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THESIS

SALT TOLERANCE IN THE FORAGE LEGUME *Stylosanthes guianensis* CIAT 184

VARAPORN VERAPLAKORN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Varaporn Veraplakorn 2013: Salt Tolerance in the Forage Legume *Stylosanthes guianensis* CIAT 184. Doctor of Philosophy (Botany), Major Field: Botany, Department of Botany. Thesis Advisor: Associate Professor Malee NaNakorn, Ph.D. 123 pages.

Stylosanthes guianensis CIAT 184 (Stylo 184) is a highly valued forage legume containing high protein. However, it is not salt tolerant and therefore not suitable for planting in areas affected by salinity. This thesis carried out selection for salt tolerant Stylo 184 and investigated its tolerance mechanisms. Sixty grams of seed (approximately 37,500 seeds) was screened for salt tolerant individuals in 2% NaCl solution. In addition, one seed was selected from seed which did not germinate at 1% NaCl, but germinated when transferred onto salt free MS medium. This clone was considered as a less tolerant clone (T1) to compare salt tolerant mechanisms with the individuals that germinated in 2% NaCl.

Ten salt tolerant clones were selected and their growth compared with clone T1 on basal MS medium containing from 0 to 1.5% NaCl. Four clones, representative of salt tolerant clones, were selected on the basis of their 50% growth reduction dose (T2, T3, T4 and T5) and their ion content and enzyme production on salt media were compared to T1. Callus of T1 displayed exclusion mechanisms as would be expected of a glycophyte and as found in nonselected seedlings; it maintained the lowest Na^+ and Cl^- content, and maintained the highest K^+ content. The salt tolerant clones (T2, T3, T4 and T5), however, accumulated Na^+ and Cl^- as well as K^+ as osmolytes; a mechanism similar to what might be expected in halophytes. Shoots of all four tolerant clones (T2, T3, T4 and T5) had significantly higher levels of SOD activity after NaCl treatment and on a recovery medium. The other enzymes, however, showed less distinct differences compared to T1 with T3 and T4 having the same levels of CAT but lower levels of POX. T2 had higher CAT but lower POX than T1. T5 was the most distinctive by having particularly high levels of both CAT and POX.

Shoot regeneration, root induction, survival in soil and relative growth rate of the clones remained high even after long term maintenance in culture. Shoots of T1 and T5 produced 70.0 and 91.0% rooting, respectively with 69.0 and 90.0% survival in after being transferred to soil. All of these plants grew well and showed normal characteristics with flowering after 7 months.

The different mechanisms among clones of Stylo 184; osmotic adjustment using different ions and antioxidant system with higher constitutive enzymes can be applied as criteria for selection in other *Stylosanthes* spp. In addition, Stylo 184 salt tolerant clones will be available for further investigation into their salt tolerant mechanisms and appropriate field investigation.

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Student's signature Thesis Advisor's signature

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Varaporn Veraplakorn
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LIST OF ABBREVIATIONS

BA	=	6-benzylaminopurine
°C	=	degree celsius
CAT	=	catalase
cm	=	centimeter
2, 4-D	=	2, 4-dichlorophenoxy-acetic acid
IAA	=	indole-3-acetic acid
l	=	liter
mg	=	milligram
MS	=	Murashige and Skoog
NAA	=	naphthalene-acetic acid
NaCl	=	sodium chloride
POX	=	Peroxidase
rpm	=	rotation per minute
µl	=	microliter
µM	=	micromolar
SOD	=	superoxide dismutase

SALT TOLERANCE IN THE FORAGE LEGUME *Stylosanthes guianensis* CIAT 184

INTRODUCTION

Salt-affected soil negatively affects world food security. It impacts upon 7% of the world's land area, amounting to 1000 million hectares in more than 100 countries and covers approximately 20% of agricultural land and 50% of cropland. Current estimates indicate that 10 – 35% of the world's agricultural land is now affected, with significant areas becoming unusable each year (Tester and Davenpot, 2003; Rengasamy, 2006; FAO, 2008). Regions that have suffered range from North and Central Asia (including Thailand), South America, Australia and the Mediterranean (Merchan *et al*., 2007; Division of soil Analysis, 2008; Yuwaniyama, 2008).

An area of 2.8 million ha in Northeast Thailand has faced several limiting factors that reduce agricultural productivity. These include low soil fertility, uncertainty of climate and natural disasters; the prevalent disaster being salinity (Wongsomsak, 1986; Nemoto and Panchaban, 1991; Tulyatid *et al*., 2008). An important study on the effects of saline soil on land use activity was conducted in Nakhon Phanom using multiple linear regression to relate the basin salinity index with land use activities (e.g. community and housing, rice farming, and livestock production). The result suggested that salinity has a major negative impact on the population density, in-season rice farming, and piggeries. However, it also has positive impacts on off-season rice farming and cow and poultry production (Seeboonruang, 2010).

Plantation development is one of the effective techniques used to ameliorate saline soil. Salt tolerant plants can help reaching salt from root zone and improving farm management practices. Growing suitable salt tolerant plants for individual areas can take into consideration (Munns *et al*. 2002; Manchanda and Garg, 2008; Al Sherif, 2009).

Legumes are a prime example of plants that have dual functions, especially deep rooted perennials as they are able to alleviate problems with rising water tables, contribute to soil nitrogen and provide products such as crop or fodder. Using legumes to address saline soil problems has therefore, been widely recommended (Betteridge and Jones 2001; Ashraf and Iram 2005; Manchanda and Garg, 2008; Al Sherif 2009; Saha *et al*. 2010; Al-Shasarani and Shetta, 2011).

Stylosanthes guianensis is a legume that is widely grown for forage production in sub-tropical and tropical regions including: Philippines, Australia, Indonesia, Malaysia, Vietnam, Laos, and Thailand. It is particularly interesting because of its exceptional forage value and high protein content (approximately 14-18% foliage), making it suitable for feeding cattle as pasture or hay (Phengsavanh and Ledin 2003; Homma *et al*., 2008). It is a short-lived perennial legume which is well adapted to a range of soil types from sandy to light clay but is sensitive to saline and sodic soils (pH>8.5). *S. guianensis* CIAT 184 also shows resistance to the fungal disease anthracnose, which causes problems with the utilisation of this genus in Southeast Asia and Australia (Homma *et al*., 2008).

In Thailand, *S. guianensis* CIAT184 (Stylo 184) has potential for the development of a *Stylosanthes*-rice relay-intercropping system. This would be most useful in North-eastern Thailand where only a single rice crop is produced each year (during the wet season), making rice fields available for other purposes (e.g. fodder) during the dry season. Stylo 184, however, has relatively low salt tolerance and this has restricted its usefulness in areas that have a history of soil salinisation (Homma *et al*., 2008). The availability of salt tolerant lines, therefore, would increase its usefulness in a *Stylosanthes*-rice intercropping system in this region.

Furthermore, the process of salt tolerance selection may be aided by the ability to screen many cell lines and determine the salt tolerance mechanisms of cells and subsequent regenerated plants. Providing the amount of cells or plants as requirement for this process may be limited. Hence, tissue culture techniques are a good option for salt tolerance selection. There are no studies that have examined selecting and improving for salt tolerance of Stylo 184. Therefore, the precise impacts of osmotic and ionic effects under salt stress as well as the mechanisms available to counteract them need to be determined. This thesis presents the selection process for salt tolerant lines of Stylo 184 *in vitro* and studies their salt tolerance mechanisms.

OBJECTIVES

1. To select salt tolerant clones of *Stylosanthes guianensis* CIAT184
2. To determine some physiological mechanisms of salt tolerance in selected clones

LITERATURE REVIEW

Saline soil

Among abiotic stresses, soil salinity is a major factor limiting sustainable agriculture. Soil salinity is measured using electrical conductivity (EC) of the soil solution. The USDA salinity Laboratory (1954) defines a saline soil as having an EC of $\geq$ 4 dSm^{-1}. Values higher than 4 dSm^{-1} would be expected to cause adverse effects on various species depending on factors such as plant type, soil-water regime and climatic condition (Munns, 2002; Manchanda and Garg, 2008).

Types of salinity

Salinity refers to the content of mineral salts dissolved in the soil solution and comprises of the major cations Na^{+}, $Ca^{2+}$and Mg^{2+} and the major anions Cl^{-}, SO_4^{2-}, HCO_3^{-}, CO_3^{2-} and NO^{3-}. Other constituents contributing to salinity in hypersaline soils and waters include B, Sr^{2+}, SiO_2, Mo, Ba^{2+} and Al^{3+} (Manchanda and Garg, 2008). The dominant sources of salt are rainfall and rock weathering. Rainfall contains low amount of salt, but salt deposited by rain over long periods of time can accumulate in the landscape. In addition, wind-transported (aeolian) material from soil or lake surfaces can also impact upon salinisation as can poor quality irrigation water. Intrusion of seawater onto land, as occurred in recent tsunami-affected regions, can cause the deposit of large amounts of salts in coastal areas. However, the particular processes for salt accumulation are usually a combination of factors influenced by climatic and landscape features and effects of human activities (Rengasamy, 2006; Manchanda and Garg, 2008).

Salinity has been classified as primary and secondary according to the causal processes. Primary salinity results from the accumulation of salts over long periods of time, from natural processes. The most important of these processes include weathering of rocks containing soluble salts of various types, mainly chlorides of sodium, calcium and magnesium, and to a lesser extent, sulfates and carbonates and the deposition of oceanic salt carried inland by wind and rain. The composition of this deposited salt is that of seawater, that is mainly sodium chloride (Munns and Tester, 2008; Manchanda and Garg, 2008). Naturally, salt-affected areas occur widely in arid and semi-arid areas. They are often obvious as salt lakes, but can also occur out of sight, in the subsoil, where they are not associated with ground water or with rising water tables. This type of salinity is named “transient salinity” because it can move up and down the soil profile, and in and out of the root zone, depending on the season. Secondary salinity results from human activities that change soil hydrology. In particular, imbalances created through changes in water input to a system (e.g. irrigation and/or rainfall) and water used by crops (transpiration). The most common causes include land clearing and the replacement of perennial vegetation with annual crops and irrigation schemes using salt containing water or having insufficient drainage (Manchanda and Garg, 2008).

Primary salinity, however, has been enumerated based on soil and ground water prosesses, to be groundwater-associated salinity and non-groundwater associated salinity

(Rengasamy, 2006). According to the feature of water table, groundwater-associated salinity, the water table is generally close to the soil surface and this leads to high accumulation of salt when the water table is less than 1.5 m. below the soil surface. On the other hand, non-groundwater associated salinity, this type is a predominant of sodic soil where the water table is deep and drainage is poor. Salt is introduced by natural processes i.e. rain weather and aerolian (Rengasamy, 2006).

Soil salinity and its management in Thailand

There are many areas where saline soil occurs in Thailand. This includes not only coastal regions, but also the inland areas mostly found in the northeastern region known as the "Knorat Plateau" (Nemoto and Panchaban, 1991). The Khorat basin of Northeast Thailand is divided into five categories with respect to the surface salinity: 1. elevated ground underlain by saline Mesozoic rocks, 2. heavily salt-affected lowland, 3. moderately salt-affected lowland, 4. slightly salt-affected lowland, and 5. non-saline (Sinanuwong and Takaya, 1974).

One approach to the management of saline soils is the rehabilitation of unproductive areas. There are reported successes of co-cultivated plantations in saline soil. For example, by planting the salt-tolerant shrub, *Maytenus mekongensis*, co-existing species can be protected from grazing and salt accumulation is suppressed. Where this has been applied, EC of the surface soil has been stabilized under vegetation protected from grazing. In addition, litter and other organic matter in the soil were able to suppress the upward movement of salt. Grazing led to an increase of bare ground where NaCl accumulated, and modified the heterogeneity of the vegetation, which was reflected in the degree of salt accumulation (Nemoto and Panchaban, 1991). In addition, Vetiver grass (*Vetiveria* spp.) planted in hedgerows as a conservation measure had been applied in cassava plantations where cassava yield increased in the third year while soil erosion decreased. Groundwater measurements confirmed the strategy as salinisaton was reduced in discharge areas (Yuvaniyama *et al*., 2005).

Stylosanthes guianensis

Stylo (*Stylosanthes guianensis*) belongs to Leguminosae and comprises of several varieties. They are perennial legumes used for forage which are native to Central and South America. Stylo is used as long and short-term pasture for grazing or hay, intercropping with rice, ground cover for erosion control in orchards, green manure as hay for leaf meal and pellets. Freshly harvested seed may have more than 70 % hard seed. Depending upon cultivar, maximum seed production ranges from 700 to 1,350 $kg.ha^{-1}$. In the seasonally dry tropics, flowering commences in September/October and peaks in November and December. By late January, 80 – 90% of the seed has fallen and seed remaining in seed heads is dislodged by beating the crop with bamboo sticks. Seed is then swept up and cleaned, yielding up to 1 $t.h^{-1}$ (Tropical forages, 2008). There are several commercial cultivars released in Australia, (viz. Schofield, Cook, Endeavour, and Graham), and reintroduced into tropical America in the 1950s, that were heavily attacked by the pathogenic fungus anthracnose (*Colletotrichum gloesporiodes*). This is thought to

have occurred due to the greater biotic pressure from this disease in tropical South America. As a result of initial multilocational trials of the RIEPT (International Tropical Pastures Evaluation Network), *S. guianensis* CIAT 184 (Stylo 184) was selected as a promising line for humid tropics in South America. It was reported that cv. Pucalla is tolerant to anthracnose under a wide range of soil; the cultivar is better adapted to low altitudes (<850 m.a.s.l.), acidic soils (<pH 5.0), low levels of organic matter (<3.4%), moderately sandy (18–56% sand), and which have rainfall accumulated in 12 weeks >800 mm. At higher altitudes (>1000 m.a.s.l.), the cultivar appears to respond to higher levels of organic matter (Amezquita *et al*., 1991).

Stylo 184 was introduced into Thailand in 1997 and named as "thapra Stylo". Here it prefers well drained, open-textured soil from sands to light clays. Found on soils with pH from 4.0 – 8.3, adaptation varying with ecotypes, moderately tolerant of high Al and Mn but not of high salinity. It is sensitive to high cutting and heavy grazing. Stylo 184 is recommended as good roughage containing high yield of dry matter approximately 9.4 – 15.6 $t.h^{-1}$ $year^{-1}$. It is also has high total protein (~ 14–18% protein) equal to concentrate which is appeopriate for substitution to reduce cost (Animal Nutrition Division, 2008).

Specific studies on the use of Stylo 184 in Thailand have shown that it is useful as fodder for pigs and cattle and useful in combination with other crops such as cassava and rice. Stylo 184 is a suitable replacement for rice bran on for the production of indigenous, Lao pigs (Keoboualapheth and Mikled, 2003). These pigs, over the live weight range of 10 to 40 kg, can consume up to 6.4% of the diet DM of Stylo 184 without any negative effects on health and with superior growth, leading to higher profits for farmers (Keoboualapheth and Mikled, 2003). In addition, trials utilising cassava hay and stylo 184 for replacement in diets for lactating dairy cows was reported as useful. These trials indicate that Cassava hay in combination with stylo 184 hay (as a supplemental protein source) can be a valuable strategy in small-holder dairy farming systems in the tropics, as an alternative to balanced concentrates for milk production. (Kiyothong and Wanapat, 2003).

Intercropping cassava with stylo 184 also has beneficial effects and can improve foliage biomass and soil fertility (Yuvaniyama *et al*., 2005). In terms of hay yield and crop production, two rows of stylo 184 to one row of cassava could be the optimal pattern for this intercropping system (Kiyothong and Wanapat, 2004). In addition, Stylo 184 has potential for the development of a *Stylosanthes*-rice relay-intercropping system (Homma *et al*., 2008). This would be very useful in areas such as Northeastern Thailand, where only a single rice crop is produced each year, where the rice fields could be utilised during the dry season for forage production. However, Stylo 184 has relatively low salt tolerance and this has restricted its usefulness in this area that has a history of soil salinisation (Homma *et al*., 2008). The availability of salt tolerant lines, therefore, would increase its usefulness in a *Stylosanthes*-rice intercropping system as well as general application for fodder production and soil degradation.

Plant physiology and mechanism under salt stress

High salinity levels can damage soil structure as the action of Na ions, when they occupy the cation exchange complex of clay particles, make the soil more compact and hamper soil aeration. As a result, salinity effects soil physicochemical properties and adversely affects the associated ecological balance of an area. In addition, salinity is a problem for agriculture because few crop species are adapted to saline conditions. Crop species show a spectrum of responses to salt, but all eventually have their yield reduced by excess salt (Bohnert *et al*., 1995, Vinocur and Altman, 2005, Jithesh *et al*., 2006).

In general, the effects of salinity on plant physiology include mechanisms to cope with hyperosmotic stress and ion imbalance (Bohnert *et al*., 1995). High salt concentrations decrease the osmotic potential of the soil solution creating a water stress in plants. Glycophytes, usually encounter ion toxicity since they cannot readily sequester Na^+ into vacuoles. Moreover, the interactions of salts with mineral nutrition may result in nutrient imbalances and deficiencies. Consequently, salinity affects growth and development processes at the cellular level as well as at the whole plant level (Sairam and Tyagi, 2004; Jithesh *et al*., 2006). The response is dependent upon species, age, the organ and cell type and the sub-cellular compartmentalisation (Bray, 1997). There are numerous cellular responses to salt and osmotic stress. For the purposes of this research plan, they have been categorized into 3 groups; ion accumulation, osmotic adjustment, and antioxidative scavenging systems.

Regulation of ion absorption and distribution

Plants are classified as halophytes or glycophytes according to their capacity to grow on high salt media. Most crop plants are glycophytes and cannot tolerate salt stress (Sairam and Tyagi, 2004). In contrast, halophytes are native flora of saline environments which distinguished from glycophytes by their ability to deal with high internal concentrations of Na^+ and Cl^-. That is keeping salt ions away by sequestering them in the vacuole. However, many extreme halophytes display Na^+ dependence for optimal growth and development. They have the capacity to accommodate extreme salinity using salt excretion represents an avoidance mechanism. Salt can be excreted directly out of the plants through their roots, shoots and leaves. Specialized structures involving in excreting salt back into environment are comprised of salt glands and bladders (Flowers, 1985; Glenn *et al*., 1999).

Another avoidance mechanism, salt exclusion, the low permeability of root cells keeps salt away from shoots. The plasmalemma of root cells is the first part which encounters the salt. There is evidence that there exists at the plasmalemma of most plant cells a sodium extrusion pump which controls the level of sodium in the cytoplasm (Chowdhury *et al*., 1995). There are indications from anatomical studies that the development of the casparian strip is important in preventing NaCl uptake in root tips (Flowers, 1985). Internal exclusion mechanisms also involve in the processes of sequestering salt ions into specialized tissues by removing them from the transport stream. This achieves by exchanging K^+ for Na^+ as they pass through the xylem. In some

plants such as *Nitellopsis obtuse* (MacRobbie and Dainty, 1958) and barley (Pitman and Saddler, 1967), excessive Na^+ has to be extruded to prevent growth reduction or cell death. Furthermore, plants can reduce Na^+ transport from roots to shoots by pumping Na^+ out of cells via Na^+-pump to limit Na^+ in the cytoplasm. Salt sensitive lines allow Na^+ accumulate in all organs while salt tolerant lines act selectively and most of the toxic Na^+ accumulate in old leaves and plants do not transport them to young leaves. K^+ accumulation is high in organs where Na^+ concentrations are low (Yasar et al., 2006). Halophytes contain more K^+ in young leaves than in old leaves; this balance is achieved by transporting K^+ in the phloem from old leaves to young leaves (Wolf *et al*., 1991).

Salinity leads to additional ion toxicity effects mainly through perturbations in protein and membrane structure (Cushman, 2001). NaCl is the principle cause of soil salinity stress and there is a focus on Na^+ and Cl^- transport systems (Yokoi *et al*., 2002). The cellular basis of salt tolerance in halophytes depends upon the compartmentation of ions necessary for osmoregulation in vacuoles and osmotic adjustment of the cytoplasm by compatible solutes (Flowers, 1985). Halophytic plants maintain low cytoplasmic Na^+ and Cl^- concentrations and high K^+: Na^+ ratios. K^+ counteracts the inhibitory effect of Na^+ therefore Na^+ and Cl^- are maintained in the cytoplasm at lower concentration than in the vacuole (Flowers, 1985; Blumwald, 2000). Na^+ negatively impacts on homeostasis of essential nutrients such as K^+ and Ca^{2+}. In the cytosol, the presence of K^+ is essential for the activation of many enzymes including those involved in pyruvate synthesis, protein synthesis, glycolysis, and osmoticum production for turgor control. Due to physiological similarities between Na^+ and K^+, excess Na^+ tends to substitute K^+ at K^+ binding sites and hence impairs cellular biochemistry (Manchanda and Garg, 2008). K^+ is probably always available as a free cation and is, with respect to amount, the dominant inorganic ion in plant cells. It should be considered as a "milieu factor" of the protoplasm and, together with antagonistically acting Ca^{2+}, influences the colloidal swelling of the plasma (Mohr and Schopfer, 1995). The negative effects find their origin in many phenonema: ionic interactions between Ca^{2+} and cellular components such as cell wall pectins and membrane phospholipids are sensitive to excess Na^+ (Munns, 2002). Ca^{2+} is, together with Mg^{2+}, a component of pectins in the cell wall and an important factor for the functional and structural integrity of biomembranes (Mohr and Schopfer, 1995). Although these sites typically have a much higher affinity for Ca^{2+} than Na^+, a large molar Na^+: Ca^{2+} ratio leads to dissociation of Ca^{2+} from its binding sites, affecting the integrity of cell walls and cell membranes. A salt-specific effect on root growth and Ca^{2+} deficiency can therefore occur severely and quickly (Munns, 2002).

Osmotic adjustment and osmoprotection

Both salinity and drought induce osmotic stress by decreasing the availability of water, causing loss of cell turgor. The cellular response to turgor reduction is osmotic adjustment; the mechanism plants use to avoid ion toxicity and maintain water uptake by accumulating large quantities of osmolytes (Bohnert and Jensen, 1996, Chen and Jiang, 2010). These osmolytes provide osmotic adjustment and osmoprotection in the cytoplasm by synthesis and accumulation of intracellular compatible solutes (osmolytes) and osmoprotectants such as glycerol, sucrose, trehalose, proline, and betaine. The

accumulation of these solutes varies between plant species (Yancey *et al*., 1982; Flowers, 1985; McCue and Hanson, 1990; Bohnert *et al*., 1995, Chen and Jiang, 2010). While the majority of osmolites are organic solutes, some species use a range of combinations of anions (e.g. Cl^-, SO_4^{2-}, and CO_3^{2-}) and cations (e.g. Na^+, K^+ and Mg^{2+}) (Yokoi *et al*., 2002; Sosa, 2005). Cells can also respond to salt stress by increasing K^+ and/or Na^+ uptake (Serrano *et al*., 1999). In addition, many organic osmolytes are presumed to be osmoprotectants, as their levels of accumulation are insufficient to facilitate osmotic adjustment. For example, glycine betaine preserves thylakoid and plasma membrane integrity after exposure to saline solutions or to freezing or high temperatures. An adaptive biochemical function of osmoprotectants is the scavenging of reactive oxygen species that are byproducts of hyperosmotic and ionic stresses and cause membrane dysfunction and cell death (Yokoi *et al*., 2002).

Antioxidative scavenging system

Oxidative stress results from the osmotic effects and ion toxicity caused by salt stress. It is determined by the overproduction of active oxygen species (AOS) represented predominantly by the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$) (Cavalcanti *et al*., 2004). The AOS, which are the major cause of oxidative stress, are counteracted by a number of antioxidative mechanisms consisting of nonenzymatic and enzymatic components. The nonenzymatic components include antioxidants such as tocopherol, carotenoids, ascorbate and glutathione that are free-radical scavenging molecules. The enzymatic components include enzymes such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT), monohydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (GR) (Jithesh *et al*., 2006). The activities of antioxidant enzymes found in plant which mostly report in scavenging system under salt stress condition are presented below.

Superoxide dismutase (EC 1.15.1.1)

The family of SOD consists of Fe-SOD, Cu/Zn-SOD and Mn-SOD the latter of which does not appear in several plants. $O_2^{\cdot-}$ is a facile product of the reduction of dioxygen that is a threat to living cells. It is scavenged by SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2 in the cytosol, chloroplast and mitochondria (Asada,1999).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Glutathione reductase (EC 1.8.5.1)

In chloroplasts, ascorbate and glutathione are essential mediators in the water-water cycle. GR completes the Halliwell-Asada pathway by regenerating the glutathione pool with NADPH as an electron donor (Foyer *et al*., 1994).

$$GSSG + NADPH \xrightarrow{GR} 2\,GSH + NADP^{+}$$

Catalase (EC 1.11.1.6)

In chloroplast stroma, no catalase has been found. However, PSII membranes have a heme catalase. This catalase would not directly participate in water-water cycle but protects water oxidase in the lumen if water-water cycle does not operate properly and H_2O_2 diffuses to the lumen (Asada, 2006). On the other hand, CAT is the primary H_2O_2 scavenger in peroxisomes (Mittler, 2002).

$$2\,H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Ascorbate peroxidase (EC 1.11.1.11)

APX is classified as a class I peroxidase. It scavenges H_2O_2, the product of SOD, which requires further detoxification. In chloroplasts, H_2O_2 is reduced to H_2O by APX by both stromal and thylakoid-bound forms (Foyer *et al*., 1994). Its responsibility is related to CAT as H_2O_2-scavenging enzymes. APX may play a role in the fine modulation of signaling, whereas CAT's role might be for the removal of excess AOS (Mittler, 2002).

$$2\,ascorbate + H_2O_2 \xrightarrow{APX} 2\,MDA + 2H_2O$$

Peroxidase (EC 1.11.1.7)

POX is widely distributed in all higher plants and protects cells against the destructive influence of H_2O_2 by catalyzing its decomposition through oxidation of phenolic and endiolic cosubstrates (Cavalcanti *et al*., 2004).

$$RH + H_2O_2 \xrightarrow{POX} RH^{\cdot} + H_2O$$

POX involves in various physiological and developmental processes. It is considered as antioxidant scavenging enzyme and associated with cell elongation processes through induction of cell wall loosening. In the same time, it is also involved with reactions that restrict growth (Passardi *et al*., 2004; Roldan *et al*., 2008). The cessation of cell elongation growth related to formation of cross-links between cell wall polysaccharides. Decreasing of cell wall extensibility by the formation of diphenyl bridges between wall polymers, such as hydroxyproline rich glycoprotein, pectins, or

hemicelluloses has been reported as a key role of POX (Fry, 1986). This cell-wall stiffening involved in growth reduction is proposed in many species such as root of rice seedling (Lin and Kao, 2002), hypocotly of pine (Sanchez *et al*., 1995).

Salt tolerant mechanisms in legumes

Legumes are usually sensitive to salinity. In general, salt tolerant plants deal with salt stress using various mechanisms that relate to morphological and physiological and biochemical processes (Greenway and Munns, 1980; Manchanda and Garg, 2008; Patel *et al*., 2010). For those salt tolerant legumes that have been identified, they are characterized by having exclusion mechanisms that maintain low concentrations of Na^+ and Cl^- in actively growing organs. In addition, both toxic ions are compartmentalized in the vacuole; this reduces disruption of cellular metabolism (Van Steveninck *et al*., 1982; Rogers *et al*., 1997; Luo *et al*., 2005). Overproduction and accumulation of osmolytes (e.g. proline and reducing sugars) can also play an essential role in osmotic adjustment (Sidari *et al*., 2008; Arulbalachandran *et al*., 2009; Amirjani, 2010). As results of osmotic stress and ion toxicity under salt stress trigger the formation of reactive oxygen species, salt tolerant legumes can also increase antioxidant enzyme activities i.e. peroxidase, catalase and superoxide dismutase to mitigate oxidative stress (Cavalcanti *et al*., 2004; Arulbalachandran *et al*., 2009; Noreen and Ashraf, 2009; Saha *et al*., 2010).

A number of studies have investigated salt tolerance mechanisms in legumes. They indicate that these plants deal with salt stress by relying on mechanism similar to those of other plants (listed above) i.e. osmotic adjustment and exclusion: ion transport and compertmentalization (Munns *et al*., 2002; Tejera *et al*., 2006). In addition, antioxidant defence systems have been reported (Munns and Tester, 2008; Arulbalachandran *et al*., 2009; Miller *et al*., 2010).

Ion exclusion

Salt tolerant legumes that are able to mitigate salt stress mainly depend on exclusion mechanisms that maintain higher ion concentrations in roots than in shoots (Munns *et al*., 2002; Tejera *et al*., 2006). Some species only exclude Na^+ (*Glycine soya*, Luo *et al*., 2005) or Cl^- (e.g. trifolium, Roger *et al*., 1997; medicago, Sibole *et al*., 2003; *Glycine max*, Luo *et al*., 2005; lotus (*Lotus tenuis*), Teakle *et al*., 2007; Sanchez *et al*., 2011) while others exclude both Na^+ and Cl^- (e.g. Lupin (*Lupinus luteus* and *Lu. angustifolius*, Van Steveninck *et al*., 1982; chickpea (*Cicer arietinum*), Abdelmajid, 2009; Krouma, 2009). These varying degrees of control depend on the salt tolerant capacity of each species as a result of three mechanisms: 1. ion selectivity of root cells, 2. loading of xylem, and 3. removing of salt from xylem in the upper part of the roots and may be retained from the upper part of the root system to lower part of the shoot (Munns *et al*., 2002).

Osmotic adjustment

Production of many compatible solutes for osmotic adjustment has been reported in legumes under salt treatment. For example, proline showed a significant increase in soybean (*Glycine max*) leaves (Amirjani, 2010), in roots of alfalfa (*Medicago sativa*) (Fougère *et al*., 1991), *M. truncatula* (Verdoy *et al*., 2006), and mungbean (*Vigna radiate* L. Wilczek) (Saha *et al*., 2010), in shoots of *Vigna unguiculata* (L.) Walp (Arulbalachandran *at el*., 2009) in roots and nodules of *Phaseolus vulgaris* and *P. aculeate* (Ashraf and Iram, 2005). Glycine betaine increased in nodules of *P. vulgaris* and *P. aculeate* (Ashraf and Iram, 2005). Increase in total reducing sugar was also reported in shoots of *V. unguiculata* (L.) Walp (Arulbalachandran *et al*., 2009). In nodules of *Lo. japonicas*, however, trehalose was produced as an osmoprotectant under salt stress (Lo´pez *et al*., 2006).

Some legumes have been classified as salt tolerant, including plants such as *Prosopis articulate*, *P. pallid* and *P. tamarugo* (which are able to tolerate seawater equivalent salinity (18,000 $mg.L^{-1}$; Felker *et al*., 1981) and *Melilotus indicus* L. (Sherif, 2009) a leguminous herb. *M. indicus* can maintain growth under salt stress conditions by accumulating high Na^+ and Cl^- in shoots and also maintaining stable water content under salt treatment (0 – 300 mM NaCl) (Sherif, 2009). In addition, chickpea was able to accumulate Na^+ and K^+ in roots to decrease root osmotic potential (Tejara *et al*., 2006). Osmotic adjustment that depends upon ion regulation (or accumulation) may be important initially as this is more energy efficient as it consumes less energy (only 3 – 4 moles of ATP per mole of ion). The equivalent synthesis of organic solutes such as proline or sucrose requires 30 – 50 moles of ATP (Raven, 1985).

Antioxidant defence system

Under salt stress, plant cells generally possess antioxidant defense mechanisms for AOS detoxification. Antioxidant enzymes are produced to scavenge AOS in order to diminish salt-induced oxidative damage (Miller *et al*., 2010). Usually, salt tolerant genotypes have higher levels of antioxidant enzymes than those of salt sensitive ones (Munns and Tester, 2008, Arulbalachandran *et al*., 2009; Saha *et al*., 2010). Salt tolerant legumes have been reported to increase antioxidant enzyme activity when exposed to salt stress (Azooz, 2009; Melchiorre *et al*., 2009). For example, a salt tolerant genotype of faba bean (*Vicia faba*) showed higher antioxidant enzymes activity (CAT, POD, APX and GR) than a sensitive genotype (Azooz, 2009). The capacity of salt tolerant lotus (*Lotus filicaulis*) to sustain growth under salt stress was correlated with enhancement of SOD and GR activity (Melchiorre *et al*., 2009).

The level of salt stress, however, causes differing amounts of oxidative damage depending on various factors such as plant variety, stress level and temperature. For example, in soybean (*Glycine max*), APX activity was significantly increased or decreased in different cultivars at 25 ºC, however, at 35 ºC different responses were produced. Under both temperatures, GR activities in the cultivars were generally increased by salt treatment, except for - 0.1 MPa. POX activities at 25 ºC decreased in

most cultivars. However, - 0.4 MPa increased the activity only in cv. Nazlican at 35 ºC (Çiçek and Çakirlar, 2008). Expression of antioxidant exzyme (SOD, CAT and POX) activity at low levels of NaCl (under 50 mM) remained stable with respect to the control, indicating that this concentration did not produced oxidative damage. When soybean plants were exposed to 100 and 200 mM NaCl, enzyme activities significantly decreased (Amirjani, 2010). Leaves of pea plants (*Pisum sativum*) showed increasing antioxidant enzyme activity (CuZn-SOD I, CuZn-SOD II and Mn-SOD) under salt stress (110-130 mol m^{-3}) while low concentration of NaCl (70 mol m^{-3}) had no effect (Hernández *et al*., 1999).

Enzyme activity also varies in different organs. For example, in soybean under mild saline stress (50 mM NaCl), antioxidant enzymes (APX, CAT, GR and SOD) were increased and glutathione, which protects nodules against activated oxygen species, was reduced (Comba *et al*., 1998). When comparing root and shoot tissue within a plant, root tissues of lentil (*Lens culinaris*) were better protected from NaCl stress compared to shoots as the roots had increased SOD and APX activity (Bandeoğlu *et al*., 2004). In addition, pretreatment with a sublethal dose (50 mM) of NaCl enhanced salt tolerance capacity of mungbean (*Vigna radiate*). This was associated with the increased activity of CAT in roots but decreased in shoots while SOD activity increased in both shoots and roots (Saha *et al*., 2010).

Some cases, have been reported where the expression of specific enzyme activity was related to plant species (Cavalcanti *et al*., 2004, Noreen and Ashraf, 2009). In mature leaves of cowpea (*Vigna unguiculata*), the ability to survive under high salinity was not aided by operating an antioxidant system involving SOD, POX and CAT activities (Cavalcanti *et al*., 2004). However, in common bean (*P. vulgaris*), the three antioxidant enzymes (APX, CAT and GR) significantly decreased while SOD increased along with increasing NaCl (Gama *et al*., 2009). In addition, nine cultivars of pea, through exposure to 0 – 120 mM NaCl expressed oxidative stress in all clones; there was no response with respect to enzymatic (CAT and SOD) and non-enzymatic (H_2O_2, melondialdehyde (MDA) and tocopherols) metabolites (Noreen and Ashraf, 2009).

In vitro salt tolerance selection

Tissue culture techniques have been widely used for breeding purposes. Large numbers of cells and cell lines can be selected *in vitro* for resistance to various stresses. This can be done by selecting survival of cells through setting stress condition *in vitro*. Stress causing agents are included in culture media containing dividing cells and act as a selective pressure and only resistant/tolerant cells survive. Many reports indicate that tolerant cell lines can be obtained using such an approach. Examples of where these have been achieved for legumes include: salt stress tolerance of *Vigna radiate* and *V. unguiculata* (Ravikumar *et al*., 2008.) and osmotic selection of alfalfa (*Medicago sativa* L.) (Dragiiska *et al*., 1996). Moreover, regenerated plants from tolerant-cells can display the same tolerant quality as the selected cells (Dixon and Gonzales, 1994). Many attempts have reported the production of salt tolerant plants selecting via tissue culture

and subsequent determination their salt tolerant mechanisms (Saleem *et al.*, 2005; Bekheet *et al*., 2006; Yacoubi *et al*., 2010).

At the cellular level, good growth on salt medium has been used as an indication of salt tolerance. A salt-tolerant callus line of *Lycopersicon peruvianum* was obtained by exposing the cells in suspension culture to increasing NaCl concentrations from 50 to 350 mM. This selected cell lines that grew better than the non-selected line at all levels of NaCl. The tolerant property was retained for 3 passages of subculturing on salt-free medium. The growth in manitol of the selected cell lines was similar to the non-selected line, which suggested that the superiority of the selected line under salt stress was not due to osmotic stress tolerance. Furthermore, the ions SO_4^{2-} and K^+ were highly toxic to *Ly. peruvianum* root callus, while Na^+, Mg^{2+} and Cl^- were less toxic (Hassan and Wilkins, 1988). Callus of salt tolerant tomato (*Ly. esculentum*) cv. Tnshet Star showed the highest callus growth and this correlated with low proline content. Shoot apices grown in salt medium had the highest shoot length while the salt sensitive cultivar (Pascal) had the highest number of leaves. In both salt tolerant and sensitive cultivars, high salt levels resulted in shortening shoot length, and reducing the number of leaves. Shoot and root fresh and dry weights were also reduced (Mohammed *et al*., 2007).

Differentiation between salt tolerant and salt sensitive lines has also been displayed when examining osmolyte production. In *Troyer citrange*, K^+ content of the selected tolerant lines was close to that of the control condition and together with greater than that of the sensitive callus. Increased vacuolar Na^+ concentrations (halophytic behavior) have been supported by increased accumulation in proline and soluble sugars, which are compatible solutes in the selected tolerant but not in the sensitive wild-type calli (Yacoubi *et al*., 2010). In addition, NaCl-selected alfalfa cells accumulated Na^+ higher than non-selected cells when NaCl introduced into their growth medium (Croughan *et a.l*, 1979). Accumulation of proline appeared only in salt tolerant callus of *Cicer arietinum* (Pandey and Ganapathy, 1985) and *Ly. peruvianum* (Hassan and Wilkins, 1988).

The independence and stability of the salt tolerance were found in species (Yacoubi *et al*., 2010). A stable salt tolerant potato cell line derived from callus which was able to grow on medium containing 120 or 150 mM NaCl. Plantlets regenerated from salt tolerant callus also exhibited salt tolerance as evidenced by their higher fresh weights when watered with 90 mM NaCl (Ochatt *et al*., 1999). Additionally, salt tolerant cell lines of *Troyer citrange* obtained by exposing callus to increasing concentrations 0 – 8 $g.L^{-1}$ NaCl maintained their growth after transferring to salt-free medium and after retransfer to salt-containing medium, respectively. Regenerated shoot buds of onion (*Allium cepa* L.) derived from tolerant callus cultures were exposed to the different levels of salts mixture. The tolerant shoot buds were *in vitro* rooted and successfully adapted to free-living conditions (Bekheet *et al*., 2006).

For some plants, however, *in vitro* selection for salt tolerant lines has been obstructed by factors such as inability to regenerate plants, stability of the salt tolerant capacity and morphology of the regenerated plants. For the NaCl-tolerant line of tobacco

(*Nicotiana tabacum/gossii*), the process of shoot regeneration was difficult and successful only in the absence of NaCl. The morphology of regenerated plants differed from those of the wild type, with abnormally short internodes, small leaves and slow growth. Cell suspension cultures derived from plants regenerated from the stable NaCl-tolerant line retained a high level of tolerance (Watad *et al*., 1991). Callus of tobacco (*N. tabacum*) adapted to low NaCl (85 mM) showed low growth with high proline content compared to callus adapted to low concentration of manitol (165 mM). Proline content was similar in both callus adapted to high NaCl (171 mM) and manitol (329 mM) but growth in the latter case was relatively low. In addition, the loss of viability of the adapted callus was comparatively less than the unadapted callus even shock-treatments with 1282 mM NaCl and 823 mM manitol (Gangopadhyay *et al*., 1997). In creeping bentgrass, the salt-tolerant plants selected from callus were unstable with regard to their tolerance, but improved the ability of plants recovered from the *in vitro* stress medium to grow under salt stress condition (Kuo *et al*., 1992).

In vitro techniques have also been used to examine salt tolerance mechanisms of plants where selection has been made on whole plants. For example, the salt tolerant line of Shamouti orange (*Citrus sinensis*) had considerably lower uptake of Na^+ and Cl^- than the sensitive lines (Ben-Hayyim and Kochba, 1985). *In vitro* shoots of For *Eucalyptus camaldulensis*, two tolerant clones were produced more proline under salt stress (Woodward and Bennett, 2005). Shoot growth and sprouting of mulberry (*Morus alba*) were determined for salt tolerance genotype. One of the screened genotypes which selected as a salt tolerant genotype has been tested under respective *in vivo* condition (Tewary e*t al*., 2000). For *In vitro* shoots of cucumber (*Cucumis sativus* L.), tolerant genotypes exhibited higher free proline and some antioxidant enzyme (superoxide dismutase and peroxidase) activities than moderately tolerant and sensitive genotypes after 20 days salinity treatments (Malik *et al*., 2010).

MATERIALS AND METHODS

Materials

Laboratory equipment

1. Media preparation

Bench, gas outlet, hot plate and magnetic stirrer, pH meter, refrigerator, freezer, water purification and storage system, dish-washing equipment, storage facilities for glassware and chemicals, autoclave, and other equipments for tissue culture technique.

2. Aseptic transfer area

Laminar air flow cabinet, forceps, scalpel and disposable blades, petri-dish.

3. Equipment for ion content determination

- Atomic absorption and flame emission spectrophotometer (Perkin Elmer, USA)
- Visible light spectrophotometer (Jenway model 6400 United Kingdom)
- UV/visible light spectrophotometer (Shimadzu, UV-1601)
- Vapour pressure osmometer (Wescor model 5100 c)

4. Plant material; seeds of Stylo 184 (*Stylosanthes guianensis* CIAT 184) obtained from Department of Livestock Thailand

Chemical

1. Chemicals for MS media (Murashige and Skoog, 1962) preparation and plant growth regulators i.e. 6-Benzyladenine (BA), α-Naphthaleneacetic acid (NAA) and 3-Indolebutyric acid

2. Chemicals for ion determination
   - Ammonium ferric sulfate ($NH_4Fe(SO_4)_2 \cdot 12\ H_2O$)
   - Calcium oxide (CaO)
   - Mercuric thiocyanate ($Hg(SCN)_2$)
   - Nitric acid (HNO_3)
   - Standard solution of K^+, Na^+, Ca^{2+}, Mg^{2+} and Cl^-

3. Chemicals for enzyme determination
   - Ethylenediaminetetraacetic acid (EDTA)
   - guaiacol ($C_7H_8O_2$)
   - Heaps
   - Hydrogen peroxide (H_2O_2)
   - 2-amino-4-(methylthio) butanoic acid (Methionine)

- Nitroblue tetrazolium
- Phenylmethanesulfonyl fluoride
- Polyvinylpyrolidone (PVP)
- Riboflavin (Vitamin B2)
- Sodiumcarbonate (Na_2CO_3)
- Sodium sulphate (Na_2SO_4)
- Triton X-100

Methods

Explants preparation

Breaking dormancy and surface sterilization

Seeds of Stylo 184 were soaked in water at 80 ^oC for 1 – 2 min in order to soften the seed coat, then immersed in 1% NaOCl for 30 min followed by 5 rinses with sterile distilled water. Surface sterlised seeds were germinated on the MS medium (Murashige and Skoog, 1962) for 2 weeks. Seedlings were used as explants in the experiments.

Culture conditions

The medium for callus induction and proliferation was MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA.

The cultures were incubated under a photoperiod of 16 hours light (40 $\mu mol\ m^{-2}s^{-1}$ of light intensity) at 25 ± 2 ^oC.

All experiments and cultures were maintained in these conditions.

Experiment 1 Effects of NaCl on growth and ion content in nonselected seedling

To clarify ion regulation in Stylo 184 seedlings, sterilised seeds were placed in Petri dishes (20 seeds per dish) and exposed to 2 mL of 0, 0.5, 0.75, and 1% NaCl for 1 week. Completely Randomized Design (CRD) was used for the experiment with 5 replicates. Germination percentage, shoot height and root length, as well as ion content (Cl^-, Ca^{2+}, Mg^{2+}, Na^+ and K^+) in shoots and roots were determined after the treatments.

Plant materials were dried at 70 ^oC for 2 days. Dried samples were then ground into a fine powder for wet digestion and dry ashing. For the wet digestion, 10 mL of 1.4 N HNO_3 were added to 0.1 g of each ground sample and kept for 1 day at room temperature. The samples were then heated at 100 – 150 ^oC until fully digested. Deionized water (50 mL) were added to the sediment and filtrated with 0.2 μm membrane filter. Ca^{2+}, and Mg^{2+} content were analyzed by atomic absorption and Na^+, and K^+ by flame emission spectrophotometer (Kim *et al*., 1999).

For the dry ashing, 0.1 g of ground sample was mixed with 0.1 g CaO and 1 mL

deionized water and then combusted at 500 ^{o}C for 3 hr. The ash was dissolved with 50 mL deionized water and then took 0.5 mL solution to mix with 4.5 mL deionized water. The solution was added to 0.5 mL mercuric thiocyanate and 1 mL ammonium ferric sulfate. Cl^- content was measured from the colour of ferric thiocyanate complex using absorbance at 460 nm compared to the standard curve for Cl^- standard solution (Adriano and Doner, 1982; Suwanwong, 2001). Molal concentration of Na^+ and K^+ were converted to osmolality by multiplying by 1.84 (Bell and O'Leary, 2003).

Experiment 2 NaCl concentration for salt tolerant seed selection

So that the optimum NaCl concentration was used to select salt tolerant individuals, Stylo 184 seeds were treated with various concentations of NaCl. After breaking dormancy and surface sterilization, seeds were placed in Petri dishes (20 seeds per Petri dish) and treated with 2 mL of 0, 0.5, 1, 1.5, 2, 2.5 and 3 % NaCl solution. They were then incubated in the same condition as describe above. Germination percentage was determined 1 week after treatment. The experiment was replicated 5 times using CRD.

For further specification NaCl concentration, seeds were germinated in NaCl solution upward from the highest concentration (1.5% NaCl) at which seeds were able to germinate as above for 1 week. The concentrations were ranged from 1.5 – 2%; 0, 1.5, 1.6, 1.7, 1.8, 1.9 and 2% NaCl.

Sixty grams of seed (approximately 37,500 seeds) was treated with 2% NaCl solution. They were incubated in the same condition as described above. The germinated seeds were considered as salt tolerant selected individuals. Seeds that germinated under these conditions were designated as salt tolerant. These tolerant seedlings were then induced to grow callus on MS medium containing 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA. Callus was proliferated by regularly subculturing onto fresh medium every 4 weeks.

Distinctive salt tolerant mechanisms were necessary to indicate salt tolerant capacity of the selected clones. The other clone derived from different selection procedure selected to make a comparison with those mechanisms of the tolerant selected clones. This clone could be a salt sensitive or/less tolerant. For the screening procedure, seeds of 1.6 g (approximately 1,000 seeds) were screened on 1% NaCl. The seed which was not capable of germinating, but germinated when transferred onto the salt free MS medium for 2 weeks was selected and used as a control clone. Further, in the thesis experiment, this seed showed good characteristics of salt tolerant glycophyte therefore it would be called less salt tolerant clone.

In order to determine the differences of salt tolerant mechanisms among the selected clones, the representative salt tolerant clones were selected. Callus of ten of the clones selected for salt tolerance and callus of the less salt tolerant clone were cultured on MS medium supplemented with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks. The criterion used to select the representative clones was

50% growth reduction dose (Gr_{50}) which was the NaCl concentration giving a 50% reduction of relative growth rate.

Experiment 3 Media and NaCl concentration for salt tolerant callus selection

For salt tolerant cell lines screening, NaCl concentration and media with or without plant growth regulators were made a comparison in Stylo 184 callus culture. Callus of Stylo 184 induced from nonselected seed were measured their growth capacity on the media including various concentrations of NaCl with or without NAA and BA.

Experiment 3.1 MS medium and NaCl concentration for salt tolerant callus selection

Callus approximately 0.2 cm^3 was cultured on MS medium supplemented with 0, 0.5, 1, 1.5 and 2 % NaCl for 4 weeks. The experiment was replicated 10 times using CRD. Relative growth rate, survival and regeneration percentage were recorded.

Experiment 3.2 MS medium added with NAA and BA and NaCl concentration for salt tolerant callus selection

Callus of 0.2 cm^3 as cultured on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA including 0, 0.5, 1, 1.5, 2, 2.5 or 3% NaCl for 6 weeks. The experiment was replicated 10 times using CRD. Relative growth rate, survival and regeneration percentage were recorded.

Callus of eight clones (clone 1, 2, 4, 5, 7, 8, 9 and 10) derived from experiment 2 was screened for salt tolerant cell lines. Callus of 2 cm^3 was cultured on the MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA and 2.5% NaCl for 6 weeks.

Experiment 4 Growth and ion content in callus (five selected clones)

In order to determine ion regulation in callus of the selected clones, selected seeds of one sensitive and four tolerant clones from experiment 2 were germinated on MS medium. Subsequently, the seedlings were cultured on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA to induce organogenic callus and proliferated on the same medium by subculturing every 4 weeks.

Callus (~0.2 cm^3) of five clones was cultured on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA and a range of salt concentrations (0. 0.5, 1.0 or 1.5% NaCl) for 2 weeks. The experiment was replicated 10 times using CRD. Fresh and dry weight, ion content (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) and cell sap osmolality were determined.

Plant materials were extracted and determined ion content as described in experiment 1.

For cell sap osmolality, fresh callus was frozen at -80^{o}C. After thawing, the callus was placed into 1 mL syringes and the cell sap was expressed by the plunger. The molality was determined by a Wescor model 5100 c vapour pressure osmometer. This value was multiplied by 2.48 to give osmotic pressure in MPa. Molal concentration of Na^{+} and K^{+} were converted to osmolality by multiplying by 1.84 (Bell and O'Leary 2003).

Experiment 5 Oxidative enzymes determination in shoot of selected clones

To examine activity of antioxidative enzymes, *in vitro* shoots of five clones (T1, T2, T3, T4 and T5) were cultured on MS basal medium supplemented with NaCl 0, 0.5 and 1% for 1 week and then transferred to the NaCl-free basal medium (recovery medium) for a further 1 week. After the one week of salt treatment and another week of recovery, enzyme activity and relative fresh weight were measured. The experiment was replicated 4 times using CRD.

Enzyme extraction for catalase, superoxide dismutase and peroxidase

Shoots of 0.5g were ground in 0.75 mL of 0.1 M potassium phosphate buffer, at pH 7.8, containing 1mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulfonylfluoride and 20 mg of polyvinyl pyrolidone. Insoluble material was removed by centrifuging at 12,000 g for 15 min at 4°C (Lokhande *et al*., 2010). All spectrophotometric analyses were conducted at 25 °C with a UV/visible light spectrophotometer (Shimadzu, UV-1601).

SOD activity assay

SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One ml reaction mixture contained 50 mM Hepes buffer (pH 7.6), 0.1 mM ethylenediaminetetraacetic acid, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, 75 µM nitroblue tetrazolium, 2 µM riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 10 min under 36W daylight fluorescent tube. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction when monitored at 560 nm. SOD activity was expressed as units g^{-1} fresh weight ($U.g^{-1}$ fwt) as Noreen and Ashraf (2009).

CAT activity assay

CAT (EC 1.11.16) activity was determined by monitoring the disappearance of H_2O_2 (Noreen and Ashraf, 2009; Amirjani, 2010). The reaction mixture was prepared by adding 0.16 mL of 33 mM H_2O_2 to 100 mL of 0.1 M potassium phosphate buffer at pH 7.0. The decrease in H_2O_2 concentration was followed as a decline in optical density at 240 nm, and the activity was calculated using the extinction coefficient of 40 mM^{-1} cm^{-1}. CAT activity was expressed as $U.g^{-1}$ fwt.

POX activity assay

POX (EC 1.11.1.7) activity was measured on the basis of determination of guaiacol oxidation at 470 nm (Noreen and Ashraf, 2009; Amirjani 2010). In the presence of H_2O_2, POX catalyzes the transformation of guaiacol to tetraguaiacol. The reaction mixture contained 0.1 M potassium phosphate buffer pH 7.0, 20 mM guaiacol and 12.3 mM H_2O_2. This reaction was recorded at 470 nm using the extinction coefficient of 26.6 mM^{-1} cm^{-1}. Enzyme specific activity was expressed as $U.g^{-1}$ fwt.

Experiment 6 Some capabilities of the selected clones after extended time in culture

To observe some characteristics of regenerants after long term maintenance, Stylo 184 callus of five selected clones (T1, T2, T3, T4 and T5) was maintained for over three years on MS with 0.01 $mg.L^{-1}$ NAA and 1.0 $mg.L^{-1}$ BA by subculturing regenerative callus monthly. Shoots regenerated from callus of five clones were cultured on MS medium for 4 weeks and used to test the secondary callus induction, shoot regeneration, rooting capacity and *ex vitro* survival.

Experiment 6.1 The secondary callus induction and shoot regeneration

Shoots of 1.0 cm in length were transferred to MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1.0 $mg.L^{-1}$ BA to test the shoot and callus regeneration capacity. Callus and shoot regeneration and morphological characteristics were recorded over 8 weeks. The experiment was replicated 6 times using CRD.

Experiment 6.2 Rooting and *ex vitro* survival

Single shoots of 1.0 cm in length were cultured on the MS medium containing 0, 0.1, 0.3, and 0.5 $mg.L^{-1}$ IBA for root induction. Root number, root length and shoot height were recorded after 4 weeks. Subsequently, shoots of clone T1 and T8 were transferred to MS medium containing 0.3 $mg.L^{-1}$ IBA to induce root formation. Rooted plants were transferred to pots containing pasteurised vermiculite, sand and peat (1:1:1 v/v). Plantlets were kept for seven days in a misting chamber with intermittent mist (75 – 90% humidity) in a greenhouse followed by 4 weeks to open benches with mist (45 – 65% humidity). Survival percentage was recorded after 5 weeks on the open benches.

Places and Duration

Department of Botany, Faculty of Science, Kasetsart University, June 2008 – January 2011.

School of Natural Science, Edith Cowan University, Australia, February 2011 – December 2011.

Expected Benefits

1. Salt tolerant clones of *Stylosanthes guianensis* CIAT 184 will be derived from selection programme.
2. Some of effective salt tolerant mechanisms of Stylo 184 will be clarified in order to use as key mechanisms to catagorize Stylo 184 salt tolerance.

Funding Source

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RESULTS AND DISCUSSION

Results

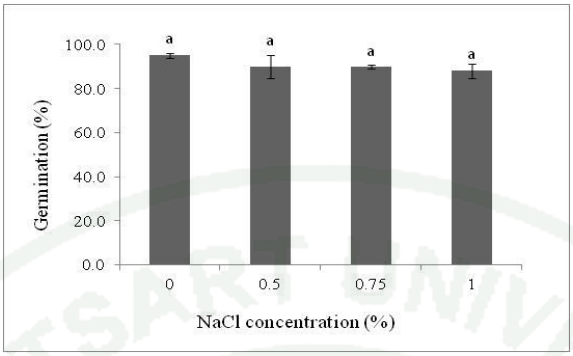
Experiment 1 Effects of NaCl on growth and ion content in nonselected seedling

Seeds of Stylo 184 were treated with 0, 0.5, 0.75 and 1% NaCl for 1 week in order to determine seedling growth and ion content. The result showed that salt treatments of 0 to 1% NaCl had no significant effect on the germination of Stylo 184 seeds and varied from 88 ± 3.2 to 95 ± 1.0% with the higher values obtained at lower NaCl concentrations (Figure 1; Appendix Table 1).The control seedlings germinated in sterilized water, produced normal growth with the highest shoot and root lengths of 2.89 ± 0.1 and 1.89 ± 0.30 cm, respectively (Figure 1; Appendix Table 1). However, NaCl treatments reduced seedling growth and induced several abnormal characteristics such as succulent stems and browning at the root tip (Figure 1). Shoot and root lengths of seedlings germinated in 0.5% NaCl (0.93 ± 0.1, 0.50 ± 0.04 cm, respectively) were significantly lower than that of the control seedlings, while seeds exposed to 0.75 and 1% NaCl showed an even greater reduction but no difference from each other (Figure 2; Appendix Table 1).

The water content in roots (88.8 ± 2.5%) was significantly higher than that of shoots (81.6 ± 2.9%). For shoots, water content was stable (against salt) under 0 to 0.75 % NaCl and ranged from 90.0 ± 1.2 to 83.1 ± 2.5%, however, it dropped significantly in the highest salt concentration to 67.8 ± 5.9%. Root water content was stable over all treatments and ranged from 92.6 ± 1.3 to 79.7 ± 7.6% (Figure 2c; Appendix Table 4).

Na^+, Cl^-, K^+, Mg^{2+} and Ca^{2+} content in shoots and roots were determined. Shoot ion content was significantly lower than that of the roots for all ions except Ca^{2+}. In addition, there was a significant interaction between shoots and roots for Na^+, Ca^{2+}, and Cl^- as shoot ion content did not vary between salt treatments but root ion content did (Appendix Table 2). As NaCl increased, mean Na^+ and Cl^- content increased significantly while K^+, $Mg^{2+}$and Ca^{2+}remained constant (Appendix Table 2). However, 0.5 – 1% NaCl did not significantly effect Na^+ content which was higher than the control in both shoots (2.47 ± 0.12 – 1.89 ± 0.41% dwt) and roots (16.79 ± 2.16 – 27.29 ± 6.68% dwt) (figure 3a; Appendix Table 3). For Cl^- content, there was no significant difference in shoots treated with 0.5 to 0.75% NaCl (2.02 ± 0.23 and 2.37 ± 0.19% dwt, respectively). The highest Cl^- content in roots was found in the 0.75% NaCl treatment for 8.84 ± 0.61% dwt. There was no significant different between Cl^- content in root treated with 0.5 and 1% NaCl (6.32 ± 0.84 and 5.19 ± 0.68% dwt, respectively) (Figure 3b; Appendix Table 3).

Na^+:K^+ ratios increased significantly at 0.5% NaCl for both shoots and roots. The ratio was also stable from 0.5 – 1% NaCl in both shoots (2.14 ± 0.15 to 1.81 ± 0.31) and roots (8.39 ± 1.01 to 10.04 ± 1.26) (Figure 4a; Appendix Table 4). The Na^+:Ca^{2+} ratios, however, did not change in the shoots (9.59 ± 0.79 - 24.51 ± 3.52) but significantly increased in the roots. High Na^+:Ca^{2+} ratio of 0.5 and 1% NaCl showed no significant different from 1276.29 ± 163.84 to 2362.56 ± 426.05 (Figure 4b; Appendix Table 4).



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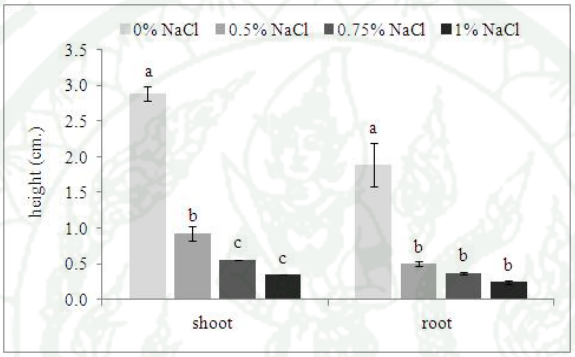

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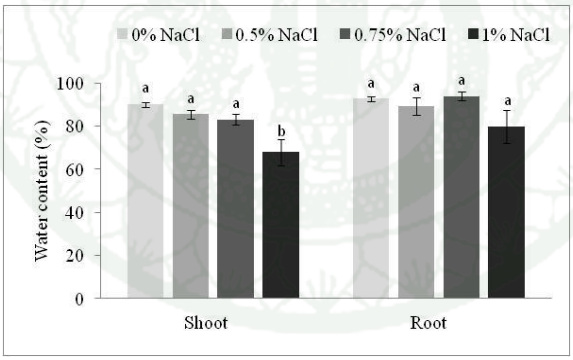


c

Figure 1 Seedling of Stylo 184 germinated in 0, 0.5, 0.75 or 1% NaCl for 1 week, (a) percent germination, (b) shoot and root height, (c) water content in shoot and root. Error bars indicate standard error; n = 5; different letters above bars for each organ indicate significant differences ($P \leq 0.05$).

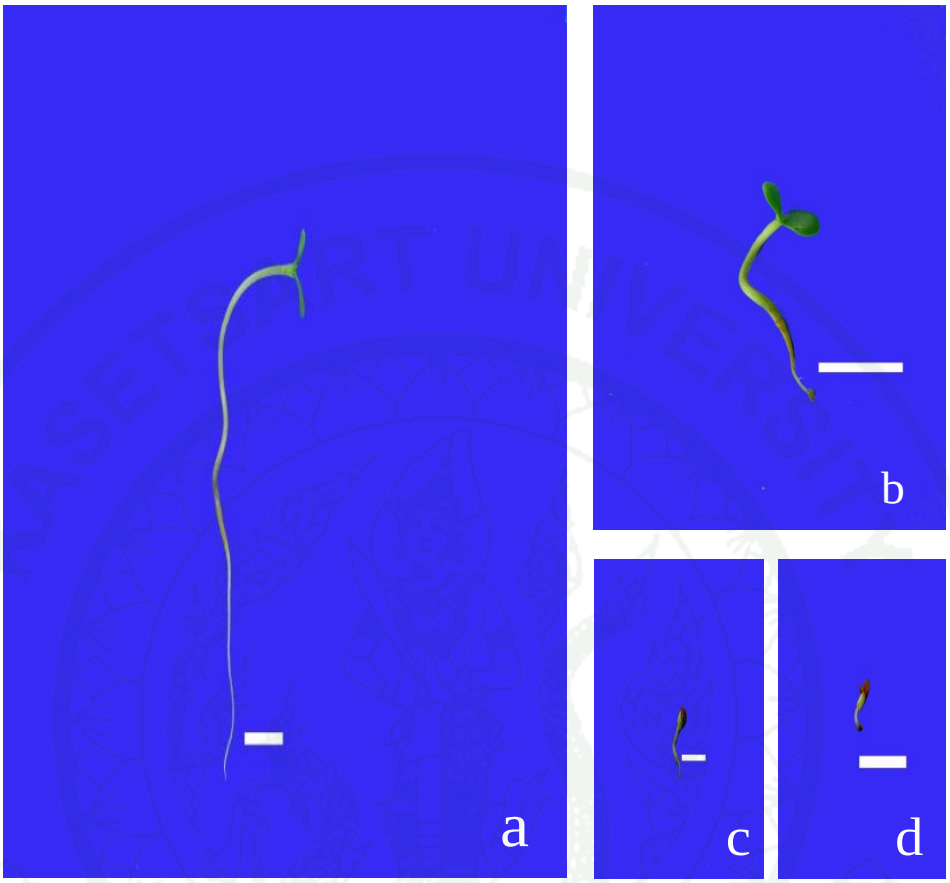


Figure 2 Seedling of Stylo 184 germinated in NaCl solution for 7 days at (a) 0% NaCl, (b) 0.5% NaCl, (c) 0.75 % NaCl, and (d) 1% NaCl. Bar scale = 0.5 cm.

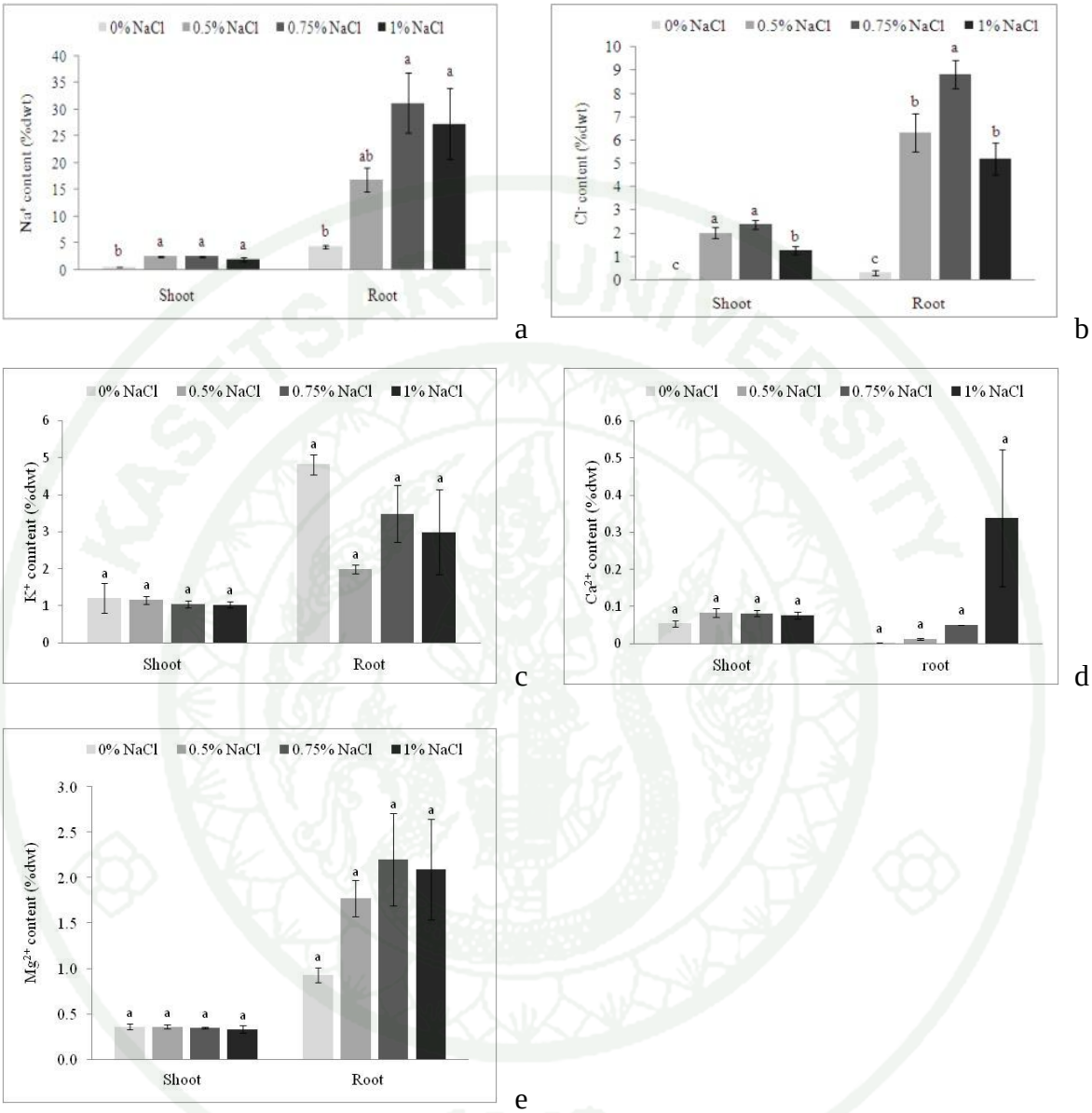
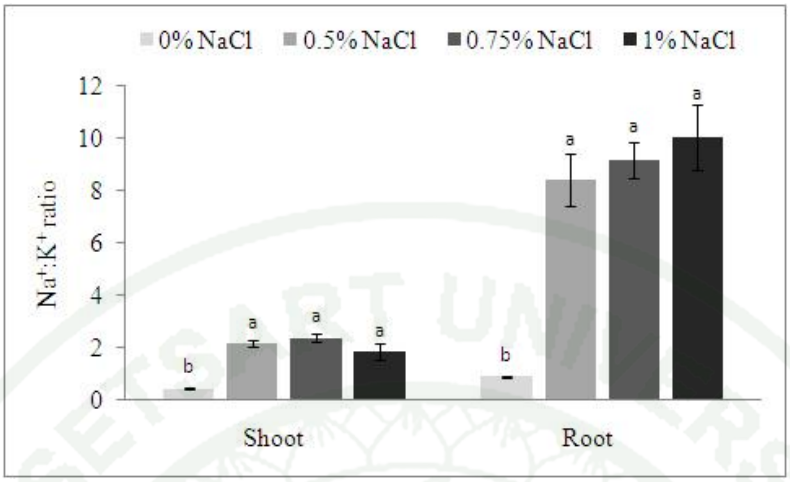


Figure 3 Ion content in seedling of Stylo 184 treated with 0, 0.5, 0.75 or 1 % NaCl solution for 1 week (a) Na^+, (b) Cl^-, (c) K^+, (d) Ca^{2+}, and (e) Mg^{2+}. Error bars indicate standard error; n = 3; different letters above bars in each organ indicate significant differences ($P \leq 0.05$).



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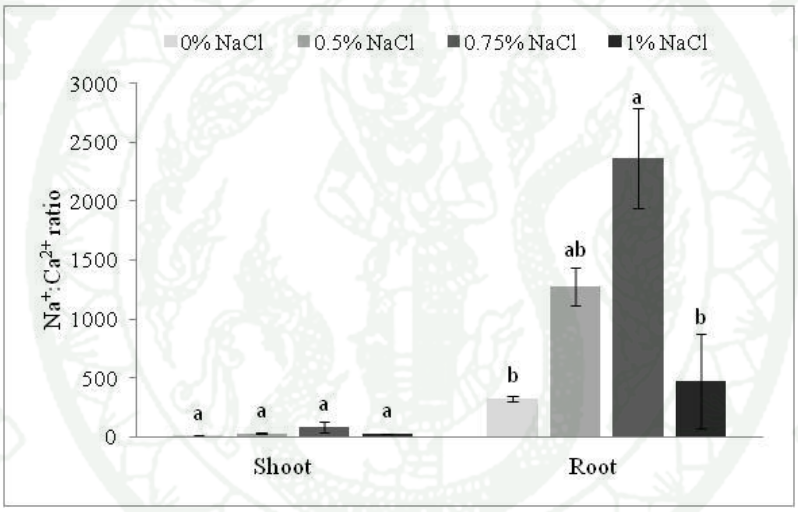

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Figure 4 Ion ratios in seedling of Stylo 184 treated with 0, 0.5, 0.75 or 1 % NaCl solution for 1 week (a) Na^+:K^+, (b) Na^+:Ca^{2+}. Error bars indicate standard error; n = 3; different letters above bars for each organ indicate significant differences ($P \leq 0.05$).

Experiment 2 NaCl concentration for salt tolerant seed selection

After heat treatment, seeds of Stylo 184 were surface sterilized and germinated in Petri dish containing 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl solution for 1 week. Germination of 95 ± 1.6% with no significant difference was found on seeds treated with 0, 0.5 or 1% NaCl. When the concentration of NaCl increased to 1.5%, germination was reduced to 40 ± 3.2% and totally inhibited when exposed to 2 or 3% NaCl solution (Figure 5a; Appendix Table 5).

In order to determine a level of NaCl concentration that could be used to select for salt tolerance, seeds were treated with 0, 1.5, 1.6, 1.7, 1.8, 1.9 and 2% NaCl solution for 1 week. In the treatment without NaCl seeds were the highest germination for 82 ± 3.4%. Germination was 22 ± 8.9% when seeds exposed to 1.5% NaCl and significantly decreased to 14 ± 3.3% in 1.6% NaCl solution, respectively. The highest concentration of NaCl where seeds germinated was 1.7% that the lowest germination percentage was found for 9 ± 3.0%. However, germination percentage found on 1.5 – 1.7% NaCl was not significantly different (Figure5b; Appendix Table 6). Consequently, seeds were germinated in 1.7% NaCl so as to screen for salt tolerant seeds.

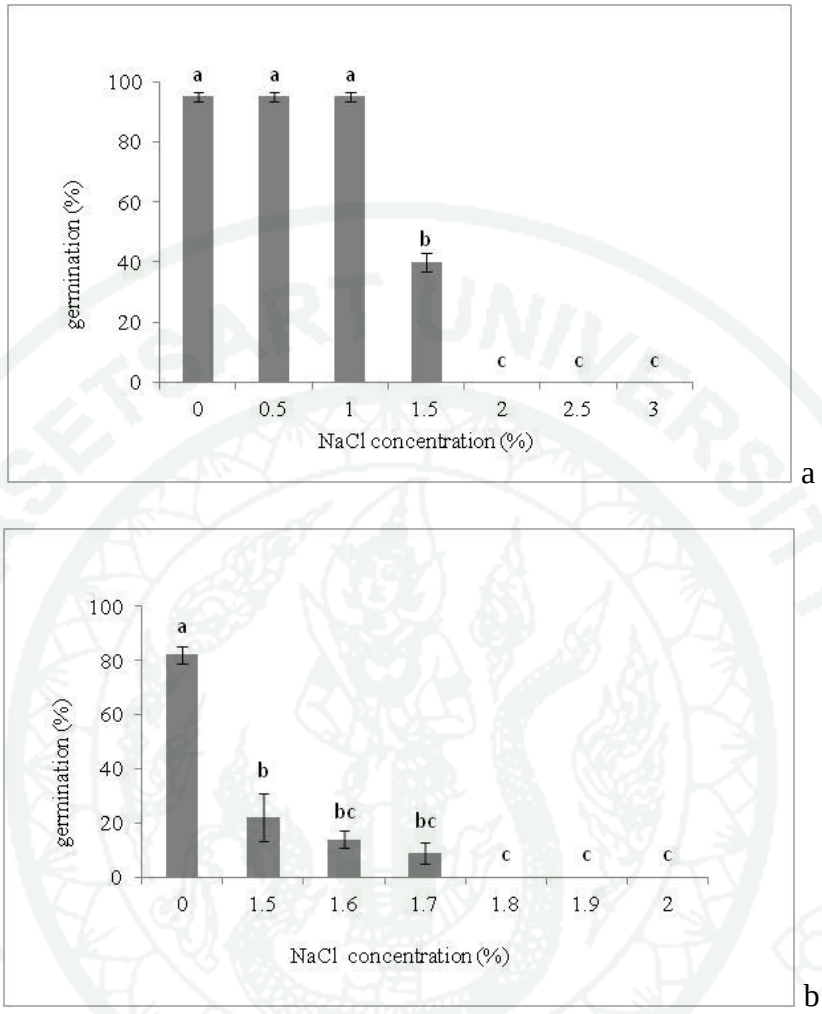


Figure 5 Percentage germination of Stylo 184 seed treated with; 0, 0.5, 1, 1.5, 2, 2.5 or 3% NaCl solution (a), 0, 1.5, 1.6, 1.7, 1.8, 1.9 or 2% NaCl solution (b) for 1 week. Error bars indicate standard error; n = 5; different letters above bars indicate significant differences ($P \leq 0.05$).

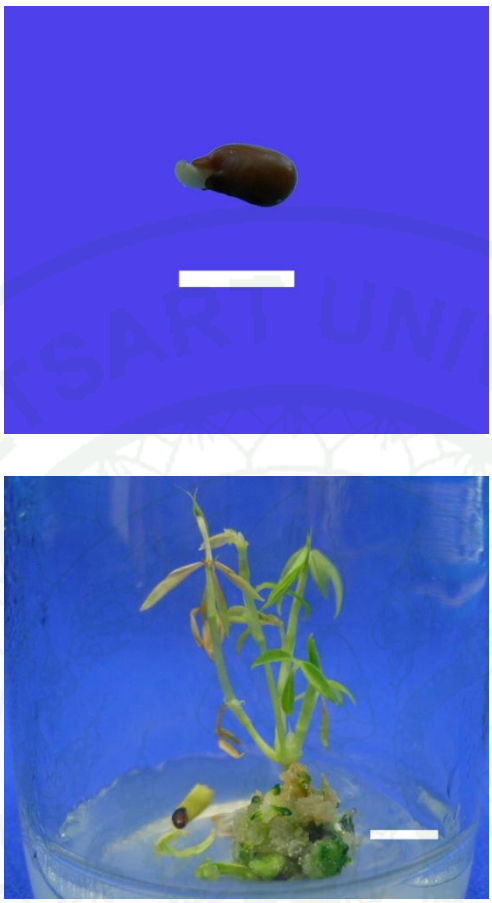
The selection programme had been conducted using 1.7% NaCl, however, large amount of selected seeds were obtained that was not appropriate to indicate their salt tolerant capacity. One germinated seed on 2% NaCl was found after extended 4 weeks treatment. Consequently, seeds of 60 g (approximately 37,500 seeds) were treated with 2% NaCl in order to screen for salt tolerant individuals. In addition, less salt tolerant seed was screened so that some salt tolerant mechanisms could be investigated and compared to those of the genotypes that germinated under high salt salt conditions. In accordance with the result that seeds equally germinated in sterilized water and the highest concentration of 1% NaCl. 1,000 seeds were treated with 1% NaCl. The less salt tolerant seed was selected from seed which was not capable of germinating on 1% NaCl, but germinated when transferred onto the salt free MS medium for 2 weeks.

Ten seeds germinated on 2% NaCl were selected as salt tolerant (Figure 6a). Salt tolerant and less salt tolerant seeds were transferred onto MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA so as to induce callus formation (Figure 6b).

Callus of ten of the clones selected for salt tolerance was investigated for salt tolerant ability in comparison to the less salt tolerant clone by culturing on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks. As a result, the selected clones were categorized into 3 groups according to their growth.

1. Group of the clone 2 and 8 had the highest NaCl level at 50% growth reduction (GR_{50}) for 0.8% NaCl.
2. Group of clone 3, 5 and 10 expressed GR_{50} for 0.7% NaCl. Clone 3, however, had higher relative growth rate (RGR) of 64.8% control at 0.5% NaCl.
3. Group of the lowest GR_{50} of clone 1, 4, 6 and 7 was 0.5% NaCl equivalent to the sensitive/or less tolerant clone. Among this group, clone 4 had the lowest RGR of 0.5% control at 1.5% NaCl which was lower than 4.9% control of the sensitive/or less tolerant clone (Table 1; Appendix Figure 1).

The salt tolerant mechanisms of the Stylo 184 selected clones would be revealed. The representative clones revealing distint characteristics were selected. They were selected from each groups of Gr_{50} level that were clone 3 and 4 from the group of Gr_{50} for 0.7% and 0.5% NaCl, respectively. Clone 2 and 8 were in the same group of the highest Gr_{50} for 0.8% NaCl, In addition, according to the result of ion content determination, they expressed distinctive characteristics to defend against salt stress, succulent of clone 2 and dominated tolerant glycophyte of clone 8. Consequently, four salt tolerant clones (clone2, 3, 4 and 8 named as T2, T3, T4 and T5) were selected as the representative of salt tolerant clones to compare salt tolerant mechanisms with the less salt tolerant clone (clone1 named T1) in the next experiment.



a

b

Figure 6 Seed selected for salt tolerance and callus induction, (a) germination of seed on 2% NaCl and (b) callus induction from selected seedling on MS medium containing 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA. Bar scale = 0.5 cm.

Table 1 Mean relative growth rate and 50% growth reduction dose (GR_{50}) of ten salt tolerant clones and one less salt tolerant selected clones. N = 10

NaCl (%)	Clone										
	1	**2 (T2)**	**3 (T3)**	**4 (T4)**	**5**	**6**	**7**	**8 (T5)**	**9**	**10**	**less tolerant (T1)**
0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.5	25.3	74.6	64.8	23.7	47.4	22.8	28.8	63.5	21.4	42.5	42.9
1.0	12.8	43.1	9.5	13.7	28.4	13.0	12.6	30.5	40.8	40.3	4.3
1.5	6.3	6.2	3.3	0.5	5.3	5.7	3.5	11.6	2.3	3.9	4.9
Gr_{50}	**0.5**	**0.8**	**0.7**	**0.5**	**0.7**	**0.5**	**0.5**	**0.8**	**0.6**	**0.7**	**0.5**

Experiment 3 Media and NaCl concentration for salt tolerant callus selection

After germination, nonselected seeds of Stylo 184 were induced callus formation on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA.

Experiment 3.1 MS medium and NaCl concentration for salt tolerant callus selection

Salt tolerant capacity of callus was determined. Callus of 2 cm^3 in size was cultured on MS medium containing 0, 0.5, 1, 1.5 and 2% NaCl for 4 weeks. Callus growth was significantly reduced with each increase in salt concentration. Relative growth rate (RGR) of callus grown on 0.5% NaCl reduced to 22.9 ± 12.3% (Figure 7a; Appendix Table 7) while he lowest growth reduction was found on 1, 1.5 and 2% NaCl for -23.8 ± 6.2, -26.2 ± 6.7 and -43.1 ± 6.9, respectively (Figure 7a; Appendix Table 7). In addition, survival percentage showed no significantly different when callus cultured on 0, 0.5 and 1% NaCl for 71.0 ± 4.1, 74.0 ± 7.8 and 58.0 ± 4.9%, respectively (Figure 7b; Appendix Table 7). Similarly, regeneration percentage was not significantly different on 0 – 1% NaCl for 54.0 ± 6.2, 57.0 ± 7.0 and 58.0 ± 4.9%, respectively. However, callus cultured on 1.5 and 2% NaCl expressed survival percentage for 33.0 ± 4.5 and 14.0 ± 4.5%, respectively, while there was no regeneration percentage found on 1.5 and 2% NaCl (Figure 7b; Appendix Table 7).

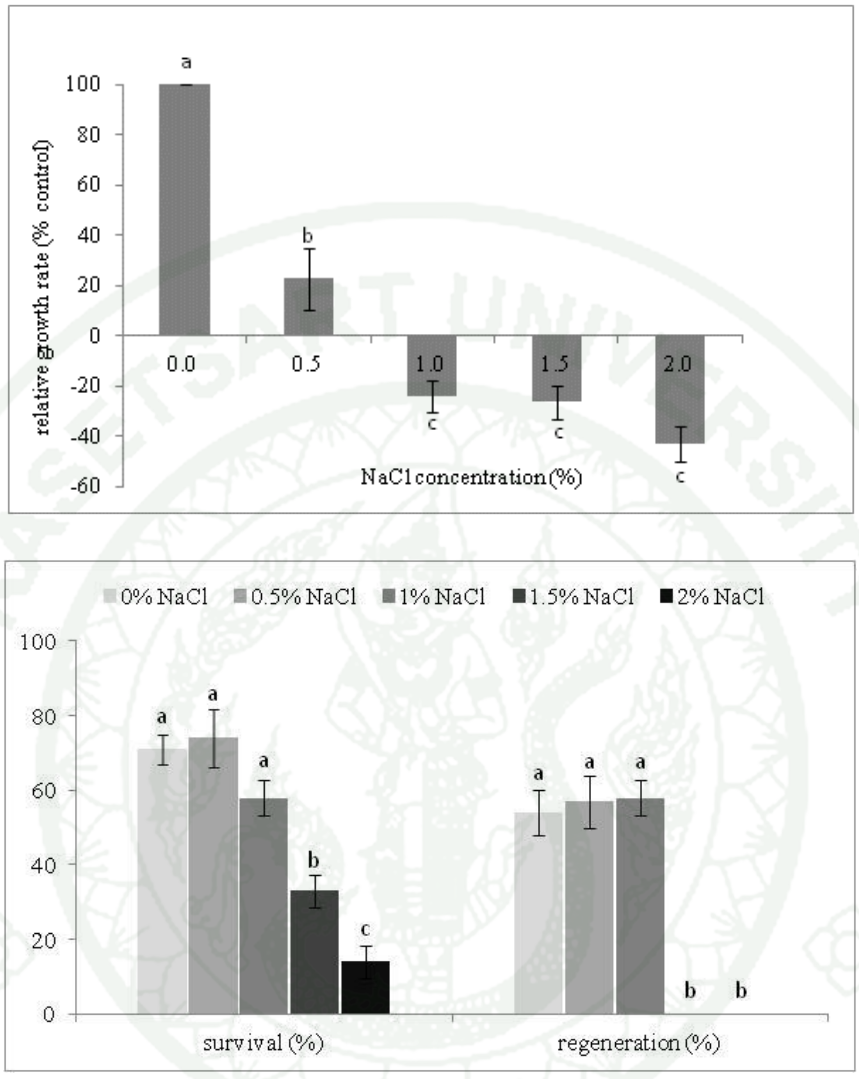


Figure 7 Callus of Stylo 184 cultured on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks; (a) relative growth rate, survival and (b) percent regeneration. Error bars indicate standard error; n = 5; different letters above bars indicate significant differences ($P \leq 0.05$).

Experiment 3.2 MS medium added with NAA and BA and NaCl concentration for salt tolerant callus selection

Callus of 2 cm^3 in size was cultured on MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA with 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl for 6 weeks. The RGR of callus on each of concentration 0 – 1.5% NaCl showed significantly reduction for 100.0 ± 0.0, 47.6 ± 5.2, 30.6 ± 4.4 and 8.7 ± 1.0, respectively (Figure 8a; Appendix Table 8). Survival percentage was not significantly difference from 0 to 1.5% NaCl for 52.0 ± 6.8, 50.0 ± 6.1, 40.0 ± 10.9 and 28.0 ± 8.1, respectively while survival percentage found on 1.5% NaCl was not significantly difference from those found on 2 and 2.5% NaCl (12.0 ± 3.6 and 11.0 ± 5.5) (Figure 8b; Appendix Table 8). Importantly, the highest concentration which callus was able to survive was 2.5% NaCl (Figure 8b; Appendix Table 7). However, NaCl concentrations which callus was capable of regenerating were from 0 – 1% for 52.0 ± 6.8, 50.0 ± 6.1 and 40.0 ± 10.9, respectively (Figure 8b; Appendix Table 8).

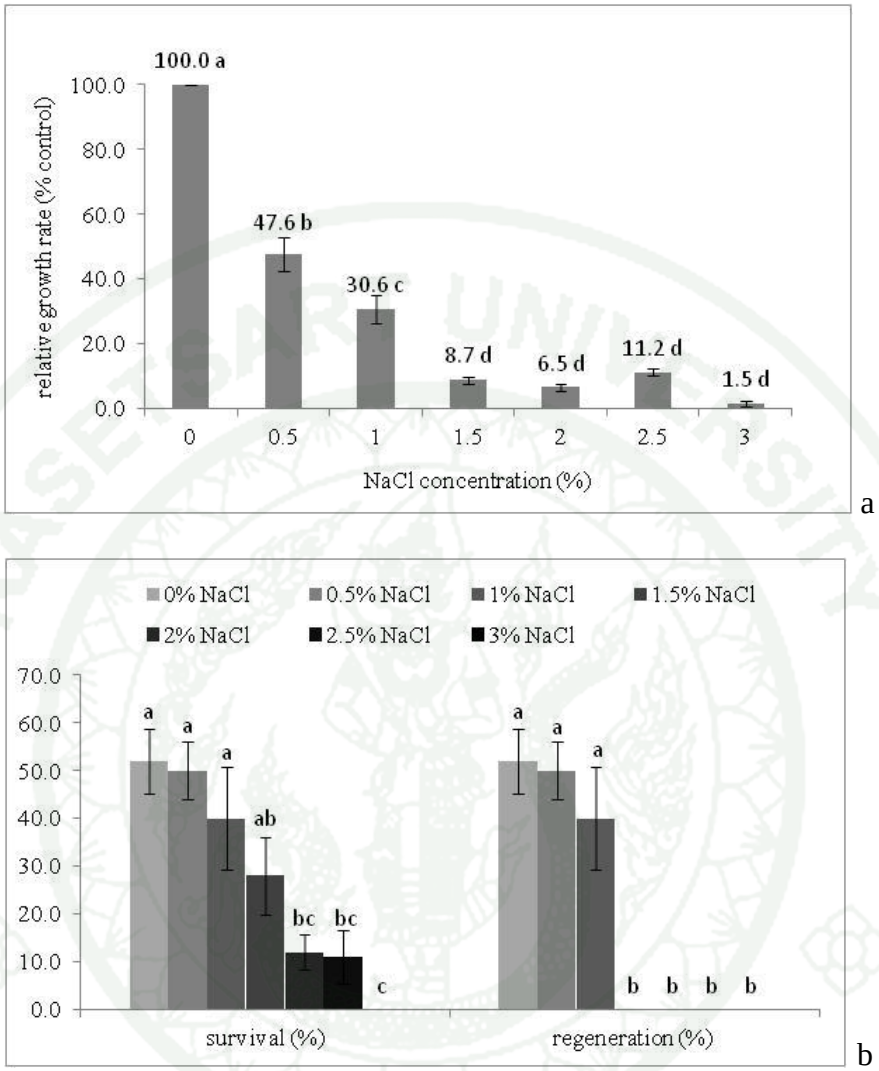


Figure 8 Callus of Stylo 184 on MS medium containing 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl for 6 weeks; (a) relative growth rate, survival and (b) regeneration percentage. Error bars indicate standard error; n = 5; different letters above bars indicate significant differences ($P \leq 0.05$).

Callus from the 8 clones selected for variation in salt tolerance derived from experiment 2 was screened for salt tolerant cell lines. Callus 2 cm^3 in size was cultured on the MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA and 2.5% NaCl for 6 weeks. Survival percentage ranged from 0 – 14.29%. Clone 8 (T5) showed the highest survival percentage for 14.29% while clone 5 and clone 7 showed no survival callus at the end of treatment time (Table 2). Subsequently, the survival callus was transferred onto the medium reduced concentration of NaCl to 1% and subcultured every 3 months. After the 2nd subculture, most of the callus turned brown and died. The survival callus, however, showed little enlargement but produced regions with green protuberances. They were able to regenerate shoots after transfer onto the fresh medium with reduced NaCl (0.5%).

Table 2 Survival percentage of salt tolerant callus cultured on the MS medium containing 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA and 2.5 % NaCl for 6 weeks.

Clone	Explants (piece of callus)	Survival percentage (%)
clone 1	80	2.5
clone 2 (T2)	60	5
clone 4 (T4)	70	5.71
clone 5	70	-
clone 7	80	-
clone 8 (T5)	70	14.29
clone 9	70	8.57
clone 10	70	12.86

Experiment 4 Growth and ion content in callus (five selected clones)

Callus of the five selected clones (T1, T2, T3, T4 and T5) derived from experiment 2 were proliferated on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA by subculturing every 4 weeks. These were subsequently transferred to MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA with varying concentration of NaCl (0, 0.5, 1 and 1.5%) for 2 weeks. Cultures were assessed for RGR, water content, Na^+, Cl^-, K^+, Ca^{2+}, Na^+:K^+, Na^+:Ca^{2+}, osmotic pressure, and contribution to osmolality (%) by Na^+ and K^+.

For each of the parameters, there was a significant difference due to both clone and salt treatment with the exception of Mg^{2+} content. In addition, there was a two-way interaction between the clones and treatment indicating that not all the clones responded in the same way (Table 3).

The relative growth rate (RGR) of the callus of the five clones under the influence of NaCl (0-1.5%) varied by both clone and NaCl concentration with growth generally being reduced with increasing salt concentration (Figure 9; Appendix Table 9). T5 displayed a significant reduction only at 1.5% NaCl for 44.4 ± 0.9% control, but there was no significant difference between 0.5 and 1% NaCl (93.3 ± 6.7 and 79.7 ± 8.8% control, respectively). T1, T2, and T4 showed the greatest reduction in growth with a decline to 70.7 ± 7.4, 65.3 ± 2.9 and 36.8 ± 2.6% control, respectively compared to the control at 0.5% NaCl. T3 had an intermediate growth response with no significant difference at 0.5% NaCl of 107.0 ± 12.8% control but drastically declined at 1.0% NaCl of 42.7 ± 3.2% of the control (Figure 9; Appendix Table 9).

Callus water content was also significantly reduced as NaCl concentration increased (Figure 10, Appendix Table 9). T2 was able to maintain water content at 0.5% NaCl (85.6 ± 0.6%) which was not significantly different from the control (89.5 ± 1.8%). It revealed a significant reduction at 1% NaCl (compared to the control) for 81.9 ± 0.5% but no further reduction at 1.5% (81.1 ± 1.6%). All the other clones, T1, T3, T4 and T5, had a significant reduction in water content at 0.5% NaCl of 76.2 ± 1.0, 82.7 ± 1.1, 82.7 ± 1.1, 83.5 ± 0.5%, respectively. The lowest water content at 1.5% NaCl was found in each clone except T5, where 1% NaCl expressed the lowest content of 81.4 ± 0.1% (Figure 10; Appendix Table 9).

All clones showed significant increases in osmotic pressure with each increase in NaCl concentration (Figure 11a; Appendix Table 10). The osmotic pressure of 0 – 1.5 % NaCl treatment ranged from 1.01 ± 0.04 to 2.73 ± 0.08 MP with the highest osmotic pressure of each clone found at 1.5% NaCl (Table 3). In addition, as NaCl concentration increased, the contribution of Na^+ and K^+ to osmolality varied between clones. The highest contribution of Na^+ was found in T2 (29.0 ± 5.7%) while the lowest was found in T1 (19.4 ± 3.4%) (Table 3). Clones T2, T3 and T5 exhibited an increasing contribution of Na^+, but T1 and T4 remained stable from 0.5 to 1.5% NaCl (21.1 ± 1.7 to 26.2 ± 2.3% for T1 and 28.4 ± 1.2 to 34.1 ± 2.2% for T4) (Figure 11b; Appendix Table 10). Additionally, K^+ contribution to osmolality in T2, T3, T4 and T5 decreased significantly, but was

stable in all levels of NaCl for T1 (38.5 ± 2.6 to 19.1 ± 0.6%) (Appendix Table 10). T1 had the highest contribution of K^+ to osmolality of 36.7 ± 4.9% (Table 3) and was also the major contributor (59.3 ± 8.6%) in the medium without NaCl. T5, however, was able to maintain the contribution of K^+ to osmolality from 0.5 – 1% NaCl of 10.3 ± 0.2 to 8.8 ± 0.7% (Figure 11b; Appendix Table 10).

The ion content of the callus depended upon the clone and the level of NaCl in the medium. Na^+ and Cl^- content significantly increased for each clone with increasing NaCl application (Figure 12a, b; Appendix Table 11). T1 had the lowest increase of 0.06 ± 0.02% dwt to 2.73 ± 0.19% dwt for Na^+ (Figure 12a) and 0.03 ± 0.00% dwt to 0.08 ± 0.00% dwt for Cl^- (Figure 12b; Appendix Table 11). On the other hand, with increasing of NaCl, T2 had the greatest of 0.45 ± 0.22% dwt to 6.83 ± 1.23% dwt for Na^+ and 0.08 ± 0.05% dwt to 0.23 ± 0.04% dwt for Cl^- (Figure 12a, b; Appendix Table 11). The other three clones showed intermediate responses (Figure 12a, b; Appendix Table 11). T3 was able to maintain Cl^- constant from 0.5 – 1.5% NaCl (0.09 ± 0.02% dwt to 0.12 ± 0.02 % dwt). T4 contained Na^+ (2.33 ± 0.09% dwt to 2.78 ± 0.12 % dwt) and Cl^- (0.08 ± 0.01% dwt to 0.08 ± 0.00% dwt) stable at 0.5 –1%, and T5 also maintained Cl^- (0.07 ± 0.01% dwt to 0.09 ± 0.01% dwt) stable at 0.5 – 1% NaCl (Figure 12a, b; Appendix Table 11).

There was a significant difference of K^+ content between the clones and the response to increasing NaCl again varied between clones (Table 3). In T1 and T2 there was no change in K^+ over the four NaCl concentrations (4.85 ± 0.03% dwt to 3.3 ± 0.04% dwt for T1, and 2.43 ± 0.14% dwt to 1.53 ± 0.31% dwt for T2) (Figure 12c; Appendix Table 11). The K^+ content; however, were twice as high in T1 compared to T3, T4 and T5 (Figure 12c; Appendix Table 11). In addition, T5 was able to stabilize its K^+ levels on 0.5 – 1.5% NaCl (1.75 ± 0.04% dwt, 1.80 ± 0.27% dwt and 1.65 ± 0.15% dwt, respectively). T4 was able to maintain K^+ stable at 0.5 – 1% NaCl from 1.73 ± 0.11% dwt to 1.22 ± 0.08% dwt while T3 had decreasing K^+ with increasing NaCl (Figure 12c; Appendix Table 11).

Despite T1 and T2 having the same trend in K^+ content, T2 was also able to maintain Ca^{2+} and Mg^{2+} at all the concentrations of NaCl. The highest Ca^{2+} was also found in T2 for 0.12 ± 0.05 % dwt, which was not significantly different from 0.06 ± 0.01% dwt of T1 (Table 3). In T1, however, Ca^{2+} was significantly reduced at 1% NaCl for 0.040 ± 0.008% dwt. For T3, T4 and T5 there was an immediate reduction in Ca^{2+} on exposure to 0.5% NaCl (0.031 ± 0.011% dwt, 0.022 ± 0.004% dwt and 0.009 ± 0.001% dwt, respectively) with no change in higher salt (Figure 12d; Appendix Table 11). For Mg^{2+}, the highest content was found on T5 for 0.591 ± 0.031% dwt (Table 3). In addition, T1 expressed limited changes all along increasing of NaCl with no significantly difference at 0.5,1 and 1.5% NaCl for 0.381 ± 0.010% dwt, 0.402 ± 0.023% dwt and 0.382 ± 0.200% dwt, respectively. T3 had abrupt decreased Mg^{2+} significantly at 1% NaCl for 0.043 ± 0.003% dwt. T2, T4 and T5 had no difference of Mg^{2+} content under NaCl influence (Figure 12e; Appendix Table 11).

The variable changes in the ion content of the clones led to significant differences in the Na^+:K^+ ratios and the Na^+:Ca^{2+} ratios. As a result of high K^+ and low Na^+, T1 had the

lowest Na^+:K^+ ratio (0.45 ± 0.1) (Table 3). T2, however, showed the opposite with a significantly higher Na^+:K^+ ratio (2.56 ± 0.6) to all the other clones (Table 1; Figure 13a, Appendix Table 12). The other clones had intermediate responses; T5 maintained a low Na^+:K^+ ratio with no significant differences from 0.5 – 1% NaCl (1.27 ± 0.08 to 1.88 ± 0.20) while the Na^+:K^+ ratio in the other clones decreased significantly. T3 had the highest Na^+:Ca^{2+} ratio (881.74 ± 357.3). Only T2 maintained a stable Na^+:Ca^{2+} ratio when exposed to NaCl range from 1.72 ± 0.17 to 1073.17 ± 808.53 (Figure 13b; Appendix Table 12).

Table 3 Relative growth rate (RGR), water content (%), ion content(%dwt), ion ratio in callus, and osmotic pressure (MP) and contribution to osmolality (%) by Na^+ and K^+ in cell sap of Stylo 184 cultured on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA including 0–1.5 % NaCl.

	Ion content				
	Na^+	**Cl^-**	**K^+**	**Ca^{2+}**	**Mg^{2+}**
Clone					
T1	1.75 ± 0.32^{c}	0.06 ± 0.01^{b}	4.42 ± 0.28^{a}	0.060 ± 0.008^{ab}	0.372 ± 0.011^{b}
T2	4.29 ± 0.85^{a}	0.15 ± 0.02^{a}	2.04 ± 0.22^{a}	0.124 ± 0.048^{a}	0.454 ± 0.062^{b}
T3	2.56 ± 0.52^{b}	0.09 ± 0.01^{b}	1.62 ± 0.12^{b}	0.037 ± 0.014^{b}	0.052 ± 0.003^{c}
T4	2.32 ± 0.42^{bc}	0.09 ± 0.02^{b}	1.70 ± 0.21^{b}	0.043 ± 0.013^{b}	0.099 ± 0.010^{c}
T5	2.85 ± 0.59^{b}	0.09 ± 0.02^{b}	1.99 ± 0.15^{b}	0.036 ± 0.010^{b}	0.591 ± 0.031^{a}
NaCl(%)					
0	0.22 ± 0.05^{d}	0.04 ± 0.01^{d}	3.00 ± 0.26^{a}	0.135 ± 0.036^{a}	0.332 ± 0.074
0.5	2.31 ± 0.21^{c}	0.08 ± 0.01^{c}	2.59 ± 0.38^{a}	0.053 ± 0.012^{b}	0.288 ± 0.051
1	3.72 ± 0.40^{b}	0.11 ± 0.01^{b}	2.03 ± 0.36^{b}	0.035 ± 0.007^{b}	0.316 ± 0.056
1.5	4.77 ± 0.43^{a}	0.16 ± 0.02^{a}	1.79 ± 0.22^{b}	0.017 ± 0.004^{b}	0.318 ± 0.064

Table 3 (continued)

	Ion ratio	
Clone		
T1	0.45 ± 0.10^{c}	48.58 ± 14.22^{b}
T2	2.56 ± 0.56^{a}	301 89 ± 218.84^{b}
T3	1.90 ± 0.42^{b}	881.74 ± 357.25^{a}
T4	1.86 ± 0.40^{b}	363.90 ± 179.51^{b}
T5	1.67 ± 0.37^{b}	171.54 ± 36.79^{b}
NaCl(%)		
0	0.09 ± 0.02^{d}	1.74 ± 0.27^{b}
0.5	1.09 ± 0.11^{c}	100.07 ± 23.50^{b}
1	2.39 ± 0.32^{b}	234.44 ± 63.09^{b}
1.5	3.18 ± 0.35^{a}	1077.87 ± 313.72^{a}

Table 3 (continued)

	RGR	Water content	Osmotic pressure	contribution to osmolality	
				Na^+	K^+
Clone					
T1	65.8 ± 6.9^{b}	73.4 ± 1.7^{d}	2.03 ± 0.25^{a}	19.4 ± 3.4^{c}	36.7 ± 4.9^{a}
T2	54.0 ± 9.5^{c}	84.5 ± 1.1^{a}	1.73 ± 0.16^{b}	29.0 ± 5.7^{a}	10.0 ± 1.4^{b}
T3	70.5 ± 10.4^{b}	78.9 ± 1.8^{c}	2.02 ± 0.24^{a}	23.9 ± 4.5^{b}	10.8 ± 1.5^{b}
T4	48.6 ± 9.2^{c}	80.8 ± 1.3^{b}	1.85 ± 0.19^{b}	22.7 ± 3.8^{bc}	11.1 ± 1.7^{b}
T5	79.4 ± 6.9^{a}	84.0 ± 0.7^{a}	1.84 ± 0.16^{b}	22.1 ± 3.9^{bc}	10.7 ± 1.2^{b}
NaCl(%)					
0	100.0 ± 0.0^{a}	86.3 ± 0.8^{a}	1.01 ± 0.04^{d}	2.1 ± 0.3^{d}	26.3 ± 4.7^{a}
0.5	74.6 ± 7.1^{b}	81.9 ± 0.9^{b}	1.62 ± 0.04^{c}	22.4 ± 1.0^{c}	16.5 ± 3.0^{b}
1	47.4 ± 4.9^{c}	77.6 ± 1.3^{c}	2.22 ± 0.06^{b}	31.6 ± 2.1^{b}	11.6 ± 2.5^{c}
1.5	32.6 ± 3.0^{c}	75.5 ± 1.7^{d}	2.73 ± 0.08^{a}	37.5 ± 2.2^{a}	9.1 ± 1.4^{c}

Number represent mean (± S.E.). Different letters within columns and factors were significantly ($P \leq 0.05$) different from each other according to Tukey's test at 5% probability level.

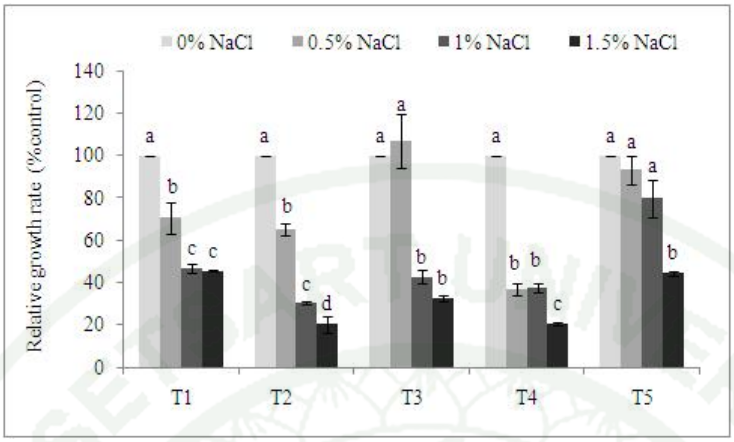


Figure 9 Relative growth rate of selected callus cultured on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks. Error bars indicate standard error; n = 10; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

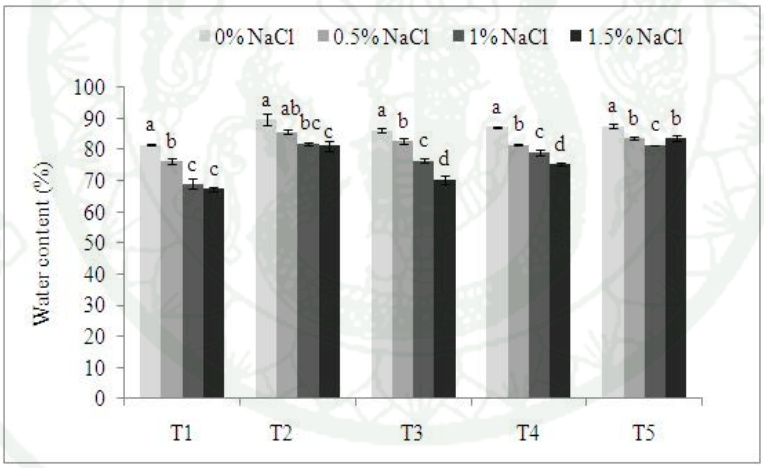


Figure 10 Water content in selected callus cultured on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks. Error bars indicate standard error; n = 10; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

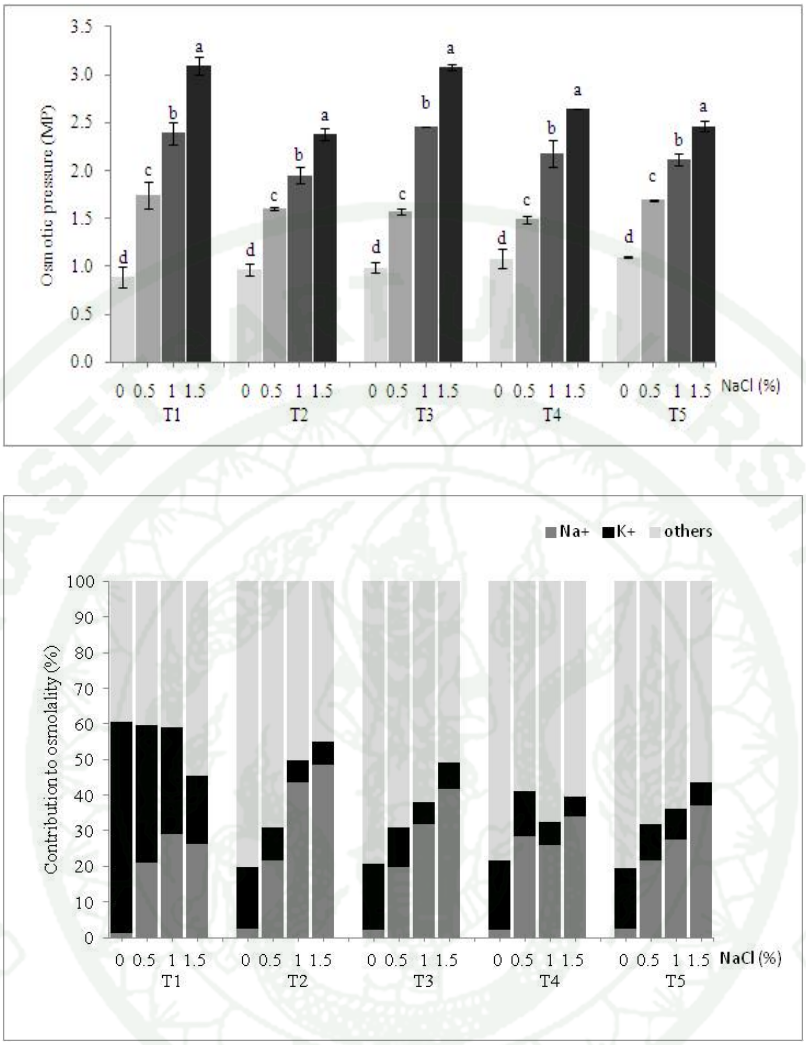


Figure 11 Cell sap determination of salt tolerant selected callus cultured on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks; (a) Osmotic pressure, (b) contribution to osmolality by Na^{+} and K^{+}. Error bars indicate standard error; n = 3; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

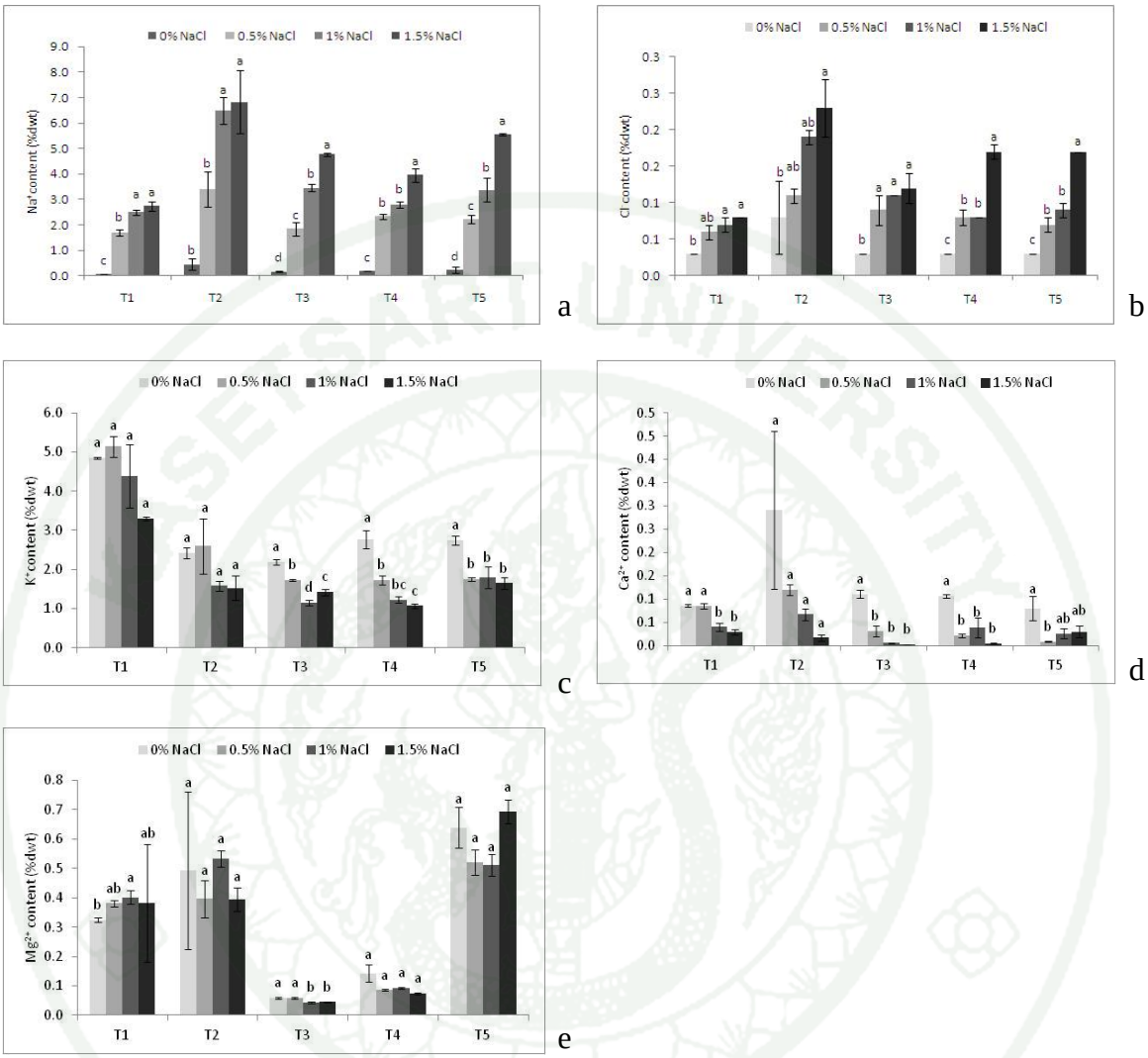
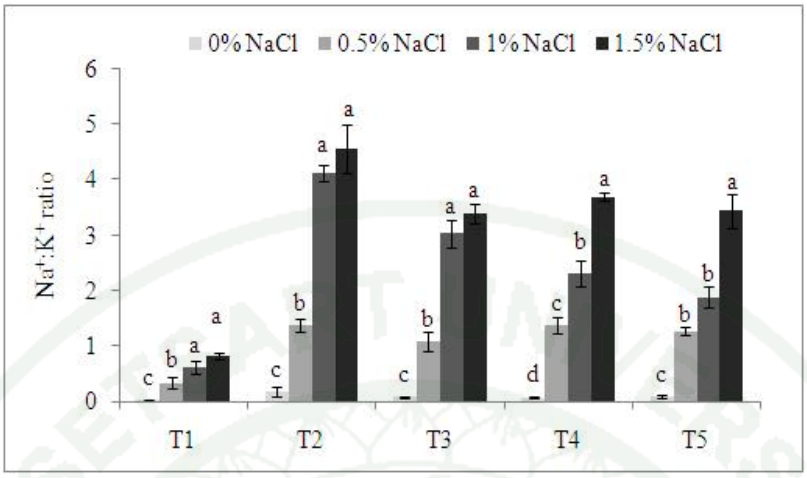


Figure 12 Ion content of salt tolerant selected callus cultured on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks; (a) Na^{+}, (b) Cl^{-}, (c) K^{+}, (d) Ca^{2+}, and (e) Mg^{2+}. Error bars indicate standard error; n = 3; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).



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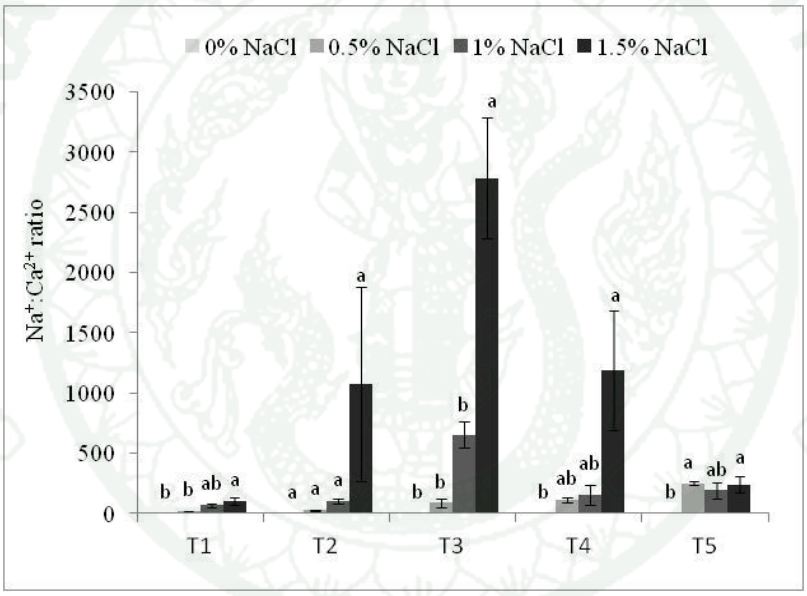

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Figure 13 Ion ratios of salt tolerant selected callus cultured on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1 and 1.5% NaCl for 2 weeks; (a) Na^+:K^+, (b) Na^+:Ca^{2+}. Error bars indicate standard error; n = 3; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

Experiment 5 Oxidative enzymes determination in shoot of selected clones

In vitro shoots of T1, T2, T3, T4 and T5 were cultured on MS basal medium supplemented with NaCl 0, 0.5 and 1% for 1 week and then transferred to a recovery medium for another week. There were significant differences in relative fresh weight (RFW) and antioxidant enzyme activities between clones, salt treatment and recovery medium. RFW increased on recovery medium while enzyme activity varied with an increase in POX, decrease in SOD and no change in CAT (Table 4). Interactions between clone, NaCl concentration and time indicated the different clones produced a variety of responses with regard to NaCl treatment and recovery (Table 4). Interaction between clone and NaCl showed significant difference in RFW, SOD and CAT but no difference in POX. While time had no effect on clones when considering RFW it was highly significant for all enzymes. Time with regard to NaCl, however, showed highly significant effect for RFW but no effect for enzymes. Three-way interaction among clones × NaCl × time was not significant (Table 4).

As the NaCl increased, RFW significantly decreased by approximately 36% (100.0 ± 0.0 to 64.4 ± 3.4%) of the control (Table 4). RFW of all clones except T1 was significantly reduced but the reduction occurred at different concentrations for the remaining four clones. T3 and T4 had a significant reduction at 0.5% NaCl (41.7 ± 6.3 and 70.8 ± 3.6% of the control) while T2 and T5 had a significant reduction at only 1.0% NaCl (57.1 ± 9.4 and 45.4 ± 3.2% of the control) (Figure 14a; Appendix Table 13). After one week on the recovery medium, however, RFW of T1 was significantly lower at 1% NaCl (64.7 ± 8.6% of the control) while there was no significant difference between of 0.5% NaCl (115.8 ± 13.0% of the control) and the control. T4 produced a partial recovery only at 0.5% NaCl (88.9 ± 11.4% of the control). Clones T2 T3 and T5 were able to recover their growth completely as indicated by no significant difference among the shoots grown on the different NaCl media (Figure 14b; Appendix Table 14).

The activity of SOD, CAT and POX significantly decreased at 1% NaCl for 40.0 ± 3.0, 2.9 ± 0.3 and 3.9 ± 0.6 Ug^{-1} fwt (Table 4) and there was a significant difference among clones for each enzyme. T3, T4 and T5 showed high SOD activity at 63.3 ± 4.6 Ug^{-1} fwt, 58.8 ± 5.6 Ug^{-1} fwt and 62.0 ± 3.6 Ug^{-1}fwt, respectively while T1 had significantly lower levels; approximately one third (19.0 ± 0.9 Ug^{-1} fwt) of these clones (Table 4). Additionally, T1 produced the lowest SOD activity which did not change on the range of NaCl concentrations or after transferring to the recovery medium (Table 4, 15; Figure 15). In the case of T2 and T3, SOD activity was stable on the NaCl media (50.71 ± 10.02 Ug^{-1} fwt to 49.01 ± 6.66 Ug^{-1} fwt and 82.30 ± 16.78 Ug^{-1} fwt to 64.90 ± 1.34 Ug^{-1} fwt) (Figure 15; Appendix Table 13). Nevertheless, SOD activity of T2 and T3 transferred from 1% NaCl to recovery medium (30.44 ± 1.20 and 34.46 ± 3.94 Ug^{-1} fwt, respectively) had a significantly decrease and difference from those activity transferred from 0 or 0.5% NaCl (Figure 15; Appendix Table 14). In the control media, SOD activity of T4 and T5 were 42.55 ± 2.09 Ug^{-1} fwt and 58.49 ± 3.63 Ug^{-1} fwt, respectively. At 0.5 % NaCl, SOD activity decreased in T4 (32.29 ± 1.42 Ug^{-1} fwt) but increased in T5 (75.70 ± 5.63 Ug^{-1} fwt) (Figure 15a, b; Appendix Table 13, 14).

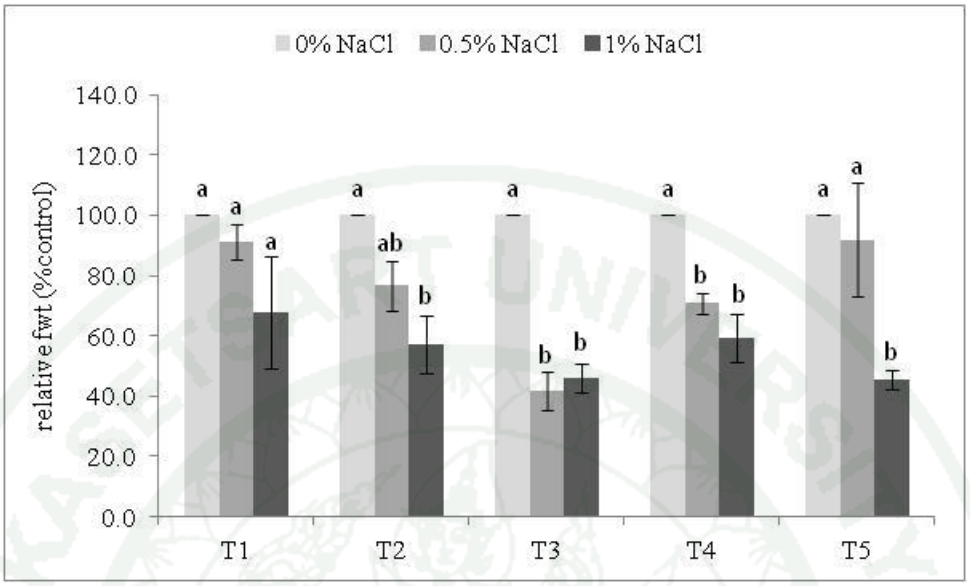
For CAT activity, T5 showed the highest activity (7.5 ± 0.8 Ug^{-1} fwt) (Table 4). T1, T3 and T4 had the same level (2.1 ± 0.2 Ug^{-1} fwt, 2.1 ± 0.3 Ug^{-1} fwt and 2.7 ± 0.4 Ug^{-1} fwt) and T2 was intermediate (4.1 ± 0.3 Ug^{-1} fwt) (Table 4). While there appeared to be differences in the CAT activity due to NaCl exposure this was not evident when individual clones were examined (Figure 16a; Appendix Table 13). After 1 week on the recovery medium, CAT activities were reduced for T5 and T3 while T2 and T4 increased and T1 did not change on the recovery medium (Figure 16a, b). CAT activity of each clone, however, was not significantly different on the range of NaCl concentrations (Figure 16; Appendix Table 14).

T5 had significantly higher POX activity than all the other clones (8.6 ± 1.2 Ug^{-1} fwt; Table 4). This activity was reduced on the 1% NaCl medium (1.91 ± 0.08 Ug^{-1} fwt) but this difference was not apparent after recovery (Figure 17; Appendix Table 15, 16). POX activity of the other clones varied by having lower levels of the enzyme and having a reduction on 0.5% NaCl (T2 and T3 for 2.69 ± 0.11 Ug^{-1} fwt and 4.39 ± 0.76 Ug^{-1} fwt, respectively).There was no change in POX activity for T1 and T4 due to either NaCl or recovery (Figure 17; Appendix Table 13, 14)

Table 4 Mean of shoot relative fresh weight (RFW) and activity of antioxidant enzymes activity; SOD, CAT, and POD treated with NaCl and then recovery by transferred to the medium without NaCl.

Clone	RFW	Activity (U g^{-1} fwt)		
	(%control)	SOD	CAT	POX
T1	89.9 ± 5.3 [a]	19.0 ± 0.9 [c]	2.1 ± 0.2 [c]	6.0 ± 0.6 [b]
T2	85.4 ± 4.9 [ab]	47.4 ± 2.9 [b]	4.1 ± 0.3 [b]	2.6 ± 0.2 [c]
T3	73.5 ± 5.4 [b]	63.3 ± 4.6 [a]	2.1 ± 0.3 [c]	3.8 ± 0.5 [c]
T4	80.5 ± 4.4 [ab]	58.8 ± 5.6 [a]	2.7 ± 0.4 [c]	3.8 ± 0.3 [c]
T5	89.7 ± 5.3 [a]	62.0 ± 3.6 [a]	7.5 ± 0.8 [a]	8.6 ± 1.2 [a]
NaCl (%)				
0	100.0 ± 0.0 [a]	55.4 ± 4.3 [a]	4.0 ± 0.4 [a]	5.8 ± 0.6 [a]
0.5	87.0 ± 4.5 [b]	54.8 ± 4.1 [a]	4.1 ± 0.6 [a]	5.1 ± 0.7 [a]
1	64.4 ± 3.4 [c]	40.0 ± 3.0 [b]	2.9 ± 0.3 [b]	3.9 ± 0.6 [b]
Time				
Treat NaCl	76.5 ± 3.4 [b]	57.4 ± 3.5 [a]	3.9 ± 0.5	4.1 ± 0.3 [b]
recovery	91.1 ± 2.8 [a]	42.8 ± 2.6 [b]	3.5 ± 0.3	5.9 ± 0.6 [a]
Interactions				
Clone x NaCl	*	*	**	ns
Clone x time	ns	**	**	**
NaCl x time	**	ns	ns	ns
Clone x NaCl x Time	ns	ns	ns	ns

Number represent mean (± S.E.). Different letters within columns and factors were significantly ($P \leq 0.05$) according to Tukey's test at 5% probability level (*, ** -significant at $p \leq 0.05$ and 0.01, respectively).



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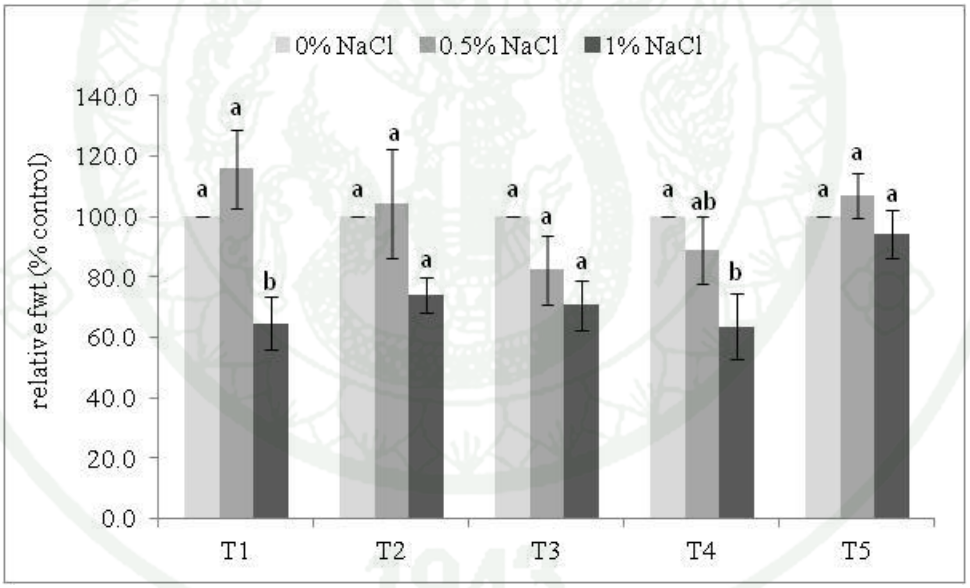
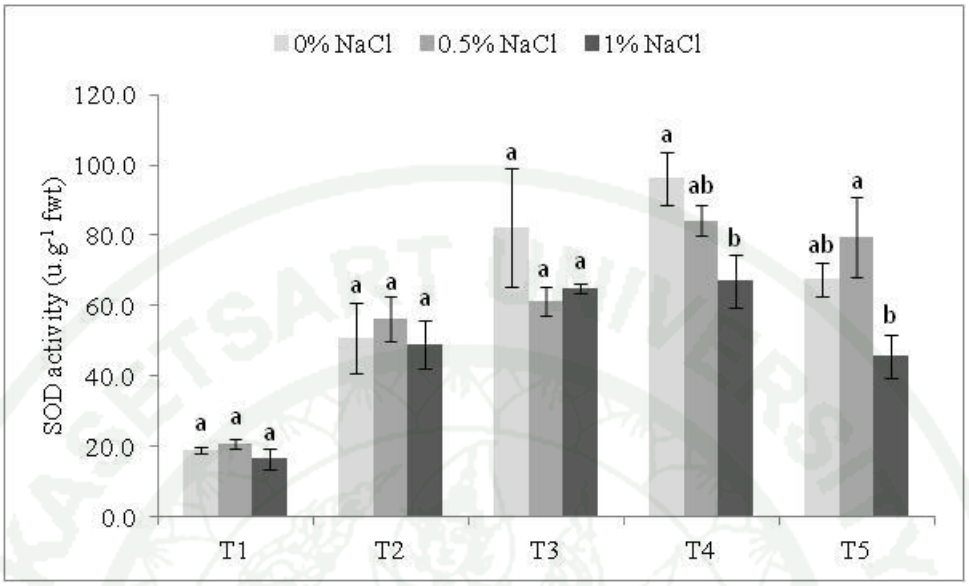


b

Figure 14 Relative fresh weight of shoot cultured on MS medium. (a) treated with 0, 0.5 and 1% NaCl for 1 week, (b) recovered on medium without NaCl for another week. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).



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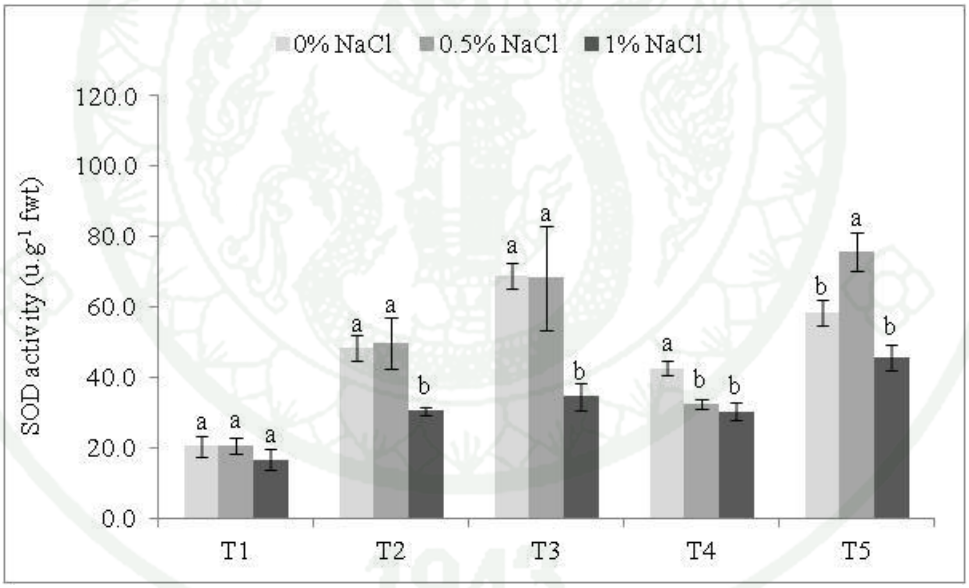

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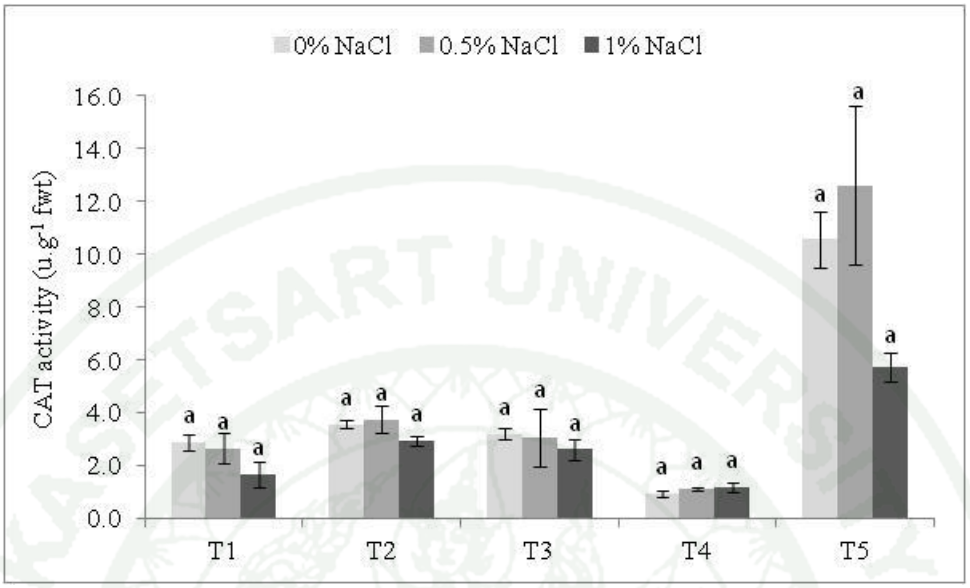
Figure 15 SOD activity of shoot cultured on MS medium. (a) treated with 0, 0.5 and 1% NaCl for 1 week, (b) recovered on medium without NaCl for another week. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).



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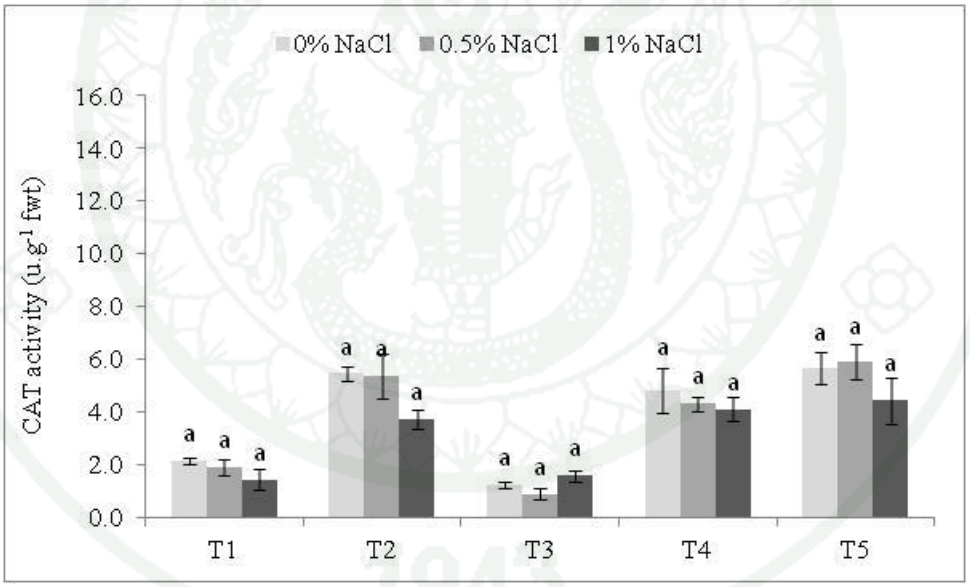

b

Figure 16 CAT activity of shoot cultured on MS medium. (a) treated with 0, 0.5 and 1% NaCl for 1 week, (b) recovered on medium without NaCl for another week. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

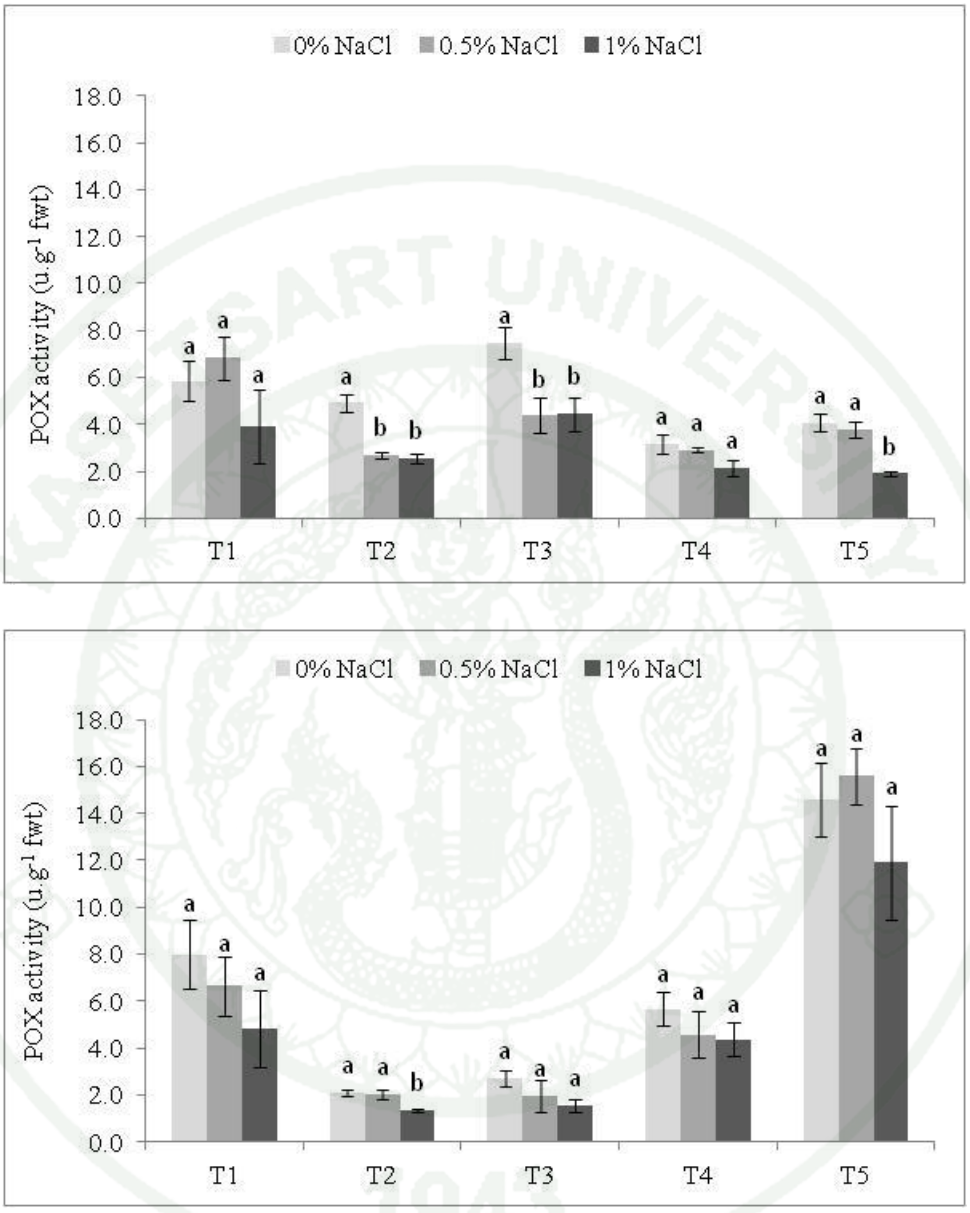

Figure 17 POX activity of shoot cultured on MS medium. (a) treated with 0, 0.5 and 1% NaCl for 1 weeks, (b) recovered on medium without NaCl for another week. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

Experiment 6 Some capabilities of selected clones after extended time in culture

Experiment 6.1 The secondary callus induction and shoot regeneration

Shoot of Stylo 184 was able to produce callus on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA (Figure 18a). By using the same medium, callus of the selected clones were induced and maintained by regularly subculturing onto fresh medium every 4 weeks (Figure 18b and c). Proliferating and regenerating of callus of the selected clones (T5 and T1) was effective on the same medium as found in nonselected callus. They were able to produce organogenic callus with green spot and protruding shoots along with the maintenance period over 3 years (Figure 18d, e and f). In addition, regenerating shoot by growing on the same medium without subculturing for 8 weeks showed normal characteristics did not difference from nonselected shoots (Figure 18g, h and i). Plantlets of T5 and T1 growing in groonhouse showed normal characteristic as general Stylo 184; green expand leaves, pinnately trifoliate with elliptic leaflets, stems hairy (Figure 18 j, k and l). This suggested that Stylo 184 was able to maintain through callus culture without somaclonal variation.

In order to confirm callus induction and shoot regeneration after long term maintenance, shoots of five selected clone were cultured on MS medium supplemented with 0.01 $mg.L^{-1}$ NAA and 1 $mg.L^{-1}$ BA. Callus and shoot production remained active but the time required for regeneration and the amount of regeneration varied between clones. Clones T2 and T3 had rapid callus formation, with 100% of the explants producing callus after 5 and 6 weeks, respectively. Two other clones (T4 and T5) produced callus (100%) by week 7 while T1 only produced callus in 67.5% of the explants for the duration of the experiment. More importantly, shoot number and percent shoot regeneration varied significantly between clones (Figure 19). The highest shoot regeneration was obtained from T4 (7.1 ± 1.3 shoots/explant), however, this was only significantly different from T3 (Figure 19). The highest percent shoot regeneration occurred in T4 (93.3 ± 4.2) but this was only significantly different from T1 (Figure 19).

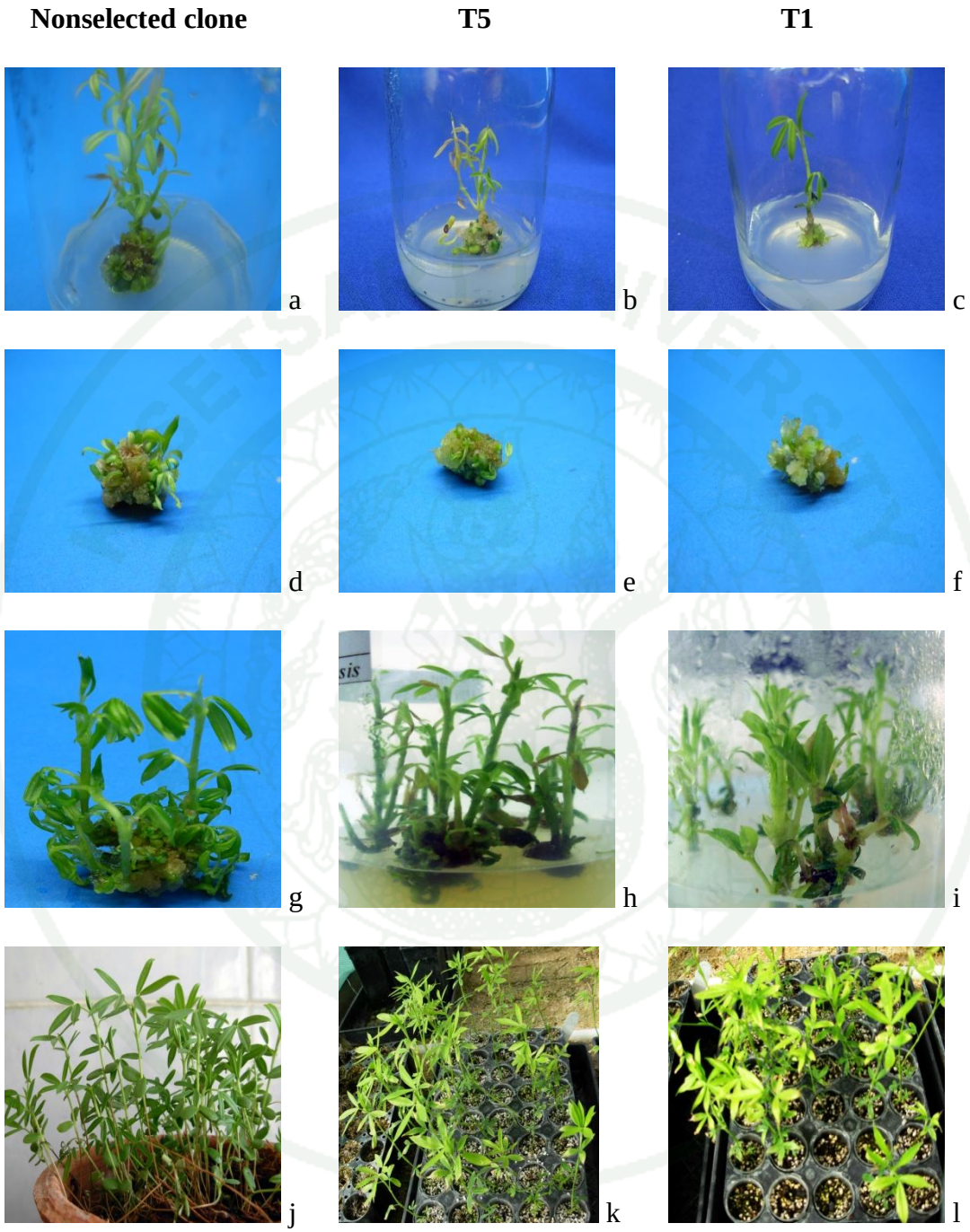

Figure 18 Callus induction of (a) nonselected clone, (b) T5 and (c) T1, callus proliferation of (d) nonselected clone, (e) T5 and (f) T1, shoot regeneration of (g) nonselected clone, (h) T5 and (i) T1, and plants in natural condition of (j) nonselected clone, (k) T5 and (l) T1

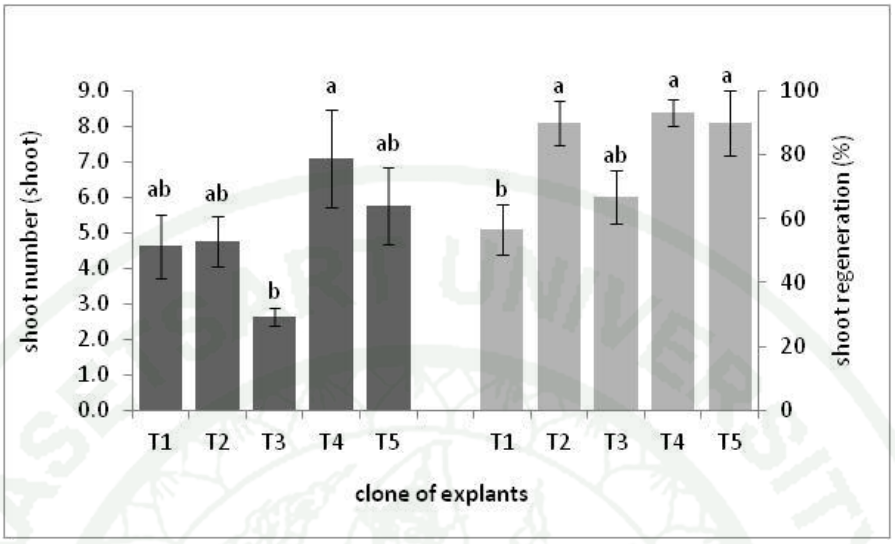

Figure 19 Shoot regeneration of Stylo 184 after maintaining for 3 years on MS medium with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA for 8 weeks. Error bars indicate standard error; n = 6; different letters above bars indicate significant differences ($P \leq 0.05$).

Experiment 6.2 Rooting and *ex vitro* survival

Stylo 184 shoots regenerated from callus maintained over 3 years were cultured on MS medium supplemented with 0.3 mg.L^{-1} IBA for 2 weeks. A comparison between the clone T1 and a representative of the salt tolerant selected clone (T5) was determined. Shoot of T1 and T5 produced 70.0 and 91.0% rooting (figure 20a, b), respectively. When they were transferred to pots containing vermiculite, sand and peat under greenhouse conditions, after 5 weeks, they had 69.0 and 90.0% survival, respectively (Figure 18k, l). All of these plants grew well and showed normal characteristics; branching upright stems, stem hairy, becoming woody at the base with age, leaves pinnately trifoliate with elliptic leaflets (Figure 18k, l and Figure 20c, d). Several plants flowered 7 months after planting; inflorescence of several spikes of a few flowers crowded into terminal heads; spikes sessile in unifoliate bracts and hairy; flower yellow (Figure 20e, f).

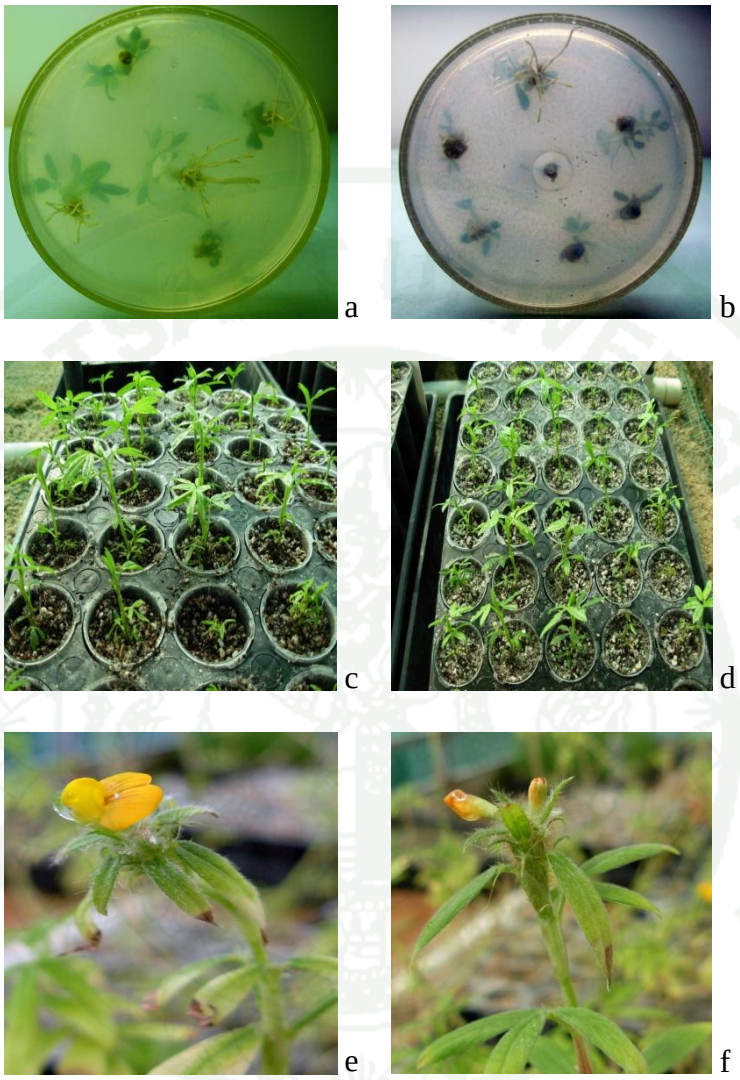

Figure 20 Root induction of (a) T5 and (b) T1, one week plantlets in greenhouse of (c) T5 and (d) T1, and flowering of 7 months plant of (e) T5 and (f) T1.

Discussion

Experiment 1 Effects of NaCl on growth and ion content in nonselected seedling

When Stylo 184 seeds were germinated in 0, 0.5, 0.75 and 1% NaCl solution for 1 week NaCl (up to 1%) had no effect on germination; a result similar to that reported for a range of other species (Chartzoulakis and Klapaki, 2000; Shannon *et al*., 2000; Bayuelo-Jimenez *et al*., 2002). For other legumes, NaCl of ~2% or more generally reduces germination and growth; in soybean (Hosseini *et al*., 2002) and cowpea (Patel *et al*., 2010). The reduction in water content achieved at 1% NaCl can be attributed to osmotic effects caused by a reduction in water uptake (Shannon *et al*., 2000; Bayuelo-Jimenez *et al*., 2002; Jamil *et al*., 2007).

Na^+ and Cl^- content in shoots and roots of Stylo 184 seedlings increased sharply when treated with NaCl. This result reflects what has been frequently reported; Na^+ and Cl^- content increases with increasing salinity (Chartzoulakis and Klapaki, 2000; Hosseini *et al*., 2002; Patel *et al*., 2010). In addition, Stylo 184 displayed an exclusion mechanism by having higher Na^+ and Cl^- content in roots than in shoots indicating that it was able to prevent the accumulation of the toxic ions in the shoots. Similarly, Lupins (*Lupinus luteus* and *Lupinus angustifolius*) also reduce the translocation of both Na^+ and Cl^- to the leaves and stems (Van Steveninck *et al*., 1982; Teakle *et al*., 2007), while clover (T*rifolium repens*; Rogers *et al*., 1997; Wang *et al*., 2010) and glycine (*Glycine soja;* Luo *et al*., 2005) control the translocation to the growing shoots of only Cl^- or Na^+, respectively.

As a result of salt stress, plants usually face potassium deficiency, as K^+ uptake is limited by high concentrations of Na^+ and xylem translocation is restricted (Tavakkoli *et al*., 2010; Patel *et al*., 2010). In Stylo 184, K^+ content was significantly higher in the roots than in the shoots and the total content was 5 – 7 times lower than Na^+ content. This is similar to other legumes such as cowpea (*Vigna unguiculata*), faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.), soybean (*Glycine max* L.), and common bean (*Phaseolus vulgaris* L.) (Cordovilla *et al*. 1995; Patel *et al*. 2010). Germination percentage of Stylo 184 was stable when NaCl increased, possibly due to effective competition of K^+ uptake. The significant increase of toxic ions and the reduction of K^+ translocation to shoots, however, appeared to have led to a reduction of seedling growth.

At the higher levels of salt (0.5-1%), Stylo 184 was able to maintain constant K^+ content and Na^+ : K^+ratios as well as Ca^{2+} at 0.75–1 % NaCl. Plants are able to cope with salt stress by maintaining a high cytoplasmic K^+:Na^+ ratio (Blumwald, 2000; Marcum *et al*., 2007). As reported in soybean, higher tissue Na^+ concentration was associated with higher K^+ and Ca^{2+} concentration in the embryo axis (Hosseini *et al*., 2002). This agreed with the report of Farhoudi and Sharifzadeh (2006) that primed NaCl canola seeds showed high K^+ and Ca^{2+} accumulation including a balanced Na^+:Ca^{2+} ratio which can prevent toxicity and nutrient deficiency.

The present study found that the water content in shoots of Stylo 184 decreased significantly at 1% NaCl. This also leads to the reducing of fresh weight similar to many reports as the result of salinity (Shannon *et al*., 2000; Jeannette *et al*., 2002). Salt may inhibit root and shoot elongation reducing water uptake by seeds; attributable to osmotic effects, the secondary effect of salt stress (Shannon *et al*., 2000; Jamil *et al*., 2007). As a result of osmotic stress during germination, Stylo 184 reduced growth when exposed to salt. K^+ accumulation, however, has been frequently observed as an effective osmolyte (Parida *et al*., 2007; Naureen and Naqvi, 2010; Haq *et al*., 2011) and ion regulation may have played an important role in osmotic adjustment for Stylo 184, leading to germination in NaCl as high as 1%.

Experiment 2 NaCl concentration for salt tolerant seed selection

The range of 0 – 3% NaCl was applied to Stylo 184 seeds to determine a suitable concentration for salt tolerance selection. Germination was remained steady from 0 to 1% NaCl and inhibited at the concentration higher than 1.7% NaCl. These related to the result of experiment 1, the NaCl concentration up to 1% only effected seedling growth. Salinity had greater effect on seedlings growth than seed germination, similar to pepper hybrid where low concentrations of NaCl delayed germination but did not reduce the final germination percentage (Chartzoulakis and Klapaki, 2000). In addition, root and shoot growth are the most important parameters under salinity stress since salt stress can inhibit root and shoot elongation due to the reduction of water uptake (Jamil *et al.*, 2007). Salt tolerant seed selection for this work was carried out 1.7% NaCl, however, many germinated seeds were obtained; this was not appropriate to clarify their salt tolerant ability. After 4 weeks, one seed was able to germinate on 2% NaCl, subsequently, 2% NaCl was the concentration used for this selection programme.

Determination of the salt concentration suitable for developing salt tolerant cells lines was made on the basis of relative growth rate. The threshold that is widely used for the ranking of plant salt tolerance is 0.4 – 0.6% NaCl (Yacoubi *et al*., 2010). For Stylo 184, variation of growth reduction dose (Gr_{50}) ranged from 0.5–0.8% NaCl were found on ten selected clones. Interestingly, some of the selected clones revealed variation in RGR, T3 expressed GR_{50} at 0.7% NaCl with high RGR of 64.8% control at 0.5% NaCl. T4 expressed GR_{50} equal to the sensitive clone at 0.5% NaCl with the lowest RGR of all the clones for 0.5% control at 1.5% NaCl. Retarded growth of T4 perhaps revealed the lowet salt tolerance among the selected clones. In addition, the explicit characteristic of salt tolerance in T2 and T5 were found after determining ion content, subsequently, T2, T3, T4 and T5 were determined salt tolerant mechanisms in comparison to the clone T1.

Experiment 3 Media and NaCl concentration for salt tolerant callus selection

Callus of Stylo 184 cultured on MS medium with or without NAA and BA supplemented with various concentrations of NaCl showed reduction of increasing fresh weight, survival and percent regeneration. The reduction in plant growth exposed to saline environments could be due to either the effects of specific ions on metabolism or adverse water relations (Javid *et al*., 2011). In addition, cells which cultured on the

medium containing NaCl are able to compartmentalise the excess salts in vacuoles, and survive by adjusting the osmotic pressure. This adaptation causes reduction of cell division and expansion (Jain *et al.*, 1985).

In almost all cases reported, the medium for salt tolerant selection contained growth regulators, however, in some case growth regulators have not been included. In these cases, the authors point out that growth regulators can produce physiological and sometimes genetic effects that could make salt-tolerant cells appear salt sensitive; perhaps by increasing the metabolic rate and uptake of salts and vice versa (Javid *et al*., 2011, Jaiwal *et al.*, 1997). Callus of Stylo 184 produced shoots in both media with or without plant growth regulators including NaCl upto 1% NaCl. The medium without plant growth regulators, however, affected callus growth. The callus cultured on MS medium containing NAA and BA could survive on upto 2.5% NaCl showed higher ability to grow than the medium without plant growth regulators.

The selected seeds derived from experiment 2 were used to induce callus and subsequently screened for salt tolerant cell lines on 2.5% NaCl for 6 weeks. On the basis of proliferation, the callus of only 8 selected clones was determined. Most callus did not survive after two subcultures even though the NaCl was reduced in the medium from 2.5% to 1%. This may due to the low capacity of the cell to adjust their osmotic potential when transfererd from high to low NaCl concentrations. This is supported by a study in hypocotyl sections of *Vigna unguiculata*, the removal of osmotic stress caused transient increase of the membrane potential of the xylem/symplast as well as an immediate and transient burst of growth. Consequently, the enhanced rate of growth fell to a steady level within 30 min (Kitamura *et al*., 1997). Transferring of Stylo 184 callus to higher osmotic potential conditions is likely to cause the cells to swell and/or undergo apoptosis because of over excessive absorption of water. In addition, cells usually exhibit sensitive characteristics when the selection pressure is removed. The critical concentration of NaCl could induce a reversible habituation instead of stable mutations. (Jain *et al.*, 1985).

Furthermore, during a long period waiting for callus growth, some callus of each clone (T1, T2, T3, T4 and T5) was analised for ion content and showed distinct regulation among clones. This supported salt tolerant capacity of each selected clone. In addition, the evidence that callus of Stylo 184 induced from salt tolerant selected seed showed low survival percentage on high salt concentration indicated differences in salt tolerant capability between seed and callus. Surviving callus had a reduction of growth and regeneration and only a small callus was able to survive on very high (2.5%) NaCl. These results suggested that selection for Stylo 184 salt tolerant clone through seed screening was appropriate by no need double screening from callus.

Experiment 4 Growth and ion content in callus (five selected clones)

Callus induced from five selected seeds (T1, T2, T3, T4 and T5) was examined for growth and ion content under the influence of 0 – 1.5% NaCl for 2 weeks. Examination of callus growth clearly showed a difference in growth rates (among clones and NaCl concentrations) and an apparent difference in salt tolerance as indicated by relative growth

rate, ion regulation and osmotic adjustment. These are important factors in contributing to the level of salt tolerance of individual genotypes (Flowers, 2004; Zakharin and Panichkin, 2009; Zhou and Yu, 2009).

In this study, callus of T1 displayed the highest K^+ content and consequently the lowest Na^+:K^+ ratio. It also contained the highest percent K^+ contribution to osmolality, with high osmotic pressure resulting in higher average dry weight of T1 than T2 and T4. This may have been achieved by T1 successfully adjusting osmotic potential by accumulating K^+ in cells. This consumes less energy than the production and accumulation of organic osmolytes and would be beneficial to its salt adaptation (Zhou and Yu, 2009).

T2 was able to stabilize K^+, Ca^{2+} and Mg^{2+} but increased in Na^+ and Cl^- content with increasing NaCl; this clone may have been using the Na^+ and/or Cl^- to adjust osmotic potential. The low osmotic pressure but high Na^+ and Cl^- with highest mean water content of all the clones suggested that T2 was able to adjust cell osmotic potential to lower than of surrounding medium by using these toxic ions. This may be considered as succulence which is one of the physiological mechanisms used to survive under salinity stress (Khan *et al*., 1999). While succulence is generally seen as an anatomical adaptation it also involves increasing vacuolar volume and permits the accumulation of water at the cellular level. It is seen as an increase in cell size, decrease in extension of growth, decrease in surface area per tissue volume, and high water content per unit of surface area (Vicente *et al*., 2004). High succulence (particularly in the whole plant) can be used as a key characteristic to evaluate the potential germplasm in selection and breeding programmes for salt tolerance (Ottow *et al*., 2005; Lacerda *et al*., 2006; Gulzar and Khan, 2006). This was different to the response obtained from the other clones which had lower water content. T2's growth was also reduced at higher salt levels compared to the other clones selected as "tolerant" (T3, T4 and T5). This may be due partly to facing an excess of toxic ions in the cytoplasm and losing high energy through the accumulation of these ions in the vacuole.

Callus water content was also significantly reduced as NaCl concentration increased. However, while there was a difference between clones the differences did not reflect those seen in callus growth. In addition, the differences in the water content between T1 and T3 and the similarity between the dry weight (growth) and osmotic pressure may be due to different adjustments in cell osmotic potential. Higher osmotic pressure of T3, but lower water content compared to the other tolerant clones, may have been achieved through insufficient balance of the osmotic potential across the tonoplast or decreasing cell wall extensibility (Çiçek and Çakirlar, 2002). Due to the highest Na^+:Ca^{2+} ratio occurring in T3, its membrane function could have been disrupted, causing accumulation of excess ions in intracellular spaces. Higher Na^+ uptake has been associated with the higher Ca^{2+} and K^+ leakage (Atak *et al*., 2006; Akbarimoghaddam *et al*., 2011). In addition, sequestering of salt from the cytoplasm into the vacuole may have created a strong osmotic gradient across the tonoplast which may have been balanced by an increase in compatible solutes in the cytosol. The excess salt ions may accumulate outside the vacuole, either in the cytoplasm or in the intracellular spaces. In the former case, the cell succumbs directly to ion toxicity while in the latter, cell expansion ceases

entirely, because water diffuses out of the cell. The loss of water from the cell further concentrates cell solutes to a level where cellular metabolism is irreversibly affected (Volkmar *et al*., 1998; Çiçek and Çakirlar, 2002; Hessinia *et al*., 2009). This may have caused the reduced growth of T3 at 1% NaCl.

T5 was the slowest growing of all the tolerant selected clones (Appendix figure 2), but, it displayed the highest tolerance to salt in terms of having the lowest reduction in growth when exposed to NaCl. The regulation of K^+, Ca^{2+} and Mg^{2+} in this clone led to stable Na^+:K^+ and Na^+:Ca^{2+} ratios. Marcum *et al*. (2007) suggested that a high Na^+:K^+ ratio can disrupt various enzymatic processes in the cytoplasm. Salt tolerant plants respond to elevated Na^+ concentrations by maintaining low cytosolic Na^+ concentrations with high cytosolic K^+:Na^+ ratios through extrusion and/or the intracellular compartmentalization (Blumwald, 2000). For example, callus of egg plant (*Distichlis spicata*) tolerant genotypes had higher K^+:Na^+ than sensitive genotypes (Marcum *et al*., 2007). Under the current conditions, T5 appeared to have better control of K^+ and hence was able to maintain better growth at high NaCl. Other plants such as wheat, which display low-Na^+ uptake depends upon higher K^+ and organic solute accumulation (Rivelli *et al*., 2002). The last tolerant clone (T4) possessed ion regulation similar to that of T5, with a cell osmotic pressure equivalent to that of T2 and T5; indicating its ability to adjust cell osmotic potential.

The present results indicated that exclusion mechanisms play an important role in Stylo 184 to survive under salt stress condition. Callus of five selected clones employed different ion regulation to adjust cell osmotic potential under the influence of NaCl. The clone (T1) showed persistent exclusion mechanisms as a general glycophyte by taking up the highest K^+ and maintaining the lowest Na^+ and Cl^-. On the other hand, the salt tolerant clones (T2, T3, T4 and T5) revealed a salt resistance glycophyte mechanism by accumulating Na^+ and Cl^- as osmolytes. These results indicate that determination of ion content in callus was able to distinguish between salt tolerant and less salt tolerant clones of Stylo 184. As a result, the tolerant clones may be used to increase the salt tolerance of this plant for its inclusion in the restoration of salt effected soil as well as its use as a fodder plant.

Experiment 5 Oxidative enzymes determination in shoot of selected clones

Oxidative stress impairs plant growth and development when antioxidative capacity and ROS is imbalanced (Munns and Tester, 2008; Ellouzia *et al*., 2011). Increasing of antioxidant enzyme activity to reduce oxidative stress under the influence of salt is always reported (Arulbalachandran *et al*., 2009; Azooz, 2009). However, it appears that for some species the enzyme increases are insufficient to overcome oxidative stress (Radyukina *et al*., 2007; Ellouzi *et al*., 2011). In addition, a correlation between antioxidant enzyme activity and salt stress has been frequently found; higher increases are not always associated with salt tolerance. For example, potato, wheat and rice salt sensitive cultivars were associated with higher ROS production leading to higher antioxidant enzyme activity (Rahnama and Ebrahimzadeh, 2005; Mandhania *et al*., 2006; Khan and Panda, 2008; Munns and Tester, 2008).

SOD activity in T1 was stable when NaCl increased as in T2 and T3; a response similar to that reported for cowpea where SOD activity was stable throughout the experimental period (Cavalcanti *et al*., 2004). However, low SOD activity may be associated with salt sensitivity, as T1 had lower constitutive SOD levels compared to the other four clones. This may be similar to reports comparing maize and wheat where maize was able to resist the potential oxidative damage without requiring additional SOD while wheat, which had lower initial SOD levels, produced SOD when exposed to NaCl (Stepien and Klobus, 2005).

The capacity to maintain SOD activity in Stylo 184 shoots may relate to preferential K^+ selectivity and stability along with increasing of NaCl up to 1% as revealed in experiment 4. According to Cakmak (2005), increase in severity of K^+ deficiency under abiotic stress was associated with enhanced activity of enzymes involved in detoxification of H_2O_2 (APX) and utilization of H_2O_2 in oxidative processes (POX). In T4 and T5, however, SOD activity was high but decreased significantly at 1% NaCl. In addition, all tolerant clones showed decreasing SOD activity after recovery. This result was similar to reducing of SOD activity in pea and green gram stressed and recovered tissues (Hernández and Almansa, 2002; Panda and Khan, 2009) and leaves of soybean (Amirjani, 2010). This may be caused from the scavenging mechanism of the clones (T4 and T5) containing high H_2O_2 which results in a reduction and inactivation of SOD (Bray *et al*., 1974; Khan and Panda, 2008; Panda and Khan, 2009).

The lack of response in CAT activity due to salt treatment in all clones is similar to what has been reported for cotton, sorghum, cowpea and lentil (Bandeoğlu *et al*., 2004; Cavalcanti *et al*., 2004; Freitas *et al*., 2011). However, the innate differences in levels of CAT activity between the clones may confer some level of salt tolerance. High CAT activity may be similar to the responses that have been reported for green bean where CAT activity had a greater increase in salt tolerant genotypes (Yasar *et al*., 2008). Within Stylo 184, T5 has the highest constitutive levels of both CAT and SOD. This may be a reflection of a salt tolerant mechanism that has explained differences between relatively salt sensitive plants such as cowpea and less salt sensitive species such as sorghum and cotton. The low SOD and CAT activities in cowpea can at least partially explain its susceptibility to salt stress (Freitas *et al*., 2011). This relationship has been related to many reports of greater salt tolerance being conferred with higher constitutive antioxidant enzyme levels, including: legumes (e.g. Türkan *et al*., 2005; Freitas *et al*., 2011), oilseed rape (Abedi and Pakniyat, 2010), maize (Stepien and Klobus, 2005) and rice (Demiral and Türkan, 2005).

On exposure to NaCl, POX activity increased in walnut leaves and peaked on the seventh day after exposure to NaCl and this prevented H_2O_2 accumulation (Goharrizi *et al*., 2011). Peroxidases are involved not only in scavenging of H_2O_2 produced in chloroplasts but also in growth and development (Panda and Khan, 2009). Increasing of POX activity in Stylo 184 even in the control after recovery may be caused from aging of plants or associated with cell wall stiffening (Sanchez *et al*., 1995; Roldán *et al*., 2008). No change in POX activity in the shoots either during salt treatment or on recovery medium in T1 or T4 is similar to the result seen in leaves of the glycophyte green gram

(*Vigna radiata*) (Panda and Khan, 2009). On the other hand, POX activity in T2, T3 and T5 was reduced as NaCl increased. This response was similar to the report in sunflower shoots and soybean leaves (Santos *et al*., 2001; Amirjani, 2010). After recovery, POX activity increased reach at the control treatment for T3 and T5 and at 0.5% NaCl for T2 through the range of NaCl. This was also reflected in RFW recovery and is similar to what has been reported for green gram where POX activity significantly decreased in stressed roots but increased in recovered tissues (Panda and Khan, 2009). Increasing activity in recovered tissue can indicate a higher capacity of H_2O_2 decomposition generated during the stress. This may be attributed to increased activity of POX encoding genes or increased tissue specific isozyme activity (Khan and Panda, 2008; Panda and Khan, 2009).

Though CAT and POX activity in Stylo 184 were not increased along with increasing of NaCl, they were constitutively higher in tolerant clones (T5) than the sensitive one. In addition, SOD activity in the latter was the lowest (Table 1). SOD activity plays a vital role in catalysing the conversion of O_2^- to H_2O_2 and O_2 (Panda and Khan, 2009). Catalases decompose H_2O_2 to water and O_2, and are mostly confined to peroxisomes and glyoxysomes. Peroxidases also decompose H_2O_2 but, unlike catalase, they rely on various organic electron donors to reduce H_2O_2 to water. Plants usually contain many isoenzymes of peroxidases with nonspecific activity. These isoenzymes are involved in lignin and ethylene synthesis and in plant development and organogenesis (Becana *et al*., 1998). Increasing of H_2O_2 induced antioxidant enzyme activity, glutathione reductase and catalase but no cause effect on SOD (Sairam and Srivastava, 2000). On the other hand, SOD can be reduced and inactivated due to high H_2O_2 (Bray *et al*., 1974). High levels of CAT and POX activity in Stylo 184 salt tolerant clone (T5) appears to have been essential for scavenging high H_2O_2 content. This provides a negative feedback that decreased SOD activity when exposed to the higher salt concentrations hence maintaining an efficient scavenging system in the tolerant clones. In addition, the antagonistic phenomenon of CAT and POX displayed in Stylo 184 is also seen in oilseed rape where high POD is associated with CAT inactivation, which might be considered a key point for the decomposition of H_2O_2 (Abedi and Pakniyat, 2010).

Restoration after recovery indicated salt tolerant capacity as found variation among Stylo 184 selected clones. Short term (8 – 96hr) salt stress interrupted leaf growth of pea with delay in restoration after 8h recovery in relation to control (Hernández and Almansa, 2002). All salt tolerant clones except T4 showed effective recovery while T1 and T4 had reduced recovery capacity as evidenced by decreased RFW after removing high salt treatment. It was noted that the sensitive clone T1 showed no change in RFW along with all salt treatments suggesting that ion toxicity has no effect on T1 under short term stress. This may be a general phenotypic of the drought tolerant Stylo 184. According to the experiment 4, callus of the sensitive clone was able to adjust osmotic potential successfully by regulating salt ion uptake. On the contrary, callus of the tolerant clones accumulated high content of salt. Difference in the osmotic adjustment mechanisms of the tolerant clones may be result of growth reduction during salt treatment. After recovery, however, RFW of T1 was significantly reduced; this may have been caused from low levels of SOD and CAT.

Experiment 6 Some capabilities of selected clones after extended time in culture

Experiment 6.1 The secondary callus induction and shoot regeneration

Shoot regeneration from callus after long-term maintenance has been reported in many species although with different responses. For example, callus of *Asparagus officinali*s was able to proliferate and regenerate 89% shoot primordia after more than 18 months in culture and regenerated plants had conspicuous somaclonal variation *i.e.* aberrant flowers and cladodes, larger flower size and glaucous foliage (Pontaroli and Camadro, 2005). In contrast, Egyptian wheat cultivars cultures lost their regenerative capacity after 32 and sorghum after 6 weeks (Fahmy and Shisy, 2004; Pola *et al*., 2009). Callus of Stylo 184 retained its organogenic and shoot regenerative capacity over prolonged period in culture. The regenerated shoots and subsequent plantlets showed no obvious abnormalities; stems hairy and pinnately trifoliate with elliptic leaflets. According to Pontaroli and Camadro (2005), higher frequency of somaclonal variation could be attributed to callus age in comparison between 19 and 7 months. There was no abnormal characteristic in the regenerants of Stylo 184 five selected clones after long term maintenance over 3 years indicating genetically conserved via callus culture. In addition, regenerated shoots of five selected clones were able to reproduce callus and shoot on the same condition as the original shoots. This suggested that Stylo 184 was able to maintain via callus culture and regenerate into shoot available for long term production of salt tolerant genotype.

Experiment 6.2 Rooting and *ex vitro* survival

Stylo 184 plantlets of both salt tolerant and less salt tolerant clone produced roots and grew well under greenhouse condition. They showed normal characteristics; branching upright stems, stem hairy, becoming woody at the base with age, leaves pinnately trifoliate with elliptic leaflets (Skerman *et al*., 1998). After 7 months planting, several plants flowered; flower was inflorescence of several spikes of a few flowers crowded into terminal heads; spikes sessile in unifoliate bracts and hairy; flower yellow (Skerman *et al*., 1998). Similarly, plantlets of many legumes regenerated from various explants were able to acclimatize and establish in soil *i.e. Vigna mungo* regenerated from cotyledon and embryonal axis explants (Ignacimuthu and Franklin, 1999), *Dalbergia siscoo* derived from cotyledons (Singh *et al*., 2001), Persian clover (Trifolium resupinatum) developed from cotyledonary nodes (Uranbey *et al*., 2005), and butterfly pea (*Clitoria ternatea*) directly regenerated from auxillary shoots (Barik *et al*., 2007).

After long term growing, however the tolerant clone (T5) showed higher survival percentage than the sensitive one (T1) which died over 50%. This could persist that the tolerant clone was more vigorous than the sensitive clone under *ex vitro* condition.

General Discussions

This research aimed to select Stylo 184 salt tolerant clones and investigate their salt tolerant mechanisms. Development of tissue culture techniques was also carried out to support the selection programme and assist in determining the tolerance mechanisms. Protocol of *in vitro* technique of shoot proliferation and transplanting to soil reported in this thesis is appropriate for both *in vitro* and *ex vitro* salt tolerance determination in *Stylosanthes* spp.

In accordance with callus ion content and shoot antioxidant enzyme activity, the tolerant selected clones revealed distinct salt tolerance mechanisms. T2 expressed succulence by accumulating high Na^+ and Cl^- as osmolytes with high SOD activity in shoots. Succulence may be appropriate for developing into wet saline soil if whole plant mechanism is consistent as in callus. T5 regulated ions and stabilized Na^+:K^+ and Na^+:Ca^{2+} ratios with high K^+ contributing to osmolality. The constitutive enzymes activity, SOD, CAT and POX of T5 was also high. These indicated that T5 expressed preferential characteristics of salt tolerant and tended to well adapt in saline soil effectively. As a result, T5 is the clone most suitable for studying its mechanisms as a model of a salt tolerant legume. T3 and T4 showed intermediary salt tolerance as shown by their relative growth rates as they had high SOD activity equivalent to T5 as well as the same ion regulation. T1 expressed general glycophyte mechanism as ion exclusion with low activity of SOD and CAT in shoots. In the future, if the selected clones (especially T5) are able to maintain their salt tolerance in whole plants then they will be a forage legume that can be utilised to improve soil fertility. In addition, they will be an alternative fodder in combination with other fodder crops because of low accumulation of toxic ions in shoots. They will also replace regular salty fodder from halophytes i.e. *Atriplex* spp., *Suaeda* spp. and, *Salicornia bigelovii* which have toxic organic compounds and a high salt load in their foliage caused of the digestion process disturbance and increases the thirst of the animals in an arid area.

According to the mechanisms of T1; Na^+ and Cl^- exclusion with high K^+ accumulation and high RGR with maintainable antioxidative enzyme this may assume that because of the selection procedure. T1 was the only seed able to germinate on NaCl free medium after 1% NaCl soaking for one week indicating its capacity of osmotic stress recovery. On the other hand, the tolerant clones were selected from germinated seeds after 2% NaCl treatment for 8 weeks. This may be the result of expression of lower salt tolerant capacity in some selected clones (T3 and T4) assuming that the experiments were conducted under osmotic stress period which is the secondary effect of salt stress (two weeks for callus and one week for shoots).

All of the clones should be planted in the field in order to determine salt tolerant stability. Some organs i.e. leaves or roots will be determined ion content as well as SOD and CAT activity whether salt tolerant mechanisms of whole plants consistent with *in vitro* explants. If confirmed, ion content and enzyme activity will be suitable to select salt tolerant and further selection within *Stylosanthes* spp.

The use of molecular techniques may also provide approaches to examine and increase salt tolerance in Stylo 184. Genes controlling salt tolerant mechanisms were disclosed and had positive results after transformed i.e. *AtNHX1* and *GhNHX1* for controlling Na^+ transport, *SOS1* for Na^+/H^+ antiport, *P5CS* for proline systhesis and *mt1D* for manitol synthesis etc. (Munns, 2005). In addition, many candidate genes for salt tolerance are interesting for transformation such as genes controlling osmotic or unknown protective function and genes controlling cell and tissue growth (Munns, 2005). Further, Stylo 184 which expresses salt tolerance mechanisms i.e. exclusion, succulence and high constitutive SOD and CAT could be so good material for molecular study. This is re-enforced by the clones' capacity to proliferate and regenerate even after long term maintenance *in vitro*. This could contribute directly towards transferring some gene(s) to increase salt tolerance.

CONCLUSIONS

1. Ion exclusion mechanisms played a role in nonselected seedlings of Stylo 184 to survive under salt stress condition.

2. Seed of 60 g (approximately 37,500 seeds) were treated with 2% NaCl. Ten salt tolerant seeds were able to germinate at 2% NaCl and determined as salt tolerant. One seed which was not able to germinate at 1% NaCl, but germinated when transferred onto the salt free MS medium for 2 weeks was selected as the control for sensitive clone.

3. Callus induced from 8 clones of salt tolerant selected seeds showed low survival percentage when screen on 2.5% NaCl. The survival callus had slow growth on low salt (1% NaCl). This result suggested that screening for salt tolerant line was not necessary for Stylo 184.

4. Callus of the five selected clones of Stylo 184 contained difference ion content. Callus of the clone T1 possessed exclusion mechanisms as a general glycophyte by maintaining the lowest Na^+ and Cl^-, and taking up the highest K^+. On the other hand, the salt tolerant clones (T2, T3, T4 and T5) revealed salt tolerant glycophyte mechanism by accumulating Na^+, Cl^- and K^+ as an osmolyte.

5. Antioxidant enzyme activity in shoots of the five selected clone of Stylo 184 revealed distinct level. Low activity of SOD and CAT advocated low tolerant of the clone T1. The tolerant clone, T5, showed the highest activity of CAT, POX and SOD. In addition, all four tolerant clones (T2, T3, T4 and T5) had significantly higher levels of CAT and SOD activity than clone T1.

6. Shoot regeneration, root induction, survival in pot and relative growth rate of the tolerant clones still being high quality even after long term maintenance over 3 years cultured.

7. Plantlets of the selected clones showed well growing and flowerring in greenhouse condition that will be further tested salt tolerant mechanisms in whole plants, and also able to produce siblings so that the next generation(s) will be determined salt tolerance.

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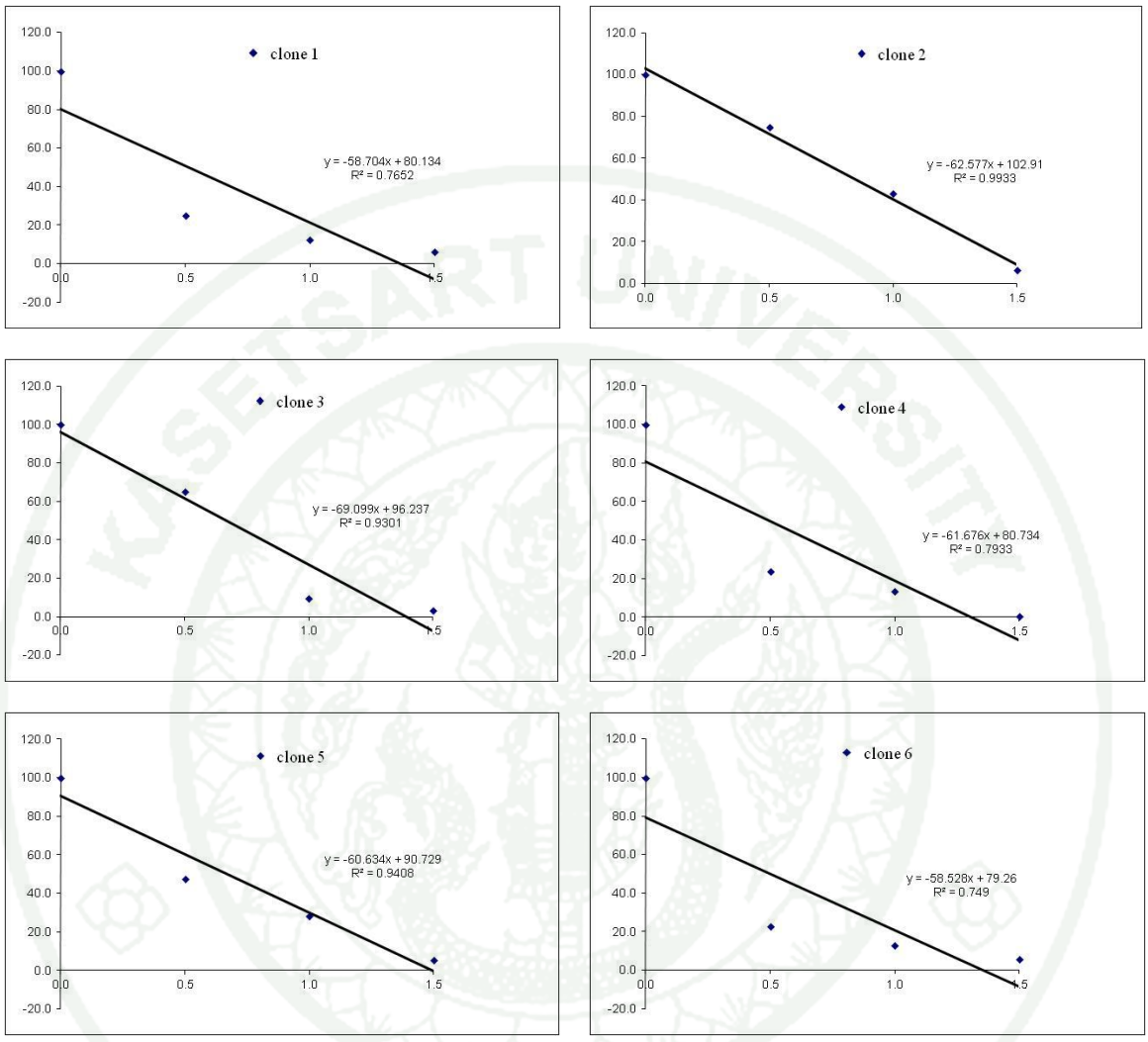
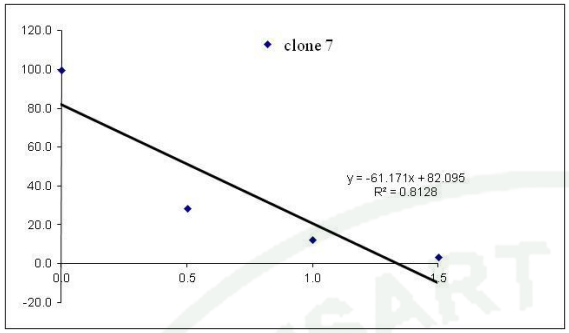
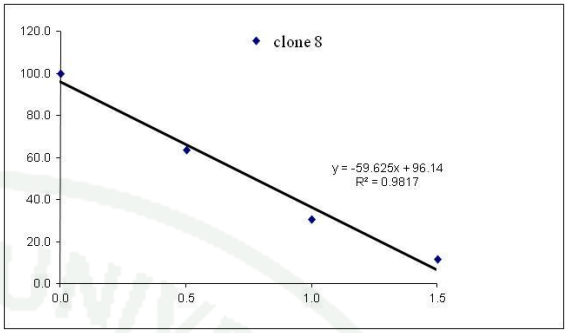
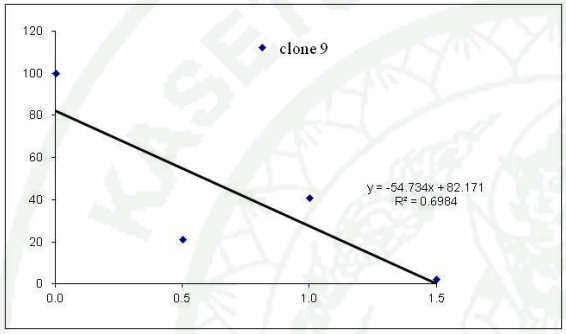
APPENDIX

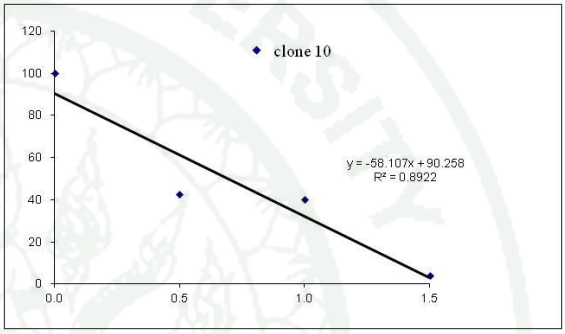


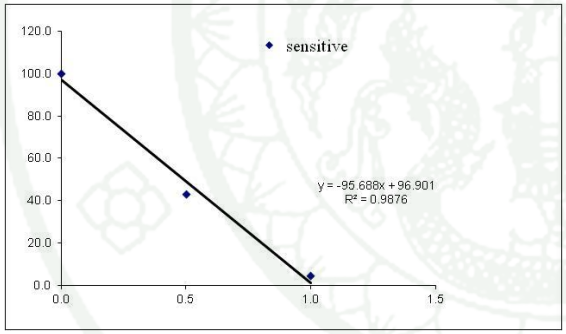
Appendix Figure 1 Relative growth rate of ten salt tolerant and one less salt tolerant selected clones.



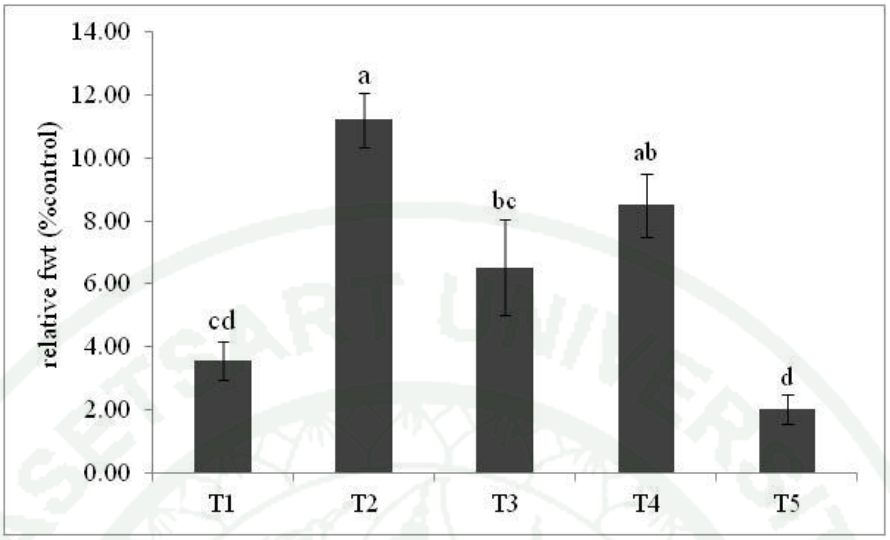








Appendix Figure 1 (continued)



Appendix Figure 2 Relative fresh weight of callus of 5 selected clones. Error bars indicate standard error; n = 10; different letters above bars indicate significant differences ($P \leq 0.05$).

APPENDIX TABLE

Appendix Table 1 Seed germination and growth of Stylo 184 seedlings treated with 0, 0.5, 0.75 and 1% NaCl solution for 1 week.

NaCl concentration (%)	Germination (%)	Shoot length (cm)	Root length (cm)
0	95.0 ± 1.0	2.89 ± 0.1^{a}	1.89 ± 0.30^{a}
0.5	90.0 ± 5.2	0.93 ± 0.1^{b}	0.50 ± 0.04^{b}
0.75	89.7 ± 0.9	0.56 ± 0.0^{c}	0.37 ± 0.02^{b}
1	88.0 ± 3.2	0.35 ± 0.0^{c}	0.24 ± 0.03^{b}

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 2 Ion content in seedling of Stylo 184 treated with 0, 0.5, 0.75 and 1% NaCl for 1 week.

NaCl concentration (%)		Ions Content (% dwt)				
		Na^{+}	Cl^{-}	K^{+}	Mg^{2+}	Ca^{2+}
Shoot	0	0.51 ± 0.07^{d}	0.03 ± 0.00^{d}	1.21 ± 0.41	0.36 ± 0.03	0.054 ± 0.009^{b}
	0.5	2.47 ± 0.12^{d}	2.02 ± 0.23^{cd}	1.16 ± 0.11	0.36 ± 0.02	0.083 ± 0.012^{ab}
	0.75	2.45 ± 0.16^{d}	2.37 ± 0.19^{c}	1.05 ±0.10	0.35 ± 0.01	0.082 ± 0.009^{ab}
	1	1.89 ± 0.41^{d}	1.27 ± 0.17^{cd}	1.03 ± 0.08	0.33 ± 0.04	0.077 ± 0.009^{ab}
Root	0	4.27 ± 0.33^{cd}	0.30 ± 0.11^{d}	4.82 ± 0.28	0.93 ± 0.08	0.002 ± 0.000^{b}
	0.5	16.79 ± 2.16^{bc}	6.32 ± 0.84^{b}	2.00 ± 0.12	1.77 ± 0.20	0.013 ± 0.003^{b}
	0.75	31.09 ± 5.61^{a}	8.84 ± 0.61^{a}	3.49 ± 0.76	2.20 ± 0.51	0.050 ± 0.000^{ab}
	1	27.29 ± 6.68^{ab}	5.19 ± 0.68^{b}	2.99 ± 1.15	2.09 ± 0.55	0.339 ± 0.184^{a}
Average						
Shoot		1.83 ± 0.26^{b}	1.42 ± 0.28^{b}	1.11 ± 0.04^{b}	0.35 ± 0.01^{b}	0.074 ± 0.006
Root		19.86 ± 3.68^{a}	5.16 ± 0.97^{a}	3.33 ± 0.43^{a}	1.75 ± 0.22^{a}	0.087 ± 0.059
Average						
0		2.39 ± 0.85^{b}	0.16 ± 0.08^{c}	3.01 ± 0.82	0.64 ± 0.13	0.027 ± 0.013
0.5		9.63 ± 3.35^{ab}	4.17 ± 1.04^{b}	1.59 ± 0.20	1.07 ± 0.33	0.043 ± 0.019
0.75		16.77 ± 6.88^{a}	5.61 ± 1.48^{a}	2.27 ± 0.65	1.28 ± 0.47	0.042 ±0.019
1		14.59 ± 6.42^{a}	3.23 ± 0.93^{b}	2.01 ± 0.68	1.21 ± 0.46	0.210 ± 0.102

Number represent mean (± S.E.). Different letter within columns and factors were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 3 Ion content in seedling of Stylo 184 treated with 0, 0.5, 0.75 and 1% NaCl for 1 week.

NaCl concentration (%)		Ions Content (% dwt)				
		Na^+	Cl^-	K^+	Mg^{2+}	Ca^{2+}
Shoot	0	0.51 ± 0.07[b]	0.03 ± 0.00[c]	1.21 ± 0.41	0.36 ± 0.03	0.054 ± 0.009
	0.5	2.47 ± 0.12[a]	2.02 ± 0.23[a]	1.16 ± 0.11	0.36 ± 0.02	0.083 ± 0.012
	0.75	2.45 ± 0.16[a]	2.37 ± 0.19[a]	1.05 ±0.10	0.35 ± 0.01	0.082 ± 0.009
	1	1.89 ± 0.41[a]	1.27 ± 0.17[b]	1.03 ± 0.08	0.33 ± 0.04	0.077 ± 0.009
F-test		**	**	ns	ns	ns
Root	0	4.27 ± 0.33[b]	0.30 ± 0.11[c]	4.82 ± 0.28	0.93 ± 0.08	0.002 ± 0.000
	0.5	16.79 ± 2.16[ab]	6.32 ± 0.84[b]	2.00 ± 0.12	1.77 ± 0.20	0.013 ± 0.003
	0.75	31.09 ± 5.61[a]	8.84 ± 0.61[a]	3.49 ± 0.76	2.20 ± 0.51	0.050 ± 0.000
	1	27.29 ± 6.68[a]	5.19 ± 0.68[b]	2.99 ± 1.15	2.09 ± 0.55	0.339 ± 0.184
F-test		**	**	ns	ns	ns

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 4 Ion ratio in seedling of Stylo 184 treated with 0, 0.5, 0.75 and 1% NaCl for 1 week.

NaCl concentration (%)		Na^+:K^+	Na^+:Ca^{2+}	Water content (%)
Shoot	0	0.42 ± 0.05[b]	9.59 ± 0.79	90.0 ± 1.2[a]
	0.5	2.14 ± 0.15[a]	30.51 ± 3.32	85.4 ± 1.9[a]
	0.75	2.36 ± 0.18[a]	80.03 ± 43.99	83.1 ± 2.5[a]
	1	1.81 ± 0.31[a]	24.51 ± 3.52	67.8 ± 5.9[b]
F-test		**	ns	**
Root	0	0.88 ± 0.02[b]	324.83 ± 25.32[b]	92.6 ± 1.3
	0.5	8.39 ± 1.01[a]	1276.29 ± 163.84[ab]	89.1 ± 4.1
	0.75	9.15 ± 0.68[a]	2362.56 ± 426.05[a]	93.8 ± 2.1
	1	10.04 ± 1.26[a]	472.44 ± 402.89[b]	79.7 ± 7.6
F-test		**	**	ns

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 5 Germination percentage of Stylo 184 seed treated with; 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl solution for 1 week.

NaCl concentration (%)	Germination (%)
0	95 ± 1.6[a]
0.5	95 ± 1.6[a]
1	95 ± 1.6[a]
1.5	40 ± 3.2[b]
2	0 ± 0.0[c]
2.5	0 ± 0.0[c]
3	0 ± 0.0[c]

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 6 Germination percentage of Stylo 184 seed treated with; 0, 1.5, 1.6, 1.7, 1.8, 1.9 and 2% NaCl solution (b) for 1 week.

NaCl concentration (%)	Germination (%)
0	82 ± 3.4[a]
1.5	22 ± 8.9[b]
1.6	14 ± 3.3[bc]
1.7	9 ± 3.0[bc]
1.8	0 ± 0.0[c]
1.9	0 ± 0.0[c]
2.0	0 ± 0.0[c]

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 7 Callus of Stylo 184 cultured on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

NaCl concentration (%)	Relative growth rate (% control)	Survival (%)	Regeneration (%)
0	100.0 ± 0.0[a]	71.0 ± 4.1[a]	54.0 ± 6.2[a]
0.5	22.9 ± 12.3[b]	74.0 ± 7.8[a]	57.0 ± 7.0[a]
1	-23.8 ± 6.2[c]	58.0 ± 4.9[a]	58.0 ± 4.9[a]
1.5	-26.2 ± 6.7[c]	33.0 ± 4.5[b]	0.0 ± 0.0[b]
2	-43.1 ± 6.9[c]	14.0 ± 4.5[c]	0.0 ± 0.0[b]

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 8 Callus of Stylo 184 cultured on MS medium added with 0.01 mg.L^{-1} NAA and 1 mg.L^{-1} BA including 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl for 6 weeks.

NaCl concentration (%)	Relative growth rate (% control)	Survival (%)	Regeneration (%)
0	100.0 ± 0.0[a]	52.0 ± 6.8[a]	52.0 ± 6.8[a]
0.5	47.6 ± 5.2[b]	50.0 ± 6.1[a]	50.0 ± 6.1[a]
1	30.6 ± 4.4[c]	40.0 ± 10.9[a]	40.0 ± 10.9[a]
1.5	8.7 ± 1.0[d]	28.0 ± 8.1[ab]	0.0 ± 0.0[b]
2	6.5 ± 1.1[d]	12.0 ± 3.6[bc]	0.0 ± 0.0[b]
2.5	11.2 ± 1.1[d]	11.0 ± 5.5[bc]	0.0 ± 0.0[b]
3	1.5 ± 0.7[d]	0.0 ± 0.0[c]	0.0 ± 0.0[b]

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 9 Realtive growth rate (RGR), water content and osmolality in callus of Stylo 184 cultured on MS medium supplemented with 0.01 mg/l NAA and 1 mg/l BA including 0-1.5 % NaCl.

Clone/ NaCl (%)		RGR (%control)	Water content (%)
T1	0	100.0 ± 0.0[a]	81.4 ± 0.3[a]
	0.5	70.7 ± 7.4[b]	76.2 ± 1.0[b]
	1	47.0 ± 2.1[c]	68.9 ± 1.6[c]
	1.5	45.5 ± 0.3[c]	67.3 ± 0.8[c]
	F-test	**	**
T2	0	100.0 ± 0.0[a]	89.5 ± 1.8[a]
	0.5	65.3 ± 2.9[b]	85.6 ± 0.6[ab]
	1	30.5 ± 0.7[c]	81.9 ± 0.5[bc]
	1.5	20.2 ± 3.8[d]	81.1 ± 1.6[c]
	F-test	**	**
T3	0	100.0 ± 0.0[a]	86.1 ± 0.6[a]
	0.5	107.0 ± 12.8[a]	82.7 ± 1.1[b]
	1	42.7 ± 3.2[b]	76.4 ± 0.8[c]
	1.5	32.3 ± 1.5[b]	70.3 ± 1.5[d]
	F-test	**	**
T4	0	100.0 ± 0.0[a]	87.2 ± 0.3[a]
	0.5	36.8 ± 2.6[b]	81.5 ± 0.1[b]
	1	37.3 ± 2.2[b]	79.1 ± 1.0[c]
	1.5	20.4 ± 0.5[c]	75.2 ± 0.5[d]
	F-test	**	**
T5	0	100.0 ± 0.0[a]	87.5 ± 0.6[a]
	0.5	93.3 ± 6.7[a]	83.5 ± 0.5[b]
	1	79.7 ± 8.8[a]	81.4 ± 0.1[c]
	1.5	44.4 ± 0.9[b]	83.6 ± 0.9[b]
	F-test	**	**

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 10 Osmotic pressure and contribution to osmolality by Na^+ and K^+ in cell sap of Stylo 184 cultured on MS medium supplemented with 0.01 mg/l NAA and 1 mg/l BA including 0-1.5 % NaCl.

Clone/ NaCl concentration (%)	Osmotic pressure (MP)	contribution to osmolality (%)	
		Na^+	K^+
T1			
0	0.889 ± 0.114^{d}	1.2 ± 0.4^{b}	59.3 ± 8.6^{a}
0.5	1.742 ± 0.143^{c}	21.1 ± 1.7^{a}	38.5 ± 2.6^{b}
1	2.390 ± 0.119^{b}	29.0 ± 3.6^{a}	29.9 ± 3.4^{b}
1.5	3.092 ± 0.097^{a}	26.2 ± 2.3^{a}	19.1 ± 0.6^{b}
F-test	**	**	**
T2			
0	0.971 ± 0.066^{d}	2.4 ± 0.4^{c}	17.4 ± 1.0^{a}
0.5	1.603 ± 0.010^{c}	21.5 ± 1.6^{b}	9.5 ± 0.1^{b}
1	1.950 ± 0.081^{b}	43.5 ± 4.7^{a}	6.4 ± 0.5^{c}
1.5	2.380 ± 0.059^{a}	48.6 ± 3.6^{a}	6.5 ± 0.5^{c}
F-test	**	**	**
T3			
0	0.990 ± 0.053^{d}	2.3 ± 0.2^{d}	18.3 ± 1.6^{a}
0.5	1.573 ± 0.026^{c}	19.7 ± 1.9^{c}	11.3 ± 0.7^{b}
1	2.453 ± 0.002^{b}	31.9 ± 2.6^{b}	6.1 ± 0.1^{c}
1.5	3.077 ± 0.036^{a}	41.6 ± 1.2^{a}	7.4 ± 0.2^{c}
F-test	**	**	**
T4			
0	1.084 ± 0.094^{d}	2.3 ± 0.2^{b}	19.4 ± 1.3^{a}
0.5	1.490 ± 0.043^{c}	28.4 ± 1.2^{a}	12.7 ± 0.8^{b}
1	2.177 ± 0.139^{b}	25.9 ± 3.6^{a}	6.7 ± 0.3^{c}
1.5	2.641 ± 0.002^{a}	34.1 ± 2.2^{a}	5.6 ± 0.3^{c}
F-test	**	**	**
T5			
0	1.099 ± 0.008^{d}	2.4 ± 1.2d	17.2 ± 0.1^{a}
0.5	1.692 ± 0.003^{c}	21.6 ± 1.1c	10.3 ± 0.2^{b}
1	2.119 ± 0.063^{b}	27.4 ± 1.9b	8.8 ± 0.7^{b}
1.5	2.464 ± 0.056^{a}	37.0 ± 1.0a	6.7 ± 0.8^{c}
F-test	**	**	**

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 11 Ion content in callus of Stylo 184 cultured on MS medium supplemented with 0.01 mg/l NAA and 1 mg/l BA including 0-1.5 % NaCl.

Clone/ NaCl (%)	Ions content (% dwt)				
	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}
T1 0	0.06 ± 0.02^{c}	0.03 ± 0.00^{b}	4.85 ± 0.03	0.086 ± 0.003^{a}	0.324 ± 0.008^{b}
0.5	1.70 ± 0.11^{b}	0.06 ± 0.01^{ab}	5.14 ± 0.27	0.085 ± 0.006^{a}	0.381 ± 0.010^{ab}
1	2.50 ± 0.10^{a}	0.07 ± 0.01^{a}	4.39 ± 0.81	0.040 ± 0.008^{b}	0.402 ± 0.023^{a}
1.5	2.73 ± 0.19^{a}	0.08 ± 0.00^{a}	3.30 ± 0.04	0.029 ± 0.006^{b}	0.382 ± 0.200^{ab}
F-test	**	**	ns	**	**
T2 0	0.45 ± 0.22^{b}	0.08 ± 0.05^{a}	2.43 ± 0.14	0.291 ± 0.170	0.493 ± 0.267
0.5	3.41 ± 0.70^{b}	0.11 ± 0.01^{ab}	2.60 ± 0.70	0.120 ± 0.012	0.396 ± 0.062
1	6.48 ± 0.53^{a}	0.19 ± 0.01^{ab}	1.58 ± 0.13	0.067 ± 0.012	0.533 ± 0.029
1.5	6.83 ±1.23^{a}	0.23 ± 0.04^{a}	1.53 ± 0.31	0.017 ± 0.007	0.394 ± 0.040
F-test	**	**	ns	ns	ns
T3 0	0.17 ± 0.01^{d}	0.03 ± 0.00^{b}	2.19 ± 0.06^{a}	0.111 ± 0.009^{a}	0.060 ± 0.003^{a}
0.5	1.85 ± 0.27^{c}	0.09 ± 0.02^{a}	1.72 ± 0.02^{b}	0.031 ± 0.011^{b}	0.059 ± 0.002^{a}
1	3.46 ± 0.14^{b}	0.11 ± 0.00^{a}	1.15 ± 0.06^{d}	0.006 ± 0.001^{b}	0.043 ± 0.003^{b}
1.5	4.77 ± 0.05^{a}	0.12 ± 0.02^{a}	1.42 ± 0.08^{c}	0.002 ± 0.000^{b}	0.045 ± 0.002^{b}
F-test	**	**	**	**	**
T4 0	0.20 ± 0.01^{c}	0.03 ± 0.00^{c}	2.77 ± 0.24^{a}	0.107 ± 0.004^{a}	0.143 ± 0.030
0.5	2.33 ± 0.09^{b}	0.08 ± 0.01^{b}	1.73 ± 0.11^{b}	0.022 ± 0.004^{b}	0.085 ± 0.003
1	2.78 ± 0.12^{b}	0.08 ± 0.00^{b}	1.22 ± 0.08^{bc}	0.039 ± 0.022^{b}	0.092 ± 0.003
1.5	3.96 ± 0.26^{a}	0.17 ± 0.01^{a}	1.07 ± 0.05^{c}	0.005 ± 0.001^{b}	0.075 ± 0.003
F-test	**	**	**	**	ns
T5 0	0.23 ± 0.11^{d}	0.03 ± 0.00^{c}	2.75 ± 0.11^{a}	0.080 ± 0.026^{a}	0.639 ± 0.070
0.5	2.23 ± 0.17^{c}	0.07 ± 0.01^{b}	1.75 ± 0.04^{b}	0.009 ± 0.001^{b}	0.520 ± 0.043
1	3.38 ± 0.48^{b}	0.09 ± 0.01^{b}	1.80 ± 0.27^{b}	0.026 ± 0.011^{ab}	0.512 ± 0.038
1.5	5.55 ± 0.05^{a}	0.17 ± 0.00^{a}	1.65 ± 0.15^{b}	0.030 ± 0.012^{ab}	0.693 ± 0.040
F-test	**	**	**	**	ns

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 12 Ion ratio of Stylo 184 callus cultured on MS medium supplemented with 0.01 mg/l NAA and 1 mg/l BA including 0-1.5 % NaCl.

Clone/ NaCl concentration (%)	Ion ratio	
	Na^+: K^+	Na^+: Ca^{2+}
T1		
0	0.01 ± 0.01^{c}	0.72 ± 0.23^{b}
0.5	0.33 ± 0.10^{b}	20.13 ± 1.24^{b}
1	0.61 ± 0.11^{a}	68.69 ± 16.67^{ab}
1.5	0.83 ± 0.06^{a}	104.78 ± 28.97^{a}
F-test	**	**
T2		
0	0.18 ± 0.09^{c}	1.72 ± 0.17
0.5	1.37 ± 0.12^{b}	28.21 ± 4.23
1	4.11 ± 0.14^{a}	104.45 ± 21.28
1.5	4.56 ± 0.44^{a}	1073.17 ± 808.53
F-test	**	ns
T3		
0	0.08 ± 0.01^{c}	1.53 ± 0.10^{b}
0.5	1.08 ± 0.17^{b}	86.95 ± 38.19^{b}
1	3.03 ± 0.25^{a}	653.90 ± 108.68^{b}
1.5	3.39 ± 0.17^{a}	2784.60 ± 504.84^{a}
F-test	**	**
T4		
0	0.07 ± 0.01^{d}	1.83 ± 0.08^{b}
0.5	1.37 ± 0.15^{c}	114.26 ± 19.39^{ab}
1	2.31 ± 0.23^{b}	154.29 ± 85.95^{ab}
1.5	3.69 ± 0.07^{a}	1185.21 ± 495.18^{a}
F-test	**	**
T5		
0	0.09 ± 0.04^{c}	2.91 ± 1.09^{b}
0.5	1.27 ± 0.08^{b}	250.80 ± 12.19^{a}
1	1.88 ± 0.20^{b}	190.89 ± 71.06^{ab}
1.5	3.43 ± 0.31^{a}	241.57 ± 67.69^{a}
F-test	**	**

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level.

Appendix Table 13 Relative fresh weight (RFW) and activity of SOD, CAT and POX of selected callus cultured on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treated with 0, 0.5 and 1% NaCl for 1 week.

Clone/ NaCl (%)	RFW (%control)	Activity (U g^{-1} fwt)		
		SOD	CAT	POX
T1				
0	100.0 ± 0.0	19.06 ± 0.81	2.86 ± 0.32	5.85 ± 0.86
0.5	91.1 ± 5.9	20.97 ± 1.39	2.65 ± 0.57	6.83 ± 0.94
1	67.9 ± 18.5	16.48 ± 2.98	1.65 ± 0.47	3.95 ± 1.57
F-test	ns	ns	ns	ns
T2				
0	100.0 ± 0.0[a]	50.71 ± 10.02	3.57 ± 0.13	4.92 ± 0.35[a]
0.5	76.6 ± 8.4[ab]	56.27 ± 6.20	3.75 ± 0.50	2.69 ± 0.11[b]
1	57.1 ± 9.4[b]	49.01 ± 6.66	2.94 ± 0.20	2.54 ± 0.22[b]
F-test	**	ns	ns	**
T3				
0	100.0 ± 0.0a	82.30 ± 16.78	3.21 ± 0.21	7.47 ± 0.68 a
0.5	41.7 ± 6.3b	61.33 ± 4.00	3.06 ± 1.11	4.39 ± 0.76 b
1	46.2 ± 4.7b	64.90 ± 1.34	2.60 ± 0.42	4.45 ± 0.72 b
F-test	**	ns	ns	*
T4				
0	100.0 ± 0.0[a]	96.23 ± 7.52[a]	0.92 ± 0.12	3.15 ± 0.41
0.5	70.8 ± 3.6[b]	84.20 ± 4.20[ab]	1.11 ± 0.08	2.91 ± 0.11
1	59.5 ± 8.0[b]	67.04 ± 7.56[b]	1.19 ± 0.18	2.14 ± 0.34
F-test	**	*	ns	ns
T5				
0	100.0 ± 0.0[a]	67.49 ± 4.69[ab]	10.56 ± 1.06	4.09 ± 0.37[a]
0.5	91.9 ± 18.8[a]	79.37 ± 11.39[a]	12.62 ± 3.00	3.78 ± 0.32[a]
1	45.4 ± 3.2[b]	45.63 ± 6.17[b]	5.74 ± 0.55	1.91 ± 0.08[b]
F-test	*	*	ns	**

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level

Appendix Table 14 Relative fresh weight and activity of SOD, CAT and POX of selected callus cultured on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on the medium without salt for 1 week.

Clone/ NaCl (%)	RFW (%control)	Activity (U mg^{-1} fwt)		
		SOD	CAT	POX
T1				
0	100.0 ± 0.0[a]	20.45 ± 3.01	2.13 ± 0.11	7.97 ± 1.46
0.5	115.8 ± 13.0[a]	20.54 ± 2.19	1.88 ± 0.30	6.64 ± 1.26
1	64.7 ± 8.6[b]	16.56 ± 2.97	1.43 ± 0.38	4.82 ± 1.67
F-test	**	ns	ns	ns
T2				
0	100.0 ± 0.00	48.22 ± 3.73[a]	5.45 ± 0.30	2.09 ± 0.12[a]
0.5	104.4 ± 17.92	49.63 ± 7.44[a]	5.35 ± 0.83	2.00 ± 0.20[a]
1	74.2 ± 5.82	30.44 ± 1.20[b]	3.71 ± 0.37	1.34 ± 0.10[b]
F-test	ns	*	ns	*
T3				
0	100.0 ± 0.0	68.82 ± 3.84[a]	1.22 ± 0.14	2.70 ± 0.32
0.5	82.4 ± 11.4	68.14 ± 14.67[a]	0.87 ± 0.22	1.96 ± 0.71
1	70.7 ± 8.2	34.46 ± 3.94[b]	1.57 ± 0.20	1.54 ± 0.26
F-test	ns	*	ns	ns
T4				
0	100.0 ± 0.0[a]	42.55 ± 2.09[a]	4.82 ± 0.84	5.66 ± 0.71
0.5	88.9 ± 11.4[ab]	32.29 ± 1.42[b]	4.30 ± 0.29	4.58 ± 0.99
1	63.7 ± 10.8[b]	30.23 ± 2.50[b]	4.09 ± 0.46	4.37 ± 0.73
F-test	*	**	ns	ns
T5				
0	100.0 ± 0.0	58.49 ± 3.63[b]	5.67 ± 0.60	14.58 ± 1.59
0.5	106.9 ± 7.4	75.70 ± 5.63[a]	5.89 ± 0.64	15.59 ± 1.19
1	94.2 ± 8.0	45.62 ± 3.79[b]	4.42 ± 0.87	11.91 ± 2.41
F-test	ns	**	ns	ns

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level

Appendix Table 15 Shoot regeneration of Stylo 184 after maintaining for 3 years on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA for 8 weeks.

Clone	Shoot number (shoot)	Shoot regeneration (%)
T1	4.6 ± 0.89^{ab}	56.7 ± 8.03^{b}
T2	4.8 ± 0.72^{ab}	90.0 ± 6.83^{a}
T3	2.6 ± 0.24^{b}	66.7 ± 8.43^{ab}
T4	7.1 ± 1.39^{a}	93.3 ± 4.22^{a}
T5	5.8 ± 1.08^{ab}	90.0 ± 10.00^{a}

Number represent mean (± S.E.). Different letter within columns were significantly different from each other according to Tukey's test at 5% probability level

Appendix Table 16 Statistical analysis of germination percentage Stylo 184 seeds treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	82.00	3.0	27.33	0.93	0.47
error	234.67	8.0	29.33		
total	316.67	11.0			

Appendix Table 17 Statistical analysis of shoot height of Stylo 184 seeding treated with 0, 0.5, 0.75 and 1% of NaCl for 1week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	12.15	3.0	4.05	291.56	.00
error	0.11	8.0	0.01		
total	12.26	11.0			

Appendix Table 18 Statistical analysis of root height Stylo 184 seedling treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5.28	3.0	1.76	25.14	.00
error	0.56	8.0	0.07		
total	5.83	11.0			

Appendix Table 19 Statistical analysis of shoot water content Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	835.77	3.0	278.59	8.02	0.01
error	277.90	8.0	34.74		
total	1113.67	11.0			

Appendix Table 20 Statistical analysis of root water content Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	368.00	3.0	122.67	2.04	0.19
error	481.58	8.0	60.20		
total	849.58	11.0			

Appendix Table 21 Statistical analysis of Na^+ content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	7.61	3.0	2.54	16.00	0.00
error	1.27	8.0	0.16		
total	8.88	11.0			

Appendix Table 22 Statistical analysis of Na^+ content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1300.92	3.0	433.64	7.15	0.01
error	485.32	8.0	60.67		
total	1786.25	11.0			

Appendix Table 23 Statistical analysis of Cl^- content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	9.65	3.0	3.22	37.51	0.00
error	0.69	8.0	0.09		
total	10.34	11.0			

Appendix Table 24 Statistical analysis of Cl^- content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	115.72	3.0	38.57	32.90	0.00
error	9.38	8.0	1.17		
total	125.10	11.0			

Appendix Table 25 Statistical analysis of K^+ content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.07	3.0	0.02	0.98	0.45
error	0.18	8.0	0.02		
total	0.25	11.0			

Appendix Table 26 Statistical analysis of K^+ content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	12.31	3.0	4.11	2.75	0.11
error	11.93	8.0	1.49		
total	24.25	11.0			

Appendix Table 27 Statistical analysis of Ca^{2+} content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.002	3.0	0.001	2.15	0.172
error	0.002	8.0	0.000		
total	0.004	11.0			

Appendix Table 28 Statistical analysis of Ca^{2+} content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.264	3.0	0.088	3.46	0.07
error	0.203	8.0	0.025		
total	0.467	11.0			

Appendix Table 29 Statistical analysis of Mg^{2+} content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.001	3.0	0.000	0.204	0.89
error	0.019	8.0	0.002		
total	0.020	11.0			

Appendix Table 30 Statistical analysis of Mg^{2+} content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2.993	3.0	0.998	2.196	0.17
error	3.634	8.0	0.454		
total	6.627	11.0			

Appendix Table 31 Statistical analysis of Na^{+}: K^{+} content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	6.87	3.0	2.29	19.77	0.00
error	0.93	8.0	0.12		
total	7.79	11.0			

Appendix Table 32 Statistical analysis of Na^{+}: K^{+} content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	159.60	3.0	53.20	23.17	0.00
error	18.37	8.0	2.30		
total	177.98	11.0			

Appendix Table 33 Statistical analysis of Na^{+}: Ca^{2+} content in shoot of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	8392.20	3.0	2797.40	1.90	0.21
error	11756.74	8.0	1469.59		
total	20148.94	11.0			

Appendix Table 34 Statistical analysis of Na^{+}: Ca^{2+} content in root of Stylo 184 treated with 0, 0.5, 0.75 and 1% of NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	7858601.85	3.0	2619533.95	9.41	0.01
error	2227940.52	8.0	278492.56		
total	10086542.36	11.0			

Appendix Table 35 Statistical analysis of percent germination Stylo 184 seed treated with 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl solution for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	67928.6	6.0	11321.4	905.7	0.00
error	350.0	28.0	12.5		
total	68278.6	34.0			

Appendix Table 36 Statistical analysis of percent germination Stylo 184 seed treated with 0, 1.5, 1.6, 1.7, 1.8, 1.9 and 2% NaCl solution for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	25904.3	6.0	4317.4	51.44	0.00
error	2350.0	28.0	83.9		
total	28254.3	34.0			

Appendix Table 37 Statistical analysis of relative growth rate of Stylo 184 callus on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	134587.9	4.0	33647.0	59.7	0.00
error	25357.0	45.0	563.5		
total	159944.9	49.0			

Appendix Table 38 Statistical analysis of percent survival of Stylo 184 callus on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	26660.0	4.0	6665.0	23.54	0.00
error	12740.0	45.0	283.1		
total	39400.0	49.0			

Appendix Table 39 Statistical analysis of percent regeneration of Stylo 184 callus on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	38168.0	4.0	9542.0	42.9	0.00
error	10010.0	45.0	222.4		
total	48178.0	49.0			

Appendix Table 40 Statistical analysis of relative growth rate of Stylo 184 callus on the MS medium supplemented with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA including 0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	73869.0	6.0	12311.5	171.3	0.00
error	4528.1	63.0	71.9		
total	78397.1	69.0			

Appendix Table 41 Statistical analysis of percent survival of Stylo 184 callus on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	25317.1	6.0	4219.5	9.5	0.00
error	27970.0	63.0	444.0		
total	53287.1	69.0			

Appendix Table 42 Statistical analysis of percent regeneration of Stylo 184 callus on MS medium including 0, 0.5, 1, 1.5, and 2% NaCl for 4 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	39234.3	6.0	6539.1	22.69	0.00
error	18160.0	63.0	288.3		
total	57394.3	69.0			

Appendix Table 43 Statistical analysis of relative growth rate of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5880.718	3.0	1960.239	44.626	0.00
error	351.411	8.0	43.926		
total	6232.129	11.0			

Appendix Table 44 Statistical analysis of relative growth rate of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	11810.452	3.0	3936.817	227.083	0.00
error	138.692	8.0	17.336		
total	11949.144	11.0			

Appendix Table 45 Statistical analysis of relative growth rate of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	13320.445	3.0	4440.148	33.653	0.00
error	1055.500	8.0	131.938		
total	14375.946	11.0			

Appendix Table 46 Statistical analysis of relative growth rate of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	11107.800	3.0	3702.600	410.865	0.00
error	72.094	8.0	9.012		
total	11179.893	11.0			

Appendix Table 47 Statistical analysis of relative growth rate of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5530.730	3.0	1843.577	19.922	0.00
error	740.301	8.0	92.538		
total	6271.031	11.0			

Appendix Table 48 Statistical analysis of water content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	386.892	3.0	128.964	115.382	0.00
error	8.942	8.0	1.118		
total	395.834	11.0			

Appendix Table 49 Statistical analysis of water content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	135.310	3.0	45.103	14.269	0.00
error	25.288	8.0	3.161		
total	160.598	11.0			

Appendix Table 50 Statistical analysis of water content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	439.530	3.0	146.510	146.476	0.00
error	8.002	8.0	1.000		
total	447.532	11.0			

Appendix Table 51 Statistical analysis of water content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	224.576	3.0	74.859	218.347	0.00
error	2.743	8.0	0.343		
total	227.318	11.0			

Appendix Table 52 Statistical analysis of water content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	57.644	3.0	19.215	34.259	0.00
error	4.487	8.0	0.561		
total	62.131	11.0			

Appendix Table 53 Statistical analysis of Na^+ content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	13.120	3.0	4.373	102.574	0.00
error	.341	8.0	0.043		
total	13.461	11.0			

Appendix Table 54 Statistical analysis of Na^+ content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	80.481	3.0	26.827	15.330	0.00
error	14.000	8.0	1.750		
total	94.481	11.0			

Appendix Table 55 Statistical analysis of Na^+ content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	35.687	3.0	11.896	172.732	0.00
error	0.551	8.0	0.069		
total	36.238	11.0			

Appendix Table 56 Statistical analysis of Na^+ content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	22.221	3.0	7.407	111.596	0.00
error	0.531	8.0	0.066		
total	22.752	11.0			

Appendix Table 57 Statistical analysis of Na^+ content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	44.544	3.0	14.848	71.706	0.00
error	1.657	8.0	0.207		
total	46.200	11.0			

Appendix Table 58 Statistical analysis of Cl^- content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.004	3.0	0.001	7.888	0.00
error	0.001	8.0	0.000		
total	0.005	11.0			

Appendix Table 59 Statistical analysis of Cl^- content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.043	3.0	0.014	4.729	0.04
error	0.025	8.0	0.003		
total	0.068	11.0			

Appendix Table 60 Statistical analysis of Cl^- content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.016	3.0	0.005	10.013	0.00
error	0.004	8.0	0.001		
total	0.021	11.0			

Appendix Table 61 Statistical analysis of Cl^- content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.031	3.0	0.010	51.756	0.00
error	0.002	8.0	0.000		
total	0.032	11.0			

Appendix Table 62 Statistical analysis of Cl^- content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.033	3.0	0.011	150.114	0.00
error	0.001	8.0	0.000		
total	0.033	11.0			

Appendix Table 63 Statistical analysis of K^+ content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5.832	3.0	1.944	3.525	0.07
error	4.412	8.0	0.551		
total	10.243	11.0			

Appendix Table 64 Statistical analysis of K^+ content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2.803	3.0	0.934	1.989	0.19
error	3.758	8.0	0.470		
total	6.561	11.0			

Appendix Table 65 Statistical analysis of K^+ content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.781	3.0	0.594	60.147	0.00
error	0.079	8.0	0.010		
total	1.860	11.0			

Appendix Table 66 Statistical analysis of K^+ content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5.317	3.0	1.772	30.417	0.00
error	0.466	8.0	0.058		
total	5.784	11.0			

Appendix Table 67 Statistical analysis of K^+ content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2.345	3.0	0.782	9.720	0.01
error	0.643	8.0	0.080		
total	2.988	11.0			

Appendix Table 68 Statistical analysis of Ca^{2+} content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.008	3.0	0.003	24.207	0.00
error	0.001	8.0	0.000		
total	0.009	11.0			

Appendix Table 69 Statistical analysis of Ca^{2+} content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.128	3.0	0.043	1.936	0.20
error	0.176	8.0	0.022		
total	0.304	11.0			

Appendix Table 70 Statistical analysis of Ca^{2+} content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.023	3.0	0.008	50.978	0.00
error	0.001	8.0	0.000		
total	0.024	11.0			

Appendix Table 71 Statistical analysis of Ca^{2+} content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.018	3.0	0.006	15.810	0.00
error	0.003	8.0	0.000		
total	0.021	11.0			

Appendix Table 72 Statistical analysis of Ca^{2+} content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.008	3.0	0.003	4.049	0.05
error	0.006	8.0	0.001		
total	0.014	11.0			

Appendix Table 73 Statistical analysis of Mg^{2+} content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.010	3.0	0.003	4.353	0.04
error	0.006	8.0	0.001		
total	0.017	11.0			

Appendix Table 74 Statistical analysis of Mg^{2+} content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.044	3.0	0.015	0.254	0.86
error	0.466	8.0	0.058		
total	0.510	11.0			

Appendix Table 75 Statistical analysis of Mg^{2+} content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.001	3.0	0.000	11.664	0.00
error	0.000	8.0	0.000		
total	0.001	11.0			

Appendix Table 76 Statistical analysis of Mg^{2+} content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.008	3.0	0.003	4.002	0.05
error	0.006	8.0	0.001		
total	0.014	11.0			

Appendix Table 77 Statistical analysis of Mg^{2+} content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.072	3.0	0.024	3.271	0.08
error	0.059	8.0	0.007		
total	0.131	11.0			

Appendix Table 78 Statistical analysis of Na^{+}: K^{+}content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.120	3.0	0.373	30.733	0.00
error	0.097	8.0	0.012		
total	1.217	11.0			

Appendix Table 79 Statistical analysis of Na^{+}: K^{+}content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	40.394	3.0	13.465	76.611	0.00
error	1.406	8.0	0.176		
total	41.800	11.0			

Appendix Table 80 Statistical analysis of Na^+: K^+content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	22.453	3.0	7.484	83.196	0.00
error	0.720	8.0	0.090		
total	23.173	11.0			

Appendix Table 81 Statistical analysis of Na^+: K^+content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	20.927	3.0	6.976	116.286	0.00
error	0.480	8.0	0.060		
total	21.407	11.0			

Appendix Table 82 Statistical analysis of Na^+: K^+content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	17.417	3.0	5.806	72.761	0.00
error	0.638	8.0	0.080		
total	18.055	11.0			

Appendix Table 83 Statistical analysis of Na^+: Ca^{2+}content of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	19986.888	3.0	6662.296	7.941	0.01
error	6711.974	8.0	838.997		
total	26698.862	11.0			

Appendix Table 84 Statistical analysis of Na^+: Ca^{2+}content of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2396604.266	3.0	798868.089	1.628	0.26
error	3925179.783	8.0	490647.473		
total	6321784.049	11.0			

Appendix Table 85 Statistical analysis of Na^+: Ca^{2+}content of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	15237717.812	3.0	5079239.271	25.257	0.00
error	1608794.579	8.0	201099.322		
total	16846512.391	11.0			

Appendix Table 86 Statistical analysis of Na^+: Ca^{2+}content of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2735724.555	3.0	911908.185	4.806	0.03
error	1517803.092	8.0	189725.386		
total	4253527.647	11.0			

Appendix Table 87 Statistical analysis of Na^+: Ca^{2+}content of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	119985.902	3.0	39995.301	5.452	0.03
error	58688.314	8.0	7336.039		
total	178674.216	11.0			

Appendix Table 88 Statistical analysis of osmotic pressure of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	7.926	3.0	2.642	61.579	0.00
error	0.343	8.0	0.043		
total	8.269	11.0			

Appendix Table 89 Statistical analysis of osmotic pressure of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3.187	3.0	1.062	96.709	0.00
error	0.088	8.0	0.011		
total	3.275	11.0			

Appendix Table 90 Statistical analysis of osmotic pressure of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	7.695	3.0	2.565	724.780	0.00
error	.028	8.0	0.004		
total	7.724	11.0			

Appendix Table 91 Statistical analysis of osmotic pressure of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	4.348	3.0	1.449	64.413	0.00
error	0.180	8.0	0.022		
total	4.528	11.0			

Appendix Table 92 Statistical analysis of osmotic pressure of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3.115	3.0	1.038	193.469	0.00
error	0.043	8.0	0.005		
total	3.158	11.0			

Appendix Table 93 Statistical analysis of contribution to osmolality by Na^+ of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1415.654	3.0	471.885	29.487	0.00
error	128.026	8.0	16.003		
total	1543.680	11.0			

Appendix Table 94 Statistical analysis of contribution to osmolality by Na^+ of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	4071.160	3.0	1357.053	47.989	0.00
error	226.228	8.0	28.279		
total	4297.389	11.0			

Appendix Table 95 Statistical analysis of contribution to osmolality by Na^+ of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2580.737	3.0	860.246	97.951	0.00
error	70.259	8.0	8.782		
total	2650.996	11.0			

Appendix Table 96 Statistical analysis of contribution to osmolality by Na^+ of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1770.336	3.0	590.112	39.963	0.00
error	118.130	8.0	14.766		
total	1888.466	11.0			

Appendix Table 97 Statistical analysis of contribution to osmolality by Na^+ of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1917.501	3.0	639.167	115.885	0.00
error	44.124	8.0	5.516		
total	1961.625	11.0			

Appendix Table 98 Statistical analysis of contribution to osmolality by K^+ of clone T1 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2605.378	3.0	868.459	12.473	0.00
error	557.028	8.0	69.628		
total	3162.406	11.0			

Appendix Table 99 Statistical analysis of contribution to osmolality by K^+ of clone T2 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	242.705	3.0	80.902	69.810	0.00
error	9.271	8.0	1.159		
total	251.976	11.0			

Appendix Table 100 Statistical analysis of contribution to osmolality by K^+ of clone T3 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	268.916	3.0	89.639	37.642	0.00
error	19.051	8.0	2.381		
total	287.967	11.0			

Appendix Table 101 Statistical analysis of contribution to osmolality by K^+ of clone T4 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	361.313	3.0	120.438	64.608	0.00
error	14.913	8.0	1.864		
total	376.226	11.0			

Appendix Table 102 Statistical analysis of contribution to osmolality by K^+ of clone T5 callus on 0, 0.5, 1 and 1.5 of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	187.166	3.0	62.389	74.271	0.00
error	6.720	8.0	0.840		
total	193.886	11.0			

Appendix Table 103 Statistical analysis of relative growth rate of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	7407.88	4.0	1851.97	31.42	0.00
NaCl	40072.64	3.0	13357.55	226.59	0.00
Clone X NaCl	7577.50	12.0	613.46	10.71	0.00
error	2358.00	40.0	58.95		
total	57416.03	59.0			

Appendix Table 104 Statistical analysis of water content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	976.66	4.0	244.16	197.46	0.00
NaCl	1045.07	3.0	348.36	281.72	0.00
Clone X NaCl	198.88	12.0	16.57	13.40	0.00
error	49.46	40.0	1.24		
total	2270.07	59.0			

Appendix Table 105 Statistical analysis of Na^+ content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	43.37	4.0	10.84	25.39	0.00
NaCl	174.22	3.0	58.07	136.01	0.00
Clone X NaCl	21.83	12.0	1.82	4.26	0.00
error	17.08	40.0	0.43		
total	256.50	60.0			

Appendix Table 106 Statistical analysis of Cl^- content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	5.80E-02	4.0	1.45E-02	17.96	0.00
NaCl	0.11	3.0	3.49E-02	43.16	0.00
Clone X NaCl	2.24E-02	12.0	1.87E-03	2.32	0.02
error	3.23E-02	40.0	8.08E-04		
total	0.22	60.0			

Appendix Table 107 Statistical analysis of K^+ content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	65.80	4.0	16.45	70.31	0.00
NaCl	13.31	3.0	4.44	18.96	0.00
Clone X NaCl	4.77	12.0	0.40	1.70	0.10
error	9.36	40.0	0.23		
total	93.23	60.0			

Appendix Table 108 Statistical analysis of Ca^{2+} content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	6.53E-02	4.0	1.63	3.50	0.02
NaCl	0.12	3.0	4.09E-02	8.77	0.00
Clone X NaCl	6.28E-02	12.0	5.24E-03	1.12	0.37
error	0.19	40.0	4.67E-03		
total	0.44	60.0			

Appendix Table 109 Statistical analysis of Mg^{2+} content of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	2.58	4.0	0.65	48.05	0.00
NaCl	1.51E-02	3.0	5.02E-03	0.37	0.77
Clone X NaCl	0.21	12.0	1.01E-02	0.75	0.70
error	0.54	40.0	1.34E-02		
total	3.25	60.0			

Appendix Table 110 Statistical analysis of Na^+:K^+ ratio of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	28.430	4.0	7.107	85.091	0.00
NaCl	84.604	3.0	28.201	337.631	0.00
Clone X NaCl	17.706	12.0	1.476	17.665	0.00
error	3.341	40.0	8.353E-02		
total	304.389	60.0			

Appendix Table 111 Statistical analysis of Na^+:Ca^{2+} ratio of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	4894792.437	4.0	1223698.109	6.877	0.00
NaCl	10902619.392	3.0	3634206.464	20.425	0.00
Clone X NaCl	9607400.032	12.0	800616.669	4.500	0.00
error	7117177.741	40.0	177929.444		
total	40021010.267	60.0			

Appendix Table 112 Statistical analysis of osmotic pressure of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	0.813	4.0	0.203	11.909	0.00
NaCl	25.013	3.0	8.338	488.740	0.00
Clone X NaCl	1.259	12.0	0.105	6.151	0.00
error	0.682	40.0	1.706E-02		
total	242.958	60.0			

Appendix Table 113 Statistical analysis of contribution to osmolality by Na^+ of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	595.775	4.0	148.944	10.154	0.00
NaCl	10779.085	3.0	3593.028	244.937	0.00
Clone X NaCl	976.303	12.0	81.359	5.546	0.00
error	586.768	40.0	14.669		
total	45831.377	60.0			

Appendix Table 114 Statistical analysis of contribution to osmolality by K^+ of callus of 5 clones on various concentrations of NaCl for 2 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	6522.329	4.0	1630.582	107.455	0.00
NaCl	2612.402	3.0	870.801	57.385	0.00
Clone X NaCl	1053.075	12.0	87.756	5.783	0.00
error	606.983	40.0	15.175		
total	25877.364	60.0			

Appendix Table 115 Statistical analysis of relative fresh weight of Stylo 184 shoots treated with NaCl for 1 week and then recovery after transfer to the medium without NaCl for another week.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	4621.2	4	1155.3	3.9	0.01
NaCl	26045.0	2	13022.5	44.4	0.00
Time	6320.9	1	6320.9	21.6	0.00
Clone x NaCl	4894.1	8	611.8	2.1	0.05
Clone x Time	1211.0	4	302.8	1.0	0.40
NaCl x Time	3405.4	2	1702.7	5.8	0.00
Clone x NaCl x Time	2841.5	8	355.2	1.2	0.3
error	26377.0	90	293.1		
total	918411.0	120			

Appendix Table 116 Statistical analysis of SOD of Stylo 184 shoots treated with NaCl for 1 week and then recovery after transfer to the medium without NaCl for another week.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	32796.5	4	8199.1	50.1	0.01
NaCl	6087.5	2	3043.7	18.6	0.00
Time	6388.0	1	6388.0	39.1	0.00
Clone x NaCl	3254.4	8	406.8	2.5	0.02
Clone x Time	8669.7	4	2167.4	13.3	0.00
NaCl x Time	190.2	2	95.1	0.6	0.56
Clone x NaCl x Time	1909.0	8	238.6	1.5	0.18
error	14714.7	90	163.5		
total	375246.0	120			

Appendix Table 117 Statistical analysis of CAT of Stylo 184 shoots treated with NaCl for 1 week and then recovery after transfer to the medium without NaCl for another week.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	494.3	4	123.6	55.4	0.01
NaCl	36.1	2	18.0	8.1	0.00
Time	4.2	1	4.2	1.9	0.17
Clone x NaCl	50.5	8	6.3	2.8	0.01
Clone x Time	206.1	4	51.5	23.1	0.00
NaCl x Time	7.1	2	3.6	1.6	0.21
Clone x NaCl x Time	27.3	8	3.4	1.5	0.16
error	200.7	90	2.2		
total	2675.7	120			

Appendix Table 118 Statistical analysis of POX of Stylo 184 shoots treated with NaCl for 1 week and then recovery after transfer to the medium without NaCl for another week.

Source	Sum of squares	df	Mean square	F	Sig.
Clone	553.4	4	138.3	40.8	0.00
NaCl	78.0	2	39.0	11.5	0.00
Time	95.1	1	95.1	28.1	0.00
Clone x NaCl	27.7	8	3.5	1.0	0.43
Clone x Time	716.9	4	179.2	52.9	0.00
NaCl x Time	1.4	2	0.7	0.2	0.81
Clone x NaCl x Time	19.3	8	2.4	0.7	0.68
error	304.9	90	3.4		
total	4750.2	120			

Appendix Table 119 Statistical analysis of relative fresh weight of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2201.8	2	1100.9	2.18	0.17
error	4537.7	9	504.2		
total	6739.5	11			

Appendix Table 120 Statistical analysis of relative fresh weight of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3693.6	2	1846.8	8.6	0.01
error	1924.0	9	213.8		
total	5617.6	11			

Appendix Table 121 Statistical analysis of relative fresh weight of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	8426.8	2	4213.4	51.02	0.00
error	743.3	9	82.6		
total	9170.2	11			

Appendix Table 122 Statistical analysis of relative fresh weight of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3495.3	2	1747.6	17.06	0.00
error	921.9	9	102.4		
total	4417.1	11			

Appendix Table 123 Statistical analysis of relative fresh weight of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	6933.0	2	3466.5	7.17	0.01
error	4353.1	9	483.7		
total	11286.1	11			

Appendix Table 124 Statistical analysis of SOD of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	40.6	2	20.3	1.33	0.31
error	137.4	9	15.3		
total	178.0	11			

Appendix Table 125 Statistical analysis of SOD of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	115.3	2	57.6	0.24	0.80
error	2198.8	9	244.3		
total	2314.1	11			

Appendix Table 126 Statistical analysis of SOD of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1007.4	2	503.7	1.26	0.33
error	3592.7	9	399.2		
total	4600.1	11			

Appendix Table 127 Statistical analysis of SOD of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1721.2	2	860.6	4.91	0.04
error	1576.3	9	175.1		
total	3297.5	11			

Appendix Table 128 Statistical analysis of SOD of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2342.2	2	1171.1	4.63	0.04
error	2278.6	9	253.2		
total	4620.8	11			

Appendix Table 129 Statistical analysis of CAT of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3.33	2	1.7	1.92	0.20
error	7.79	9	0.9		
total	11.12	11			

Appendix Table 130 Statistical analysis of CAT of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.4	2	0.7	1.75	0.23
error	3.7	9	0.4		
total	5.2	11			

Appendix Table 131 Statistical analysis of CAT of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.8	2	0.4	0.20	0.82
error	17.4	9	1.9		
total	18.2	11			

Appendix Table 132 Statistical analysis of CAT of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.16	2	0.08	1.15	0.36
error	0.63	9	0.07		
total	0.79	11			

Appendix Table 133 Statistical analysis of CAT of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	99.6	2	49.8	3.58	0.07
error	125.3	9	13.9		
total	224.9	11			

Appendix Table 134 Statistical analysis of POX of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	17.18	2	8.59	1.58	0.26
error	49.05	9	5.45		
total	66.23	11			

Appendix Table 135 Statistical analysis of POX of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	14.2	2	7.1	28.59	0.00
error	2.2	9	0.2		
total	16.5	11			

Appendix Table 136 Statistical analysis of POX of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	24.8	2	12.4	5.95	0.02
error	18.8	9	2.1		
total	43.6	11			

Appendix Table 137 Statistical analysis of POX of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2.21	2	1.10	2.79	1.11
error	3.56	9	0.40		
total	5.76	11			

Appendix Table 138 Statistical analysis of POX of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after treatment with 0, 0.5 and 1% NaCl for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	11.14	2	5.57	16.77	0.00
error	2.99	9	0.33		
total	14.13	11			

Appendix Table 139 Statistical analysis of relative fresh weight of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5464.1	2	2732.1	8.44	0.01
error	2911.9	9	232.5		
total	8376.0	11			

Appendix Table 140 Statistical analysis of relative fresh weight of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2137.1	2	1068.5	2.26	0.16
error	4260.9	9	473.4		
total	6398.0	11			

Appendix Table 141 Statistical analysis of relative fresh weight of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1741.1	2	870.6	3.32	0.08
error	2358.1	9	260.0		
total	4099.2	11			

Appendix Table 142 Statistical analysis of relative fresh weight of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2773.8	2	1386.9	4.24	0.05
error	2941.4	9	326.8		
total	5715.1	11			

Appendix Table 143 Statistical analysis of relative fresh weight of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	326.3	2	163.2	1.03	0.40
error	1425.1	9	158.3		
total	1751.5	11			

Appendix Table 144 Statistical analysis of SOD of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	41.3	2	20.7	0.68	0.53
error	272.6	9	30.3		
total	314.0	11			

Appendix Table 145 Statistical analysis of SOD of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	915.6	2	457.8	4.85	0.04
error	849.0	9	94.3		
total	1764.6	11			

Appendix Table 146 Statistical analysis of SOD of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3087.2	2	1543.6	4.72	0.04
error	2946.4	9	327.4		
total	6033.6	11			

Appendix Table 147 Statistical analysis of SOD of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	348.4	2	174.2	10.31	0.01
error	152.1	9	16.9		
total	500.5	11			

Appendix Table 148 Statistical analysis of SOD of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1822.1	2	911.0	11.55	0.00
error	710.2	9	78.9		
total	2532.3	11			

Appendix Table 149 Statistical analysis of CAT of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.02	2	0.51	1.53	0.27
error	3.00	9	0.33		
total	4.02	11			

Appendix Table 150 Statistical analysis of CAT of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	7.6	2	3.8	3.14	0.09
error	10.9	9	1.2		
total	18.5	11			

Appendix Table 151 Statistical analysis of CAT of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	0.97	2	0.48	3.34	0.08
error	1.31	9	0.15		
total	2.27	11			

Appendix Table 152 Statistical analysis of CAT of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.14	2	0.57	0.42	0.67
error	12.18	9	1.35		
total	13.32	11			

Appendix Table 153 Statistical analysis of CAT of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	5.04	2	2.52	1.23	0.34
error	18.45	9	2.05		
total	23.50	11			

Appendix Table 154 Statistical analysis of POX of T1 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	20.0	2	10.0	1.15	0.36
error	78.2	9	86.7		
total	98.2	11			

Appendix Table 155 Statistical analysis of POX of T2 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	1.33	2	0.66	7.79	0.01
error	0.77	9	0.09		
total	2.10	11			

Appendix Table 156 Statistical analysis of POX of T3 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	2.78	2	1.39	1.56	0.26
error	8.02	9	0.89		
total	10.80	11			

Appendix Table 157 Statistical analysis of POX of T4 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	3.87	2	1.93	0.72	0.51
error	24.05	9	2.67		
total	27.92	11			

Appendix Table 158 Statistical analysis of POX of T5 shoots on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA after recovery on medium without salt for 1 week.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	28.9	2	14.5	1.11	0.37
error	117.3	9	13.0		
total	146.2	11			

Appendix Table 159 Statistical analysis of shoot number of 5 selected clones after maintaining for 3 years on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA for 8 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	323.6	4.0	80.9	3.02	0.02
error	3889.4	25.0	26.8		
total	4212.9	29.0			

Appendix Table 160 Statistical analysis of percent shoot regeneration of 5 selected clones after maintaining for 3 years on MS medium with 0.01 mgL^{-1} NAA and 1 mgL^{-1} BA for 8 weeks.

Source	Sum of squares	df	Mean square	F	Sig.
treatment	6586.7	4.0	1646.7	4.57	0.01
error	9000.0	25.0	360.0		
total	15586.7	29.0			

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