 3A). The number of flowers per miniature rose plant also decreased relative to NaCl salt concentration (Fig. 3B). Morphological characteristics of miniature rose flowers in the control showed the flowers were well-developed and demonstrated the maturing process, while those in the plants under salt stress showed delayed flowering and abnormal flower development (Fig. 4). The reduction in physiological and morphological parameters of salt stressed plantlets may be employed as multi-indicators for salt tolerance screening in rose genotypes.

Table 4. Shoot height (SH), fresh weight (FW), dry weight (DW) and leaf area (LA) of miniature rose plantlets under salt stress for 14 d.

NaCl (mM)	SH (cm)	FW (mg)	DW (mg)	LA (mm^2)
0 (Control)	5.9 a	360.4 a	80.7 a	1628 a
25	5.1 b	270.6 b	52.2 b	1521 a
50	4.2 c	218.3 c	36.8 c	1022 b
100	3.2 d	154.9 d	29.1 c	659 c
r^2	0.980	0.935	0.820	0.949

Different letters in each column show significant difference at $P \leq 0.01$ by Least Significant Difference (LSD).
r^2: Regression coefficiency

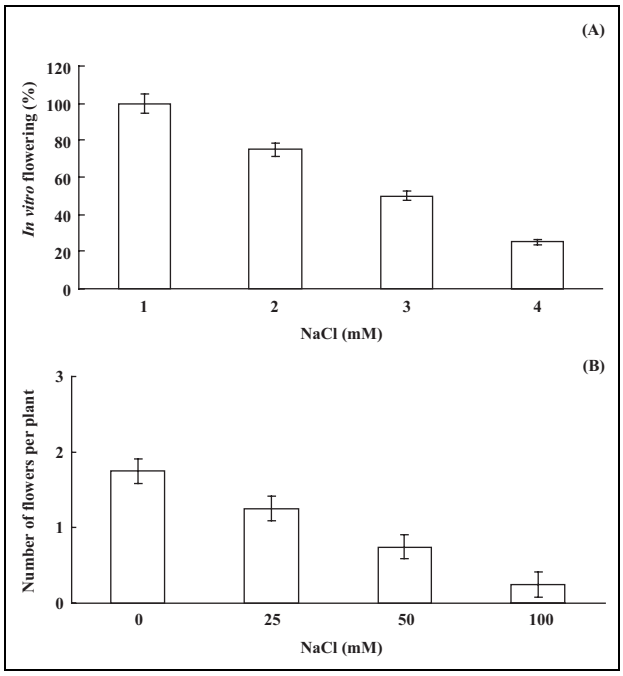


Fig. 3. *In vitro* flowering percentage (A) and number of flowers per plant (B) of miniature rose plantlets under salt stress for 14 d. Error bars represent ± SE.

Discussion

Na^+ in the leaves of salt stressed miniature roses flowering *in vitro* was enriched, relative to the exogenous NaCl treatment in the culture media, whereas the level of K^+ decreased. In *in vitro* cultivated rootstocks of roses, Na^+ has been reported as a toxic ion accumulating in the leaf tissues, the amount depending on salt concentration (0.2–0.8 % DM) and plant genotype, leading to leaf necrosis and chlorosis symptoms (Wahome et al. 2001a). Similarly, Na^+ in the leaf tissues of the rootstock roses, namely *Rosa chinensis* 'Major', peaked at 1.10 % DM, causing injury to the leaves (90.8 %) when exposed to 30 mM NaCl for 13 months in an experimental station (Wahome et al. 2001b). In hydroponic culture of roses (*Rosa×hybrida* L.), Na^+ in the salt stressed leaves reached 150 mM kg^{-1} after cultivation for 100 d in 40 mM NaCl salt treatment (Savvas et al. 2007). In addition, K^+ reduction is an inverse effect to Na^+ enrichment in the plant tissues, leading to an increased Na:K ratio, as demonstrated in cashew (Ferreira-Silva et al. 2008) and Chinese iris (Bai et al. 2008). Leaf necrosis and chlorosis symptoms are primarily evidenced when plants are subjected to salt stress, especially in salt susceptible species, including hybrid rose genotypes (Wahome et al. 2001a, b; Reezi et al. 2009). Chl_b pigment was sensitive to salt stress, and was degraded by 54.42 % when exposed to 100 mM NaCl. Similar results have been demonstrated in chrysanthemum (Pandey et al. 2009), *Populus cathayana* (Yang et al. 2009), castor bean (Li et al. 2010) and *Catharanthus roseus* (Jaleel et al. 2008), and are expressed as a sharper reduction in Chl_b than Chl_a and C_{x+c}. Chlorophyll biosynthesis has been reported as susceptible to salt stress, especially a decrease in 5-aminolaevulinic (ALA) intermediate and a regulation of chlorophyllase activity (Fang et al. 1998; Santos 2004). In senescent barley seedlings, Chl_b is very sensitive to chlorophyll b reductase (at day 2.5 of senescence) and chlorophyllase enzyme (at day 4–6 of senescence) (Scheumann et al. 1999). Also, the absorption rate of manganese (Mg^{2+}), a central pillar of the chlorophyll structure, in the salt stress condition is limited (Leidi et al. 1991). The chlorophyll content in the salt stressed leaves of *in vitro* miniature roses decreased

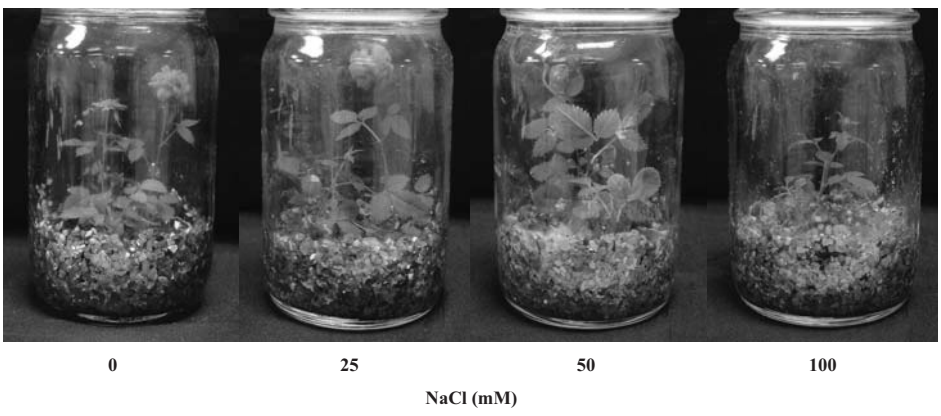


Fig. 4. Morphological characters of miniature rose plantlets cultivated under salt stress for 14 d.

significantly and was negatively related to Na^+ accumulation, while proline content gradually increased. Proline accumulation in the salt stressed leaves is a defence response of plants to salt stress (ASHRAF and FOOLAD 2007) and may play a key role as osmoregulation or osmotic adjustment in cashew (SILVEIRA et al. 2003; FERREIRA-SILVA et al. 2008) and Chinese iris (BAI et al. 2008). Alternatively, proline, well known as an antioxidant or alleviator of oxidative stress, is identified by antioxidant enzyme regulation (KUMAR et al. 2010) and malondialdehyde content (LI et al. 2010). In addition, endogenous and exogenous proline in the petals of roses may play a key role in delaying senescence in cut flowers (KUMAR et al. 2009, 2010). In 'Hot Lady' hybrid rose, the Chl_a and Chl_b pigments in the salt stressed leaves (25 mM NaCl) were damaged significantly and were lower than the control by 46.32 % (REEZI et al. 2009). Chl_a and Chl_b degradation in the salt stressed leaves of red raspberry 'Autumn Bliss' were enriched, depending on the NaCl salt concentration in the Hoagland nutrient solution (NEOCLEOUS and VASILAKAKIS 2007). The degradation of photosynthetic pigments in the leaf tissues was positively related to chlorophyll a fluorescence diminution, identified by decreased F_v/F_m, Φ_{PSII} and qP and increased NPQ, leading to P_n reduction. Photosynthetic apparati, including the chloroplast ultra structure, thylakoid membrane, electron transport proteins, total number of active reaction centres per absorption (RC/ABS), amount of active PSII reaction centres per excited cross section (RC/CS) and light harvesting complexes, as well as Rubisco activity, are damaged when plants are subjected to salt stress (MA et al. 1997, LU et al. 2002, XIA et al. 2004, YAMANE et al. 2008, MEHTA et al. 2010). F_v/F_m, Φ_{PSII} and qP diminution and P_n reduction in salt stressed plants have been investigated as sensitive indicators in plants including rose (SAVVAS et al. 2007; NIU et al. 2008), *Arbutus unedo* (NAVARRO et al. 2007), red raspberry (NEOCLEOUS and VASILAKAKIS 2007), citrus (LÓPEZ-CLIMENT et al. 2008) and tomato (ZRIBI et al. 2009). In the present study, growth parameters i.e. shoot height, fresh weight, dry weight and leaf area in salt stressed plantlets were reduced, depending on the salt concentration in the culture medium. Similar results have been well established in pot culture of 'Hot Lady' genotype roses (REEZI et al. 2009). In salt stress conditions, Na^+ is generally enriched at the cellular level, leading to induced program cell death (AFFENZELLER et al. 2009) and inhibiting growth characters, especially fresh weight, dry weight and leaf area (TAKEMURA et al. 2000, CHA-UM et al. 2010). *In vitro* flowering of miniature roses was sensitive to salt stress, leading to low numbers of flowers per plant and low flower initiation, especially in response to 100 mM NaCl. Reproductive developmental processes in salt stressed roses in pot culture have been reported as being salt susceptible (SAFI et al. 2005; REEZI et al. 2009), as represented a low number of flowers (37.50 % of the control) and a high number of rejected flowers, not of marketable value (67.21 % of the control) (SAVVAS et al. 2007). In cut flower and bulb species, the number of flowers per plant and total number of flowering plants dropped significantly when they were exposed to salt stress (8.5 dS m^{-1}) (SHILLO et al. 2002). The reproductive stage of plant development, including flower initiation, blooming pollen viability, fertilisation and seed set, has been reported as being salt susceptible (MORADI and ISMAIL 2007, VADEZ et al. 2007), which means that the accumulation of toxic ions, including Na^+ and Cl^-, in the plant cells, negatively affects flower development, leading to loss of productivity.

In conclusion, the reproductive stage of *in vitro* miniature roses was sensitive to salt stress, as demonstrated by abnormal flower development, maximum pigment degradation, low photosynthetic abilities and maximum growth reduction. Na^+ enrichment in the salt stressed plantlets directly affected pigment degradation, P_n reduction and growth performance in the salt stressed plantlets, leading to delayed flowering and blooming and induction of unfolded flowers.

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Addresses of authors: Suriyan Cha-um (corresponding author) and Chalermpol Kirdmanee, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand, e-mail: suriyanc@biotec.or.th.