Sensitivity of hybrid *Cymbidium* to salt stress and induction of mild NaCl stress tolerance

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Abstract

Research on the sensitivity of *Cymbidium* to NaCl does not exist. In this study, the protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light' were induced to form *neo*-PLBs on Teixeira *Cymbidium* (TC) medium. After initial exposure of PLBs to a range of NaCl concentrations ranging from 5 mM to 200 mM, *neo*-PLBs were unable to grow in the presence of NaCl that exceeded 20 mM in TC medium, showing reduced explant survival, fewer *neo*-PLBs per explant, and reduced fresh and dry weight. *Neo*-PLBs growing in medium containing 5 or 10 mM NaCl gradually adapted over several subcultures, to 40 mM NaCl in TC medium. In total, only eight *neo*-PLBs were found to grow on 40 mM NaCl, but none at higher levels. This simple protocol tests for NaCl stress tolerance in orchids and was found to induce mildly salt-tolerant *neo*-PLBs.

Key words: brackish water, NaCl, protocorm-like bodies, sea water, Teixeira *Cymbidium* medium.
Abbreviations: NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; PLB, protocorm-like body; TC medium, Teixeira *Cymbidium* medium.

Introduction

Competition for fresh water, a limiting natural resource, will increase, and compounded with 25 to 30% waste of fresh water (Taft 2015), will lead to further commoditization and commercialization of fresh water, such as rain or river water. This may one day place pressure on tissue culture labs and commercial greenhouse managers to seek alternative sources for the liquid base of their media (*in vitro*) or irrigation (field or in greenhouses), either as a substitute, or to reduce costs. Thus, researchers and the agricultural industry will have to increasingly turn towards brackish and sea water to fulfill this need. Limited trials that produce ornamental plants *in vitro* or in the greenhouse using brackish or salty water are already taking place (Cassaniti et al. 2013; Sánchez et al. 2015), an endeavor typically limited to halophytic plant species (Muscolo 2011). One example is *Conocarpus lancifolius* (Combretaceae), which grows optimally in Kuwait at a temperature range of 4 – 45 °C and thrives well when irrigated with brackish water (salinity >3500 ppm), suggesting that this plant is a xerohalophyte (Al-Kandari et al. 2009). Such objectives would form an important aspect of the future vision of orchid biotechnology (Hossain et al. 2013).

High salinity in soil or irrigation water is a common environmental problem affecting plant growth; salt stress can inhibit or delay germination and seedling establishment, as well as almost every aspect of the physiology and biochemistry of plants, which in turn significantly reduces yield (e.g., Ates, Tekeli 2007). Physiological mechanisms against salt stress can be divided into three strategies: activation of the ion transport system, osmotic adjustment and induction of antioxidant enzymes (Chaparzadeh et al. 2004). During salt stress, a high level of toxic ions (such as Na and Cl) is taken up into the plant cell, potentially causing metabolic disorders (Cheeseman 2007). Understanding the molecular basis of ion transporters is a key to understanding and improving tolerance to salt stress (Hamamoto et al. 2015).

This study focuses on an attempt to induce salt-tolerant *neo*-PLBs on NaCl-impregnated Teixeira *Cymbidium* (TC) medium (Teixeira da Silva 2012) using a model hybrid *Cymbidium*. At first, the growth response to a range of NaCl concentrations ranging from 5 mM to 200 mM was assessed. New protocorm-like bodies (*neo*-PLBs) forming in the presence of NaCl were then subcultured under a salt-stress environment in a bid to induce salt-tolerant neo-PLBs.

Materials and methods

Stock PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) were established and maintained as explained in detail in Teixeira da Silva (2012; 2014; and references therein). Briefly, *neo*-PLBs were induced and subcultured every two months on TC medium

supplemented with 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA) and 0.1 mg L^{-1} kinetin (Kin), 2 g L^{-1} tryptone and 20 g L^{-1} sucrose, and solidified with 8 g L^{-1} Bacto agar (Difco Labs., USA) (pH adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min). Neo-PLBs were kept in 100-mm diameter plastic Petri dishes (AsOne, Osaka, Japan) at 25 °C, under a 16-h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan).

In the first phase, to test the effect of NaCl on *neo*-PLB growth and development, *neo*-PLBs were placed on TC medium containing 0, 5, 10, 20, 50, 100, and 200 mM NaCl and subcultured every 30 days onto TC medium containing the same concentration of NaCl. In medium containing 100 and 200 mM NaCl, the medium did not solidify effectively and explants were placed on paper bridges, a strategy used and explained in Teixeira da Silva (2013). In a second stage, in a bid to induce salt stress tolerance, *neo*-PLBs growing in medium containing 5 or 10 mM NaCl were subcultured four times (30 days each subculture) onto TC medium containing 10 mM of NaCl (stress tolerance adaptation phase). After four cultures, the surviving *neo*-PLBs were cultured in a step-wise manner at 15 to 40 mM NaCl in TC medium, at 5 mM increments for each 30-day subculture (i.e., over a total period of 6 months) (Fig. 1). In *neo*-PLBs and *neo*-PLBs (control and at each concentration of NaCl), *neo*-PLB fresh and dry weight, number of *neo*-PLBs per explant and percentage survival were determined after 30 days. The timing of the sampling in *Cymbidium* can affect the quantitative outcome of the experiment (Teixeira da Silva, Dobránszki 2013).

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate. Percentage values were arcsine transformed prior to analysis. Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Correlations were performed in Excel 2010 (Microsoft Windows, Redmond, WA, USA).

Results and discussion

The *in vitro* environment can be used to induce salt stress tolerance, as has been investigated in many plant species, with each species showing vastly different levels of sensitivity and tolerance (Rai et al. 2011). To date, however, the sensitivity of orchids to salt (NaCl or other ions) *in vitro* has not yet been examined. This study aimed to not

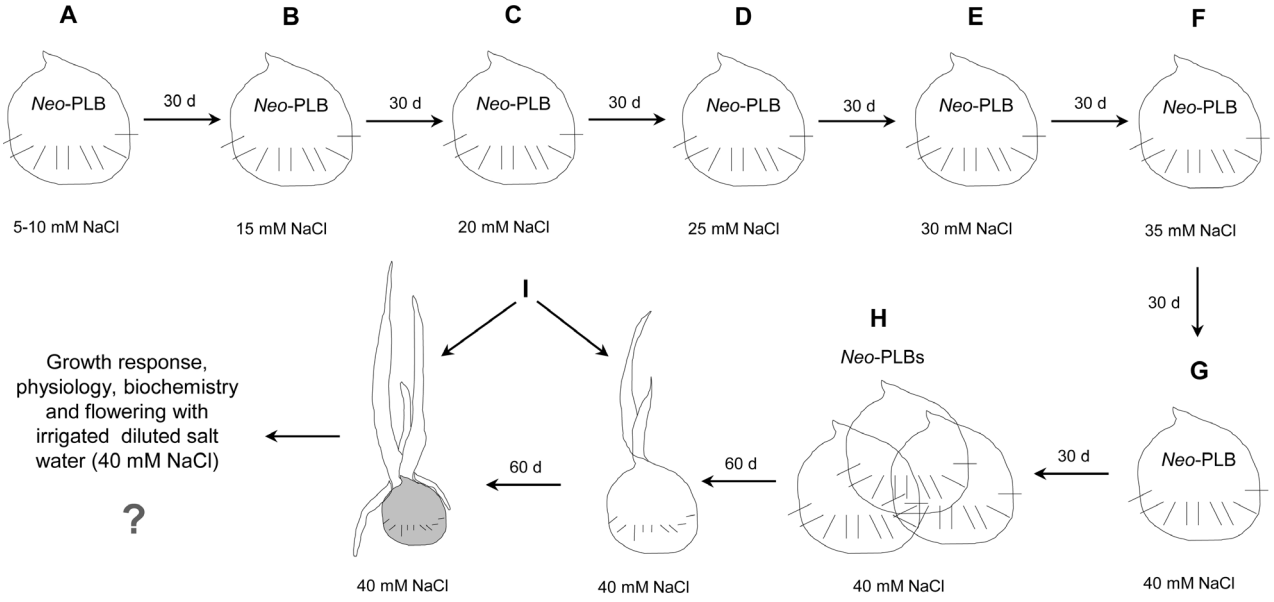

Fig. 1. How to induce mild NaCl tolerance in hybrid *Cymbidium* Twilight Moon 'Day Light'. (A) *Neo*-PLBs that form in the presence of a low concentration of NaCl (5 – 10 mM) are then transferred to a slightly higher NaCl concentration (15 mM) and left to develop or react for 30 days (B). The same procedure is repeated at 5 mM increments, each for 30 days, until the final concentration is 40 mM, i.e., 20 mM = C, 25 mM = D, 30 mM = E, 35 mM = F, and 40 mM = G. Naturally, step G will differ for different plants, but in this experiment and for this cultivar of hybrid *Cymbidium*, it was 40 mM. After a total of 6 months after initial culture (A), a total of 8 mildly NaCl-tolerant *neo*-PLBs were obtained. These can then be sustained, and multiplied, indefinitely on TC medium (Teixeira da Silva 2012) containing 40 mM of NaCl (H), or induced to form shoots and plantlets *in vitro*, also within a NaCl stress condition (I; this step was not achieved in this study, and remains a hypothetical option) to then form plants. These plants could then be constantly irrigated with diluted sea water or brackish water (in this case at 40 mM NaCl) under greenhouse conditions, although the effect on physiological and biochemical parameters needs to be assessed, to ascertain if there is any effect on flowering. Note: So as not to clutter the figure, the single PLB in A-G in fact represents multiple neo-PLBs, but has been graphically represented as a single PLB.

only examine the basic biological response of *neo*-PLB formation to NaCl, but also to attempt to induce NaCl-tolerant *neo*-PLBs.

NaCl had a significant negative effect on *neo*-PLB formation and percentage survival of explants, with necrosis occurring in as little as 20 mM of NaCl in TC medium (Fig. 2). The fresh weight of *neo*-PLBs dipped significantly with as little as 5 mM NaCl, and even more so at higher concentrations of NaCl (Table 1). All concentrations of NaCl had a significantly negative effect on *neo*-PLB dry weight (Table 1). The negative effect on percentage survival of explants and the number of *neo*-PLBs per explant was strongly ($y = -16.036x + 94.571$; $R^2 = 0.8415$) or fairly strongly ($y = -1.2179x + 6.9714$; $R^2 = 0.7771$) correlated, respectively, making hybrid *Cymbidium* a strongly salt-sensitive plant. Both parameters were strongly negatively correlated with NaCl concentration (Fig. 2A, 2B). Even though the development of *neo*-PLBs was severely hindered at 20 mM NaCl, step-wise exposure of *neo*-PLBs over 6 months at 5 mM increments, from 15 mM to 40 mM (Fig. 1), resulted in eight *neo*-PLBs that were tolerant to 40 mM NaCl, and which could be maintained on 40 mM NaCl for at least three 30-day subcultures.

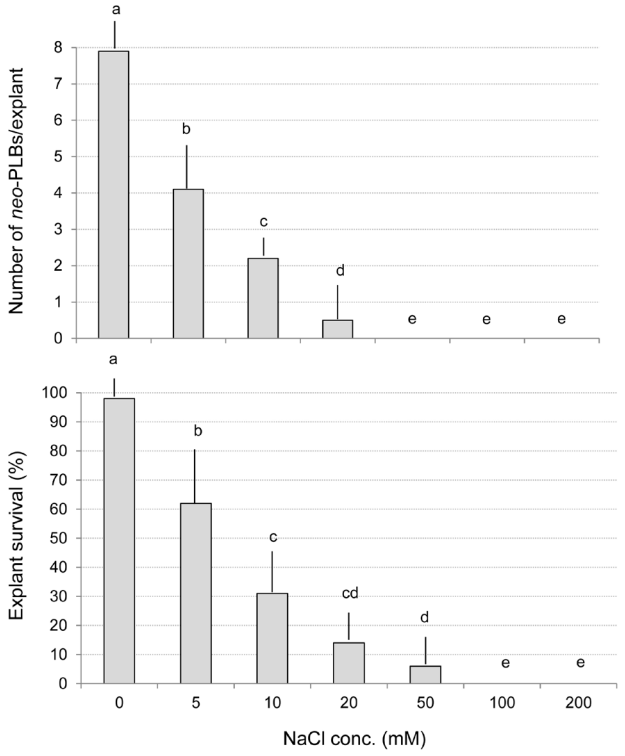

Fig. 2. Decrease in morphological responses (A, number of neo-PLBs per explant; B, % of explant survival) of PLB explants in response to salt (NaCl) stress (5 to 200 mM) after 30 days. Different letters between treatments and within each parameter indicate significant differences according to DMRT ($P \leq 0.05$); $n = 30$ per treatment.

Table 1. Change in neo-PLB fresh weight and dry weight in response to different concentrations of NaCl (5 to 200 mM), determined after 30 days of culture. Different letters between treatments and within each parameter indicate significant differences according to DMRT ($P \leq 0.05$); $n = 30$ per treatment.

NaCl concentration (nM)	Fresh weight (mg)	Dry weight (mg)
0 (control)	486 ± 27 a	54 ± 7 a
5	318 ± 42 b	31 ± 8 b
10	274 ± 31 c	28 ± 6 b
20	181 ± 16 d	21 ± 6 bc
50	68 ± 9 e	20 ± 4 bc
100	59 ± 11 e	18 ± 3 bc
200	54 ± 8 e	14 ± 3 c

This suggests that sea water, if diluted to 20 – 40 mM, could in essence be used to grow *Cymbidium* plants *ex vitro*, or could be used as the liquid for basal medium during *in vitro* experiments using PLBs as the explant source. The potential impact (in terms of economics and volumes of water employed) would be more substantial when using sea water that has a concentration of NaCl that lies between 300 – 400 mM, but less so for NaCl concentrations between 500 – 600 mM (Lin, Brown 1993). Countries in drought-affected regions of the world such as Israel that have a good ornamental industry, or countries along the north African belt, but that have limited fresh water resources, or even countries that have under-developed ornamental industries but with ample access to sea water, such as the Middle East, countries surrounding the Mediterranean Sea, or other sea-side operations, could benefit from such a finding.

This study showed that salinity stress at moderate to high levels affected the morphology and yield of *Cymbidium*, as found previously for many other plants (Ashraf 2004). However, detailed physiological and biochemical studies are required to investigate the mechanisms underlying this sensitivity and/or mild tolerance to NaCl. One of the reasons why salt (*sensu lato*) negatively affects plant growth is the increase in the level of electrolyte leakage (Parida, Das 2005). The tolerance of plants to salt is also dependent on the accumulation of different polyamines (Pang et al. 2007). HKT transporters are involved in salt tolerance in plants (Hamamoto et al. 2015) and are integrated into a complex regulatory system involving physiological, biochemical and genetic regulation (Gupta, Huang 2014). The plant literature has an abundance of studies on transgenic strategies for inducing salt stress tolerance, but induction of salt stress tolerance using a step-wise approach is less explored, and may depend on wide-ranging factors such as ploidy (Xue et al. 2015), natural somaclonal variation (López Colomba et al. 2013), or the induction of polyamines (Kuznetsov et al. 2007).

Acknowledgements

The author thanks Prof. Michio Tanaka for providing research and

laboratory facilities to conduct this research. The author declares no conflict of interest.

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Received 13 May 2015; received in revised form 22 May 2015; accepted 3 June 2015