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In Vitro Salt Stress Responses of Thai Oil Palm's Embryogenic Callus Variety 'SUP-PSU1'

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Abstract

Evapotranspiration, which includes the evaporation of water from soils, may rise as a result of climate change and rising temperatures. The outcome is that water evaporates but salt stays in the soil, raising its saltiness. The growth and development of oil palm are also affected by salt stress, but decades of time frame are required with conventional breeding due to its reproductive biological reasons and its long-time life span. However, to facilitate stress tolerance evaluation at an early stage of plant material, the *in vitro* selection was considered, and the evaluation process was optimized for innovating variant selection out from calluses over abiotic stress. Thus, the objectives of this research were to study the effect of concentrations of sodium chloride (NaCl) on the growth rate, electrolyte leakage (EL) and proline content of embryogenic callus (EC) of oil palm 'SUP-PSU1'. Was also, recovery of EC growth after treating with NaCl for further evaluation to set new variant tolerant line for breeding was established. EC was treated with different concentrations of NaCl in oil palm culture medium (OPCM) supplemented with 0.1 mg L^{-1} 3,6-Dichloro-o-anisic acid (dicamba) and 200 mg L^{-1} ascorbic acid (AA) for 4 weeks. The results showed that inhibition concentration at 50 % (IC_{50}) was 149.1 mM and NaCl at high concentrations increased EL and proline content. After transferring treated EC to solidified medium without NaCl for 4 weeks, The 200 mM condition's EC turned yellowish-brown and couldn't withstand higher (300 and 400 mM) NaCl concentrations. The lowest growth rate was obtained with NaCl at 300 and 400 mM, and the EC entirely turned brown. So, it was judged that NaCl concentration around 300 to 400 mM can be used as a selection agent for 'SUP-PSU1' new variant tolerant line in oil palm tissue culture breeding condition.

Keywords: Oil palm, 'SUP-PSU1', NaCl, Salt responses, Embryogenic callus

Introduction

Crop plants are subjected to multiple environmental stresses such as water deficit or drought [1], low and high temperature [2], flooding [3], or high salinity [4] affected plant growth and development. Soil salinity is an adverse environmental constraint that hinders land productivity worldwide [5]. In Thailand, there are 2.302 million hectares of salt-affected soils, of which 1.904 million hectares are in the interior while the remaining ones are around the shore [6]. Soil salinity also affects growth and development in many plants [7] as well as oil palm [8]. Oil palm can tolerate moderately salt-affected soils (4 - 8 dS/m) [6]. So, it is very important to find adaptation mechanisms toward salt tolerance in oil palm.

The oil palm (*Elaeis guineensis* Jacq.) belongs to the family Palmaceae or Arecaceae and the genus *Elaeis*. Oil palm is an important oil-producing perennial crop in food, non-food derivatives and biofuel industries [9], in particularly Southern Thailand. The shell thickness gene (*Sh*) allows oil palm to be divided into 3 types based on the thickness of their shell: Dura, pisifera, and tenera. Tenera type (heterozygous Sh^{+}/Sh^{-}) is a hybrid between the dura (homozygous Sh^{+}/Sh^{+}) and pisifera (homozygous Sh^{-}/Sh^{-}) type and commercial planting due to thicker mesocarp (containing > 30 % of fruit) than the dura parent [10]. The only method of general oil palm reproduction is by seed. However, oil palm has a single dominant vegetative apex and does not produce adventitious or axillary shoots. Thus, *in vitro* tissue culture is the only means of vegetative propagation.

In breeding program of oil palm, genetic diversity was required. So far, there is low or narrow genetic diversity of oil palm populations in southeast Asia [11]. Establishing oil palm sexual crosses requires a long time for filial generation growth and evaluation. For the 1st generation of oil palms, cultivation takes around 12 years, and for the next 8 generations, it takes about 40 years [12]. In addition, oil palm is a perennial crop which requires several months for germination of seeds and seedling evaluation process. *In vitro* techniques such as somaclonal variation and mutagen treatments can be used as alternative methods to obtain new genetic variation which can be assessed both in small plantlets and mature stands.

Somatic embryogenesis is the one of interesting methods for *in vitro* propagation in oil palm. Tissue culture technique via somatic embryogenesis is an efficient method that can be applied for salt *in vitro* screening. Instead, *in vitro* selection was investigated to assist stress tolerance screening at an early stage of plant material, and the evaluation procedure was designed for enabling variant selection out of callus under abiotic stress. This method can help to understand plants for their potential adaptation to salinity as reported in many plant species, such as date palm [13], rice [14], tomato [15,16]. In oil palm, salt stress altered various physiological and growth responses of 2-month-old *in vitro* plants [17]. A high concentration of sodium chloride (NaCl) resulted in a decrement of fresh weight, dry weight, dry matter and chlorophyll content. The highest concentration of NaCl (684.43 mM) gave the lowest results in all of those parameters. The growth of oil palm 'SUP-PSU1' suspension cell decreased when the concentration of NaCl increased, especially at concentrations higher than 200 mM [18]. This result suggests that oil palm cells *in vitro* couldn't stand to NaCl concentration higher than 200 mm However, non-embryogenic callus was treated, thus regeneration of tolerated cells couldn't expose.

The major parameters examined in plant studies under salt stress are growth and physiological and biochemical characteristics. For physiological analysis, electrolyte leakage was used to test the abilities of membrane permeability. Electrolyte leakage reflects damage to cellular membranes, resulting in loss of cell membrane integrity and cell death [19]. Moreover, endogenous osmolyte is reported as one of the most important selection measurements for identification of salt-tolerant plants. The most common endogenous osmolyte accumulated under salinity stress is proline. Proline accumulation is an important mechanism for osmotic regulation under salt stress [20,21]. Its accumulation in plants provides protection against salinity stress. It was hypothesized for this study that the EC of oil palm responds differently to various NaCl concentrations. Therefore, EC was chosen and employed in the current investigation to examine the impact of NaCl concentrations on EC growth rate, electrolyte leakage, and proline content.

Materials and Methods

Plant material

Embryogenic callus (EC) used in this experiment was obtained from culturing zygotic embryo of oil palm 'SUP-PSU1' ('25C3/77') at Crop Biotechnology Laboratory, Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University. Proliferation of the callus was carried out by regular subculture at 4-week intervals in oil palm culture medium (OPCM) supplemented with 0.1 mg L^{-1} 3,6-Dichloro-o-anisic acid (dicamba) and 200 mg L^{-1} ascorbic acid (AA) [22].

Culture media and conditions

OPCM supplemented with 0.1 mg L^{-1} dicamba, 3 % sucrose, 0.6 % agar and 200 mg L^{-1} AA was used. The culture medium was adjusted to pH 5.7 before adding 0.6 % agar and autoclaved at 1.05 kg cm^{-2} at 121 °C for 15 min. The cultures were maintained at 26 ± 2 °C under 14 h photoperiod (15 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps [22].

Effect of concentrations of NaCl on growth of EC

EC at 50 mg fresh weight (FW) was cultured on OPCM supplemented with various concentrations of NaCl (0, 50, 100, 150, 200, 300 or 400 mM), 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA. After 4 weeks of culture, growth rate based on an increase in fresh weight, growth inhibition concentration at 50 % (IC_{50}) and color of EC were recorded and compared statistically.

The growth rate of EC was calculated as the following formula [18];

$$\text{Growth rate (\%)} = \frac{(\text{final FW} - \text{initial FW})\ \text{treated EC}}{(\text{final FW} - \text{initial FW})\ \text{control EC}} \times 100$$

Effect of concentrations of NaCl on some physical and biochemical parameters

After 4 weeks of culture, electrolyte leakage (EL) and proline content were recorded and compared statistically.

For EL assay, it was determined according to the modified method of [23]. Briefly, EC at 100 mg from different concentrations of NaCl treated were collected and placed in 15 mL glass vial containing 10 mL deionized water. The glass vial was capped and maintained at room temperature for 15 min. Initial electrical conductivity (EC_0) was measured using an electrical conductivity meter. The EC was autoclaved at 121 °C for 15 min. After cooling down to 25 °C, the electrical conductivity (EC_1) was measured. EL was expressed as following the formula;

$$\text{EL}\ (\%)\ = \frac{\text{EC}_0}{\text{E}_1} \times 100$$

For proline content estimation, modified method of [24], was used. Briefly, EC at 100 mg derived from different concentrations of NaCl treated was ground in liquid nitrogen with a mortar and pestle. The homogenate powder was mixed with 2 mL of sulfosalicylic acid (3 % w/v) and filtered through Whatman#1 filter paper. The mixtures were added with glacial acetic acid and ninhydrin reagent and incubated at 95 °C for 1 h. After incubation, the mixture was terminated on ice bath for 5 min, added with 4 mL of toluene and mixed vigorously by vortex mixture. After phase separation, upper layer was used to measure the absorbance at 520 nm by spectrophotometer (UV-1100) and L-proline (Sigma®) was prepared and used as a standard.

Recovery rate of EC after treating with different concentrations of NaCl

For recovery rate of EC estimation, modified method of [18], was used. Briefly, 4-week-old of EC treated with various concentrations of NaCl (0 - 400 mM) was subsequent to OPCM medium without NaCl for evaluation of cell recovery rate. All culture media were supplemented with 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA. After culture for 4 weeks, growth rate, FW, color of EC, somatic embryo formation frequency and the mean number of somatic embryos were recorded.

Experimental design and statistical analysis

The completely randomized design was performed and means among treatments were separated by Duncan's multiple range test (DMRT) at 1 or 5 % probability. The experimental data were analyzed using R 2.14.0 software and subjected to ANOVA analysis.

Results and discussion

Effect of concentrations of NaCl on EC growth

After culturing EC on OPCM medium with different concentrations of NaCl, the results showed that the inhibition concentration that limits the growth of EC at 50 % (IC_{50}) was 149.1 mM (**Figure 1**) and this value could be used as criteria for screening salt tolerance in many plants such as rice [25,26]. an increase in concentrations of NaCl decreased growth rate. EC growth as well as growth rate decreased at 50 - 400 mM NaCl. The highest growth rate at 100 % was obtained from control treatment. While OPCM medium with 400 mM NaCl gave the lowest growth rate at 15.8 % (**Figure 1**). Salt stress impairs fresh weight and crop development. High NaCl concentrations led to the gradually decreasing fresh weight of EC of oil palm that was observed in this present study. Almost all the research showed that high concentrations of NaCl resulted in a decrease in plant growth. [15,16]. Our result has shown that NaCl at 0 - 200 mM gradually decreased callus growth rate but high concentration of NaCl more than 200 mM resulted in callus death. It might be because NaCl causes high Na^+ and Cl^- levels in the cytoplasm, leading to ion toxicity and osmotic stress. It also triggers the synthesis of reactive oxygen species (ROS) resulting in oxidative stress in cells [27]. A similar result was also attained from [18], which reported that when NaCl concentration increased, particularly at concentrations greater than 200 mM, the growth of the non-embryogenic callus (NEC) in the suspension of oil palm 'SUP-PSU1' decreased. According to their findings, oil palm cells in suspension cultures couldn't tolerate NaCl at concentrations more than 200 mM. A contrary result was reported by [17]. They reported that the highest concentration of NaCl that oil palm can stand was 684.4 mM, 3 times higher than the result obtained in the present study. The difference might be due to physiological factors such as varieties, ages, and types of explants.

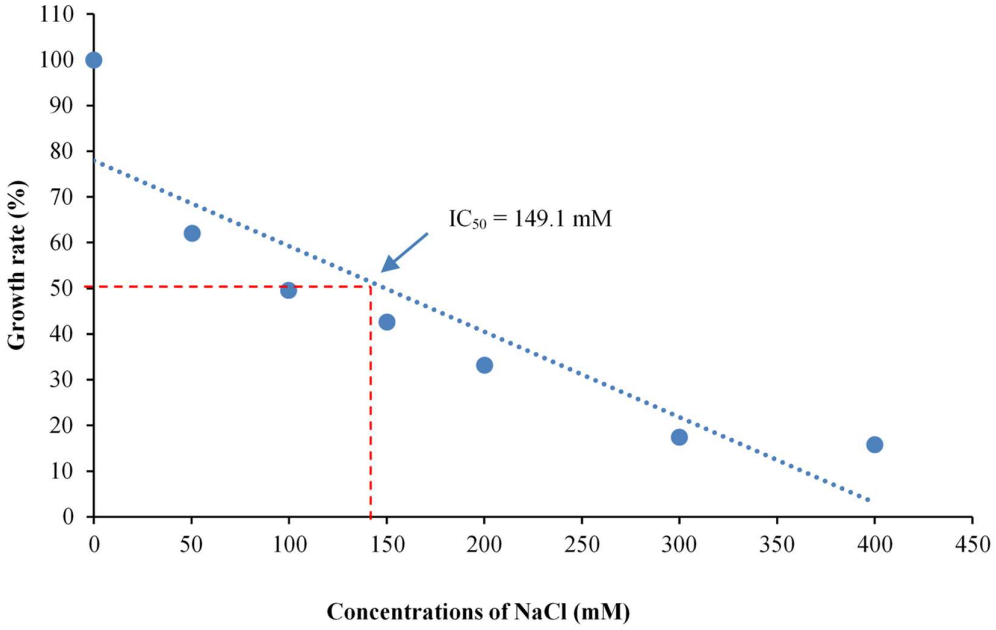

Figure 1 Effect of different concentrations of NaCl on growth rate of EC on OPCM medium with 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA after culture for 4 weeks.

Effect of concentrations of NaCl on some biochemical parameters

After culturing EC on OPCM medium with different concentrations of NaCl, electrolyte leakage of EC was significantly increased with the increment of concentrations of NaCl. The highest EL at 89.3 % was obtained from 400 mM NaCl but not significantly different from 300 mM which gave the EL at 83.2 %. However, the concentrations from 0 to 200 mM gave the EL at 9.1, 22.8, 36.3, 47.5 and 62.2 %, respectively. For the relationship between growth rate and electrolyte leakage, the results showed that increasing in concentration of NaCl resulted in decreasing in growth rate. On the other hand, the increasing of EL was following with an increasing of NaCl (**Figure 2**). Treated EC from those concentrations could grow and develop into SE. Whereas 400 mM NaCl increased 10 times of EL when compared to control treatment (without NaCl). Therefore, callus obtained from 400 mM NaCl containing medium was completely died (**Figure 2**). High concentrations of NaCl gave high toxicity and caused the formation of ROS [28] which stimulates lipid peroxidation in cell membranes, DNA destruction, protein denaturation etc. [29]. EL reflects damage to cellular membranes, and EL was commonly measured between control and stress conditions for testing in the tolerance of the explant against stress (30). The result indicates that an increment in concentrations of NaCl increased EL in *Spinacia oleracea* [31], petunia [32], *Coleus* species [33], barley [34], potato [35], lettuce, New Zealand spinach and common purslane [36]. For salt stress by treating oil palm EC with NaCl in the present study, revealed that high concentration of NaCl more than 200 mM causes cell damage and death due to high EL.

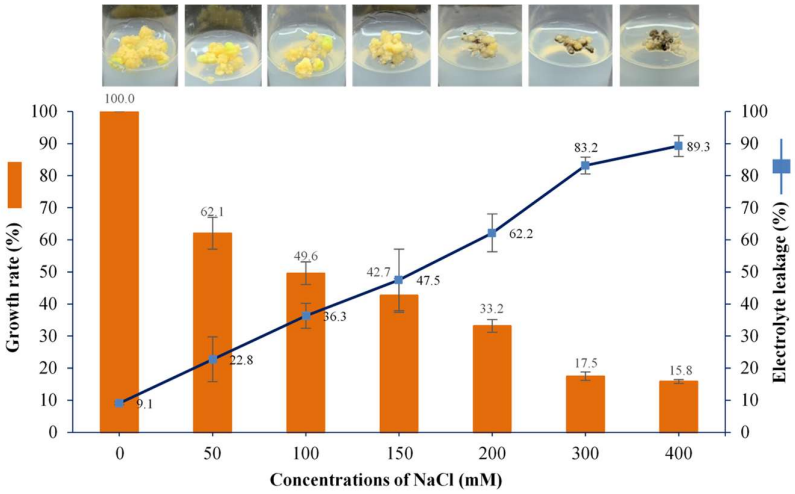

Figure 2 Growth rate, electrolyte leakage and characteristics of EC on OPCM medium with 0.1 mg L^{-1} dicamba, 200 mg L^{-1} AA and different concentrations of NaCl after culture for 4 weeks (bars indicate standard error).

For proline content, it was measured from EC treated with different concentrations of NaCl after 4 weeks of treatment. The result showed that proline content in EC increased drastically when concentration of NaCl increased from 50 to 150 mM. Concentrations at higher than 150 mM gave decrement content of proline. Concentrations from 200 to 400 mM gave proline content not significantly different with control treatment (without NaCl). Among NaCl's concentrations, 150 mM gave the highest proline content (32.6 mg g^{-1} FW), while 400 mM gave the lowest proline content (7.9 mg g^{-1} FW). However, culture medium without NaCl showed the lowest proline content at 3.5 mg g^{-1} FW (**Table 1**). Proline is 1 kind of osmoprotectant that are produced in numerous plants along with increased salinity in the environment and could be assessed as a salinity tolerance index [37]. It can assist plants to retain their cell turgor, preventing protein denaturation, and protect membrane integrity under various stresses [38]. In this study, the highest proline content was obtained from EC treated with 150 mM NaCl, concentration higher than this caused the reduction of proline. It might be confirmed that concentration of NaCl higher than 150 mM makes severe toxicity of ions, and overgeneration of ROS induces oxidative damage in plant cells leading to a decrease in proline content. Proline might play a remarkable role in the adjustment of osmotic pressure.

Table 1 Proline content of EC on OPCM medium supplemented with 0.1 mg L^{-1} dicamba, 200 mg L^{-1} AA and different concentrations of NaCl after culture for 4 weeks.

Concentrations of NaCl (mM)	Proline content (mg g^{-1} FW)
0	3.5 ± 0.5^{d}
50	20.6 ± 2.0^{b}
100	30.7 ± 2.4^{a}
150	32.6 ± 3.3^{a}
200	14.7 ± 2.3^{bc}
300	8.6 ± 1.4^{cd}
400	7.9 ± 1.6^{cd}
F-test	**
C.V. (%)	21.1

**significantly different ($p \leq 0.01$)

Mean values ± standard error followed by the same letter within the column are not significantly different according to DMRT.

Recovery of EC after treating with different concentrations of NaCl

After treating EC with various concentrations of NaCl containing in OPCM medium with 0.1 mg L^{-1} dicamba for 4 weeks and transferring to the same medium without NaCl for 4 weeks, the recovery of EC was recorded. The results showed that EC treated with 50 and 100 mM NaCl gave the higher results in both growth rate and increased FW than those of control. While EC treated with 150 to 400 mM NaCl gave the lower results in both parameters than control. The highest growth rate and increased FW among the NaCl concentrations were attained at 50 mM, at 127.6 % and 583.9 mg, respectively. The lowest growth rate and increased FW of EC occurred at 400 mM NaCl among the investigated concentrations, at 10.7 % and 1.7 mg, respectively (**Table 2**). For characteristics of EC, EC treated with 0 to 150 mM NaCl have the normal yellow color. While the EC from 200 mM changed to yellowish and brown color and EC from 300 and 400 mM showed brown and dark brown color (**Figure 3**). So, the result found that EC treated with NaCl at concentration of 50 to 200 mM could recover growth on medium without NaCl. While EC treated with 300 - 400 mM was completely incapable of growing. (**Figure 3**). Additionally, it has been discovered that EC can develop into SE. The results showed that low concentration of NaCl can improve SE formation in oil palm 'SUP-PSU1'. NaCl at concentration of 50 mM gave the highest results in SE formation at 83.3 % and the average number of SEs at 2.8 embryos/tube. NaCl at concentrations higher than 50 mM resulted in a decrement of both parameters (**Figure 4**). However, SE formation wasn't recovered from NaCl at higher concentration than 200 mM (**Figure 4**). From the result, indicated that low concentration of NaCl In this study, we found that EC treated with NaCl at concentrations of 50 to 200 mM could undergo growth on medium without NaCl but EC from NaCl at higher concentration than 200 mM couldn't recover. NaCl at 50 mM gave the best results in recovery of growth in term of growth rate, increased FW of EC and SE formation whereas NaCl at higher concentration than 200 mM couldn't develop into SEs. Similar results were obtained from [17], who reported that cells in suspension from treating NaCl of oil palm couldn't stand to NaCl at concentration higher than 200 mM but the result was different in the development of SE. In this study, SE was well developed from NaCl-treated EC and possible to germinate into plantlets while non- embryogenic callus couldn't develop into SE. Different results depended on different clones of oil palm.

Table 2 Recovery of EC after treating with different concentrations of NaCl for 4 weeks and transfer to OPCM medium without NaCl for 4 weeks. (The medium was supplemented with 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA).

Concentrations of NaCl (mM)	Growth rate (%)	Increased FW of EC (mg)	Color of EC
0	100.0 ± 0.0[a]	450.6 ± 25.5[a]	Yellow
50	127.6 ± 8.6[a]	583.9 ± 16.4[a]	Yellow
100	116.6 ± 16.9[a]	529.4 ± 99.1[a]	Yellow
150	28.1 ± 6.1[b]	437.0 ± 13.1[a]	Yellow
200	22.0 ± 0.7[b]	120.6 ± 34.5[b]	Yellowish, brown
300	13.1 ± 0.8[b]	1.7 ± 1.7[c]	Brown
400	10.7 ± 0.3[b]	1.7 ± 1.7[c]	Dark brown
F-test	**	**	
C.V. (%)	21.9	23.8	

**significantly different ($p \leq 0.01$)

Mean values ± standard error followed by the same letter within a column are not significantly different according to DMRT.

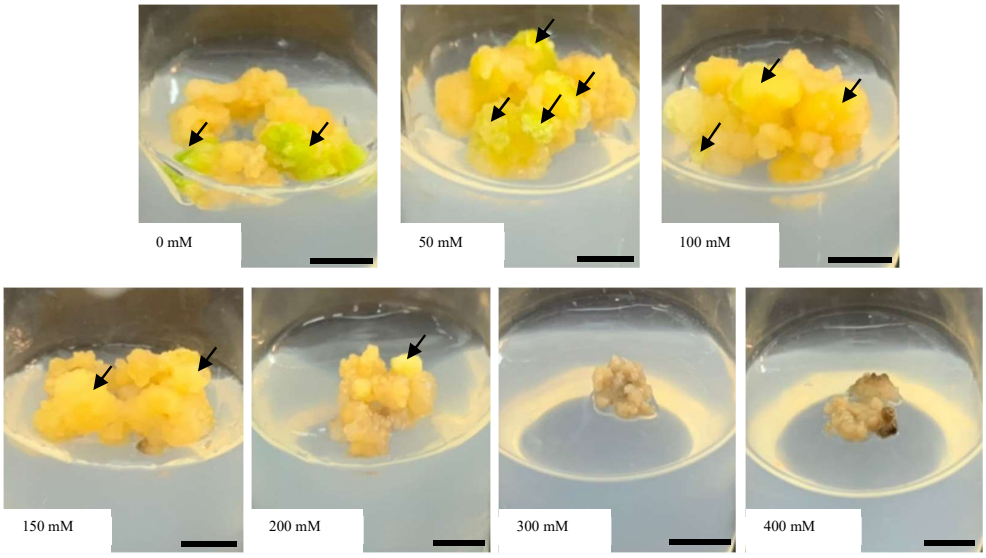

Figure 3 Characteristics of EC and SEs (arrows) obtained from EC treated with various concentrations of NaCl containing OPCM with 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA for 4 weeks subsequent to transfer to the same medium without NaCl for 4 weeks (bars = 0.5 cm).

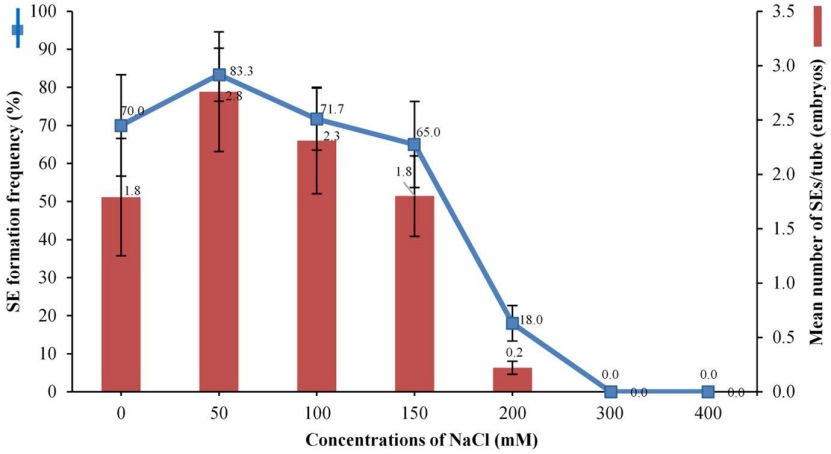

Figure 4 Effect of NaCl on SE formation frequency and mean number of SEs of treating EC subsequent to recovery on NaCl-free OPCM medium with 0.1 mg L^{-1} dicamba and 200 mg L^{-1} AA for 4 weeks (bars indicate standard error).

Conclusions

In conclusion, the *in vitro* salt stress responses tested method can be used for salt-tolerant screening of the EC of oil palm 'SUP-PSU1'. Among concentrations of NaCl, 300 and 400 mM gave the lowest growth rate, the highest EL, the lowest proline content, and the EC completely turned brown. So, it was judged that NaCl concentrations around 300 to 400 mM can be used for selection of the new variant-tolerant line of EC or other early-stage plant materials for breeding of oil palm 'SUP-PSU1' in tissue culture conditions.

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