SELECTION FOR SALT TOLERANCE IN *PETUNIA GRANDIFLORA*

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In

Biosciences

By

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CERTIFICATE

I declare that the dissertation entitled “SELECTION FOR SALT TOLERANCE IN *PETUNIA GRANDIFLORA”* has been prepared by me under the guidance of Prof. R. G. Saini, invited Professor and coordinator, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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I certify that Mr. RAOOF AHMAD NAJAR has prepared his dissertation entitled "SELECTION FOR SALT TOLERANCE IN *PETUNIA GRANDIFLORA*", for the award of M.Phil. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Bioscience, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

SELECTION FOR SALT TOLERANCE IN *PETUNIA GRANDIFLORA*

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Petunia grandiflora a native of South America is a popular and high value ornamental plant in the world. Soil salinity is the major abiotic stress in semi-arid Malwa region of Punjab, adversely affecting its productivity, survival and quality. In order to develop salt resistant varieties of *P. grandiflora*, an attempt was made to select salt tolerant seedlings from varieties Violet Blue, Giant California and Nana compecta by using *ex-vitro* and *in-vitro* methods. Seedlings were subjected to salt treatments of 100 mM, 200 mM, 300 mM, 400 mM, 500 mM and 600 mM for durations of 4 hr, 6 hr, 12 hr and 24 hours. Salt treatment reduced shoot length, leaf number and survival percentages and delayed days to 50% flowering. Variety Nana Compecta was identified as tolerant to salt and variety Giant California was most sensitive to soil salinity. In *in-vitro* method of gradual increase in NaCl concentration (0, 25, 50, 75 and 150 mM) from low to high level was found to be a better approach for selecting salt tolerant calli as compared to direct method in which direct transfer of calli to high salt concentrations (50, 100, 150, 200, 250 and 300 mM) was found to be detrimental to callus survival and growth. Treatment of calli upto 50 mM salt concentrations was good for shoot regeneration in all the three varieties. Protocol was standardized for callus induction, direct shoot induction from leaves, shoot regeneration from callus and root induction from shoots. For callus induction, 2 mg/l 2, 4-D and 0.5 mg/l kinetin was optimum for variety Violet Blue and 1 mg/l both BAP and NAA was optimum for varieties Giant California and Nana Compecta. For direct shoot induction from leaves 2 mg/l BAP and 0.1 mg/l NAA were good for all the three varieties. For shoot regeneration from calli, 2 mg/l BAP in combination with 0.1 IAA for variety Violet Blue and 1 mg/l both BAP and NAA for varieties Giant California and Nana Compecta were ideal. For root induction, 0.5 mg/l NAA and 0.1 mg/l IBA were ideal for all the three varieties. Seeds from plants grown *ex-vitro* showing tolerance to salt were harvested and stored for further testing.

(Raoof Ahmad Najar) (R. G. Saini)

Signature of student Signature of supervisor

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Raoof Ahmad Najar

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviation
1	Ascorbate Peroxidase	APX
2	Bicarbonate	HCO_3
3	Benzyl Adenine	BA
4	Benzyl Amino Purine	BAP
5	Brilliant Blue G	BBG
6	Calcium ion	Ca^+
7	Chloride ion	Cl^-
8	Chloride Channels	CLC
9	Catalase	CAT
10	Carbon dioxide	CO_2
11	Calcinurin B Like	CBL
12	Centimeter	cm
13	Deoxyribose Nucleic Acid	DNA
14	Diammonium Phosphate	DAP
15	Deci Siemens	ds
16	Degree Celsius	^oC
17	2,4-Dichlorophenoxy Acetic Acid	2,4-D
18	Electric conductivity	EC
19	Farm Yard Manure	FYM
20	Glycinebetaine	GB
21	Gama Amino Butyrate	GABA
22	Glutathione Reductase	GR
23	Glutathione Disulfide (oxidized form of GSH)	GSSG
24	Glutathione	GSH
25	Hydro Chloric Acid	HCl
26	Hydrogen ion	H^+
27	Histidine Kinase Transporter	HKT
28	Hydroxyl Radical	HO^-
29	Hydrogen Peroxide	H_2O_2

30	Hectare	Ha
31	High Voltage Activated	HVA
32	Vaculor Protein Pyrophosphatase	HVP
33	Hour	Hr
34	Indole Acetic Acid	IAA
35	Indole Butyric Acid	IBA
36	Kilogram	Kg
37	Litre	L
38	Load Bearing System	lbs
39	Magnesium ion	Mg^{+}
40	Meter	m
41	Milli Molar	mM
42	Micro Molar	µM
43	Milli gram	mg
44	Mercuric Chloride	$HgCl_2$
45	Murashige and Skoog	MS
46	Milli litre	ml
47	Milli meter	mm
48	Mitogen Activated Protein	MAP
49	Napthalene Acetic Acid	NAA
50	Normal	N
51	Oxygen	O_2
52	Potassium Ion	K^{+}
53	Phospholipase D	PLD
54	Private Limited	Pvt Ltd.
55	Plant Growth Regulator	PGR
56	Power of Hydrogen	pH
57	Phosphatidic Acid	PA
58	Reactive Oxygen Species	ROS
59	Ribose Nucleic Acid	RNA
60	Reverse Osmosis	RO
61	Standard Error of Mean	SEM
62	Sodium ion	Na^{+}

63	Sulphate ion	SO_4^{-2}
64	Superoxide Radical	O_2^-
65	Na^{+}/H^{+} antiporters	NHXs
66	Superoxide Dismutase	SOD
67	Salt Overly Sensitive	SOS
68	Salt Overly Sensitive Calcium Binding Protein 8	SCaBP8
69	Weight/volume	w/v

CHAPTER I

INTRODUCTION

The term “ornamental plants” is generally used to describe the plant species which are primarily cultivated for aesthetically pleasant characteristics. The ornamental plants are grown worldwide for their aesthetic value. Over the last few decades, the monetary value of ornamentals has increased manifold and the market for ornamentals has steadily increased in a developing country like India (Hossain *et al.*, 2007). The flowers from Indian florists are in great demand in traditional markets, such as Europe, Australia, West Asia and Japan. But the Indian produce accounts for less than one per cent of the global floriculture trade, which is dominated by Kenya, Ethiopia, Ecuador and Colombia. However, regions around Bangalore and Pune are the major production centre’s in India for cut flowers like roses, carnations etc. (Kulkarni, 2013). The use of flowers for various purposes is gaining momentum and especially in India where every auspicious occasion starts with exchange of flowers. Therefore, now days it seems to be the most wanted item in any social occasion for conveying one’s status and aesthetic sense. The genus *Petunia* which is not native to India has 14 species occurring exclusively in South America. Thirteen of these species are found in Southern and South eastern Brazil (Stehmann *et al.*, 2009).

Petunia plants belonging to family Solanaceae are most popular bedding plants of the world due to their versatility, variety and range of color. *Petunia grandiflora* is a fast growing, non-edible plant with beautiful flowers, available in large amount during several months of year, and has no environmental hazard (Watharkar *et al.*, 2013). *Petunia* species are usually annual and are herbs with non woody stems. *Petunia* produce a nonstop parade of lightly fragrant, funnel shaped flowers which scent the air with lovely fragrance. They are excellent choices for window boxes or hanging baskets and when massed in borders they are showy beyond compare. *Petunia* got its name from French, which took the word ‘Petun’ meaning ‘tobacco’ from Tupi-Guarni language. It is considered as genetically interesting plant of applied value as it is also a model plant particularly, for studies in gene regulation and genome structure (Ganga *et al.*, 2011). It has anti-microbial activity and it has mild acting medicinal value also (Rahman *et al.*, 2008) in addition to its mild anti oxidation activity. Its leaves are widely used as natural insecticide (Kays *et al.*, 1994). Recently it has also been reported that *P. grandiflora* has phytoremediation potential because its wild as well as tissue cultured plants show decolorization of Brilliant Blue

G (BBG), a textile effluent (Watharkar *et al.*, 2013). *Petunia* has 4 months life cycle from seed to seed. It bears large number of flowers which make biochemical sampling easier, transformation is easy and regeneration from leaf disc protoplast is also easy.

The wild type of flowers of *Petunia* are typically purple having four concentric whorls; calyx with five sepals, corolla gamopetalous, epipetalous five stamens and two carpels. When pollination occurs in these flowers, fruit can be harvested after about four weeks (Colombo *et al.*, 1997). The seeds are very small (less than 1.4 mm), foveolated with variable seed coat morphology (Stehmann *et al.*, 2009). The amenability of *Petunia* to experimentation has allowed researchers to uncover the molecular, biochemical and physiological basis of several plant processes, both at cellular and whole plant level as it becomes easy to examine genetically controlled changes in its morphological characteristics. At present 20-25 academic groups use *Petunia* for research purpose while a number of smaller and larger companies are developing further new varieties (Ganga *et al.*, 2011). However, no systematic effort has been made for its cultivation in salt affected soil to meet the increasing demand of floriculture industry in salt affected regions like Bathinda. Several grain, crops, cotton and vegetables are grown in Bathinda District of Malwa region of Punjab but ornamentals being very sensitive to both biotic and abiotic stresses are difficult to grow. Bathinda district has arid brown and siezoram soils. The arid brown soil is calcareous in nature and salinity and alkalinity is the principal problem of this soil. Siezoram soils contain accumulated calcium carbonate which is the main problem of this soil. Out of the total 2,110 hectares under ornamentals in Punjab, Patiala district has got the maximum of 315 hectares area under floriculture and produces 1900 metric ton flower seeds per year. District Bathinda has only 17 hectares under flower cultivation and it produces 125 metric ton seed every year (Mann, 2014). In Bathinda district the availability of fresh water is reduced due to rapid increase in salinity and the pH value of which ranges from 7.42 to 9.0. Under these conditions growing of ornamentals is difficult in this region.

Plants have evolved complex multiple mechanisms to overcome stress due to high concentration of salt in soil and water, the effect of which include, formation of compatible solutes like proline, ROS detoxification, Na^+ ion compartmentalization and

selective ion uptake and exclusion (Yang *et al.*, 2013), suggesting involvement of several genes controlling resistance to salt stress. Simple selection under salt stress conditions will enable identification of plants having tolerance/resistance to salt. The selection inclusion will further increase if the selection is treated at cellular level.

Development of salt tolerant plants through *in-vitro* culture has been reported in many crop plants, legumes and ornamentals (Rai *et al.*, 2011) and it is gaining momentum as experiments under *in-vitro* conditions need less space and allow better control over environment and nutritional conditions which is difficult through traditional experiments. Therefore, the present investigations have been planned with following objective:

Isolation of stable salt tolerant plants of *Petunia grandiflora* using

(I) Selection through exposure of seedlings to different concentrations of salt

(II) Regeneration of salt tolerant seedlings from salt treated callus tissue.

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CHAPTER II

REVIEW OF LITERATURE

Salt stress may be defined as an excess of ions of soluble salts such as calcium (Ca^{+}), Chloride (Cl^{-}), Magnesium (Mg^{+2}), sodium (Na^{+}), Sulphate (SO_4^{-2}) and bicarbonate (HCO_3^{-}), in soil, water or in the culture medium that may have deleterious effect on plant growth. Salinity is the saltiness or dissolved salt content of a body of water. Soil salinity is the salt content in the soil; the process of increasing the salt content is known as salinization. Salinity has adverse affects on plant vigour, germination and yield. Soil salinity affects plants in many ways like ion toxicity, nutritional disorders, water stress, disorganization of membranes, oxidative stress, and reduced cell division and expansion. Over all, salinity reduces plant growth and survival (Carillo *et al.*, 2011). Salinity hampers the soil structure especially when the Na^{+} ions cause soil aggregation by occupying the cation complex of clay particles. Hence the soil becomes compact with reduced porosity and aeration (Carillo *et al.*, 2011).

The salinity of soil is mostly due to NaCl accumulation and it is a threat to crop yields particularly, in arid and semi arid areas. Soil salinity is one of the abiotic stresses which is responsible for worldwide loss of agricultural productivity and is considered as the major threat to plant production. The main limiting factor to the extension of agriculture is the quality of irrigation water available in arid and semi arid region especially when it is salty (Munns, 2002). It has been estimated that by 2050, half of the irrigated land will be lost due to salinity and among stresses, salinity is thought to be most important (Tuteja, 2007). Irrigation malpractices, intensive industrialization, extensive use of chemicals in farming leads to secondary soil salinity (Munns and Tester, 2008). Salinity has affected 20% of the global area and 2% of the world's rainfed areas which account for over 800 million ha worldwide. With the changing climatic conditions and poor practices of cultivation, it is expected that this area will enlarge extensively with a rate of 10% annually in future (Bennett and Khush, 2003).

To overcome the effect of salt stress in plants, main focus of plant biology research is in studying the mechanism of salt tolerance in plants. It has been noticed that plants have evolved complex mechanism to overcome this effect which include, formation of compatible solutes e.g. proline, ROS detoxification, Na^{+} ion compartmentalization, selective ion uptake and ion exclusion (Yang *et al.*, 2013).

When we compare water potential of normal soil and saline soil, water potential of root cells in normal soil is lower than the surrounding environment and hence influx of water occurs. As compared to saline soil water potential is inverted and therefore, water uptake is reduced which leads to growth inhibition and tissue damage (Hauser and Horie, 2010). Actually salt induced signal transduction, activation of stress related genes, alter metabolism and protein expression which results in alteration of metabolic pathways like mitogen activated and calcium dependent protein kinase and histidine kinases signaling (Zhang *et al.*, 2011).

Salt tolerance is a complex genetic trait regulated by multiple genes. For salt tolerance plant cell needs ion compartmentalization, synthesis of compatible solutes, resisting the leakage across apoplast and tolerance to low potassium and sodium ratio within the cell. Plants have been classified as salt sensitive (glycophytes) and salt tolerant (halophytes) based on their ability to grow on salinity. Various strategies have been adapted by the glycophytes to respond to salinity. One of the strategy is that plants reduce their growth to save energy for defence purpose and also to limit the risk of heritable damage (May *et al.*, 1998). By gradual adaptation to higher level of Nacl, cell cultures derived from many glycophytes were made salt tolerant in tobacco. Cell cultures were inoculated on media containing salt near to seawater level over tens of thousands generations to achieve this (Ali *et al.*, 1999).

The common metabolic response towards salinity is the accumulation of various compatible solutes like proline, glycinebetaine (GB), Gama amino butyrate (GABA), glucose and myoinositol. These maintain osmotic balance and also protect the cells from death (Ashraf and Foolad, 2007). Osmoprotectant genes coding biosnthesis of GB proline and manitol has been introduced in *Nicotiana tobaccum* as it is most common model in developing salt tolerant plants. Among osmolytes proline is considered as common metabolite in response to higher water deficit and salinity stress that protects the plant cell membrane, proteins and scavenges ROS (Zhang *et al.*, 2011). Exogenous application of compatible osmolytes like proline, glycinebetaine has gained tremendous importance in plants for salt tolerance which can protect the cellular damage occurring due to salt stress. Also it has been observed that exogenous application of proline regulates osmotic potential under osmotic stress (Ashraf and Foolad, 2007). Osmoprotectants like proline, glycine betaine etc. help

maintaining the membrane structure and also helps in scavenging free radicals to prevent the lipid peroxidation. It has been observed that salt stress increase production of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot -}$) (Apel and Hirt, 2004). The plant processes such as photosynthesis, respiration and photorespiration also produce reactive oxygen species which are partially reduced form of atmospheric oxygen (Mittler *et al.*, 2004). Water is produced in these processes by five electrons which are required for perfect reduction of oxygen and ROS typically resulting from transference of one, two and three electrons respectively, to O_2 to form superoxide ($O_{2.}^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-) (Mittler *et al.*, 2004). These react with lipids, protein's, nucleic acid causing lipid peroxidation, protein denaturing and mutations in DNA (Quiles and Lopez, 2004). Retaining of water content by plant helps in salt tolerance by mitigating an excessive ion concentration by dilution effect (Romero-Aranda *et al.*, 2006). Generally salt in soil cause imbalance in ionic equilibrium, nutritional uptake and osmotic potential (Munns, 2002). Imbalance in osmotic potential results in loss of turgidity, cell degradation and finally cell death (Cicek and Cakırlar, 2008). Due to salt stress germination of seeds is also inhibited because of imbalance in water uptake limiting the hydrolysis of food reserves and its immobilization from storage tissue to developing embryo axis (de Lacerda *et al.*, 2003). The toxic ion accumulation due to salinity retards the seed germination (Okcu *et al.*, 2005).

2.1. Selection for salt tolerance through *In-vitro* culture

Totipotency of plant cell is exploited during *in-vitro* culture which is important tool in plant biotechnology. By *in-vitro* culture many commercial varieties of ornamental plants are being propagated in culture medium. By *in-vitro* culturing of cells, desired salt tolerant cells are selected and used for recovery of whole plants tolerant to salt. Plant tissue culture technique is generally considered as an important technique to select tolerant clones from non tolerant clones (Gandonou *et al.*, 2006). In this technique a population of cells is subjected to suitable salt selection pressure and the cell lines showing resistance or tolerance are recovered to generate whole plants from such lines. There are several reports available on NaCl tolerant plants produced through *in-vitro* technique using callus (Kumar and Sharma, 1989; Patnaik and Debata, 1997; Tal, 1994; Vajrabhaya *et al.*, 1989). However, the loss of regeneration potential or genetic instability is the only hurdle to develop NaCl tolerant plants. For studying the physiological effects of salt on plants at cellular level, plant tissue culture is the best tool Olmos *et al.*, (1994) especially for species having long reproduction cycles. In this technique, propagation of shoots is done and the plants selected from the salt stressed culture are grown on saline soils (Shatnawi *et al.*, 2010). It has been reported that in *Nicotiana tabaccum* over expression of superoxide dismutase in chloroplast has enhanced tolerance to salt stress (Badawi *et al.*, 2004). For the recovery of stable plants with improved salt tolerance, callus line selection procedure plays important role (Winicov, 1996). There are several reports available on selection of salt tolerant cell lines in various crops during recent years. Salt tolerant line have been reported in potato (Sabbah and Tal, 1990), in *Hordeum vulgare* (Sibi and Fakiri, 2000), and wheat (Barakat and Abdel-Latif, 1996). Actually certain plants contain gene complexes which help them adapt to salinity by developing some physiological changes which make them resistant to salt stress.

2.2. Selection of salt tolerant cell line

Callus is proliferative mass of predominantly thin walled parenchyma cells which is in unorganized form (Bhojwani and Razdan, 1986). Callus has important role in plant tissue culture as it is used to study physiological phenomenon including tolerance to various environmental stresses. To get the best callus culture, selection

of parent material explant, and choice of culture media are the prerequisite for its successful establishment (Yadav and Tyagi., 2006). While physiological and structural variations are revealed during improvement for salinity tolerance under field conditions (Hawkins and Lips, 1997), now a day's plant tissue culture techniques are successfully utilized for isolation of salt tolerant cell lines and to elucidate the mechanism of salt tolerance at cellular level (Elkahoui *et al.*, 2005).

The first salt tolerant line was isolated from *Capsicum annum* L. by Dix and Street, (1975). Since then much attention is paid to get salt resistant plants and the result of which is that till now, number of reports are available on selection of salt tolerant cell lines in different economically important plants. Several cell lines have also been isolated e.g. Tobacco (Nabors *et al.*, 1980), *Cymbopogan maritini* (Patnaik and Debata, 1997), and sugarcane (Gandonou *et al.*, 2006). Most of these plants belong to family Solanaceae, Poaceae and Fabaceae. Selection of salt tolerant cell line involves two strategies i.e direct and indirect. In direct treatment the callus is treated with different salt concentrations directly while in the indirect method, gradual treatment is given to callus. Several researchers suggest that direct selection is a better approach it resembles the field conditions (Aghaei *et al.*, 2008; Sabbah and Tal, 1990). Stepwise selection is also preferred by several researchers (Ochatt *et al.*, 1998; Queiros *et al.*, 2007). They suggest that the stepwise selection of callus allows physiological and biochemical adjustments, which are the basis for a new cellular homeostasis compatible with the imposed stress (Leone *et al.*, 1994; Patnaik and Debata, 1997; Queiros *et al.*, 2007). As the selected cell lines consist of mixture of adapted cells, they loose tolerance when shifted to salt free medium and only true genetic variants retain their tolerance (Hassan and Wilkins, 1988). To get true salt tolerant genetic variants the only way seems to regenerate salt tolerant plants followed by testing the inheritance at whole plant level. Plants from salt tolerant cell lines have been regenerated on NaCl containing medium (Beloualy and Bouharmont, 1992; Reddy and Vaidyanath, 1986). However regeneration was extremely difficult in most cases. To overcome this difficulty It is suggested that plant regeneration from selected salt tolerant cell lines should be done in salt free medium (Ben-Hayyim and Goffer, 1989; Heszky *et al.*, 1992).

It has been observed experimentally that in *Chrysanthemum morifolium*, increase in salinity reduces shoot proliferation, number of leaves, carotenoids and chlorophyll content while proline and sodium content increases (Shatnawi *et al.*, 2010). Salt concentration effects plant height and it has been observed that with increase in salt concentration there is a significant decline in growth (Zhani *et al.*, 2013). In rice crop, it has been experimentally proved that increase in concentration of salt causes reduction in root and shoot growth and also reduces chlorophyll content. Seed germination was also reduced and the cause of inhibition in germination of seeds occurred due to imbalance in water uptake which limits the hydrolysis of food reserve and immobilizes their translocation from storage tissue and developing embryo axis (de Lacerda *et al.*, 2003). Elevation in salinity levels leads to decrease in osmotic potential which results in accumulation of toxic ions which may retard seed germination (Yagmur and Kaydan, 2008). Actually when Na^+ and Cl^- sequesters in vacuole, internal osmotic potential is decreased resulting in dehydration in cytoplasm (Alloing *et al.*, 2006). Such dehydration impairs cellular metabolism and hence cell death (Deivanai *et al.*, 2011). In wheat it has been found that increase in saline concentration Na^+ content elevates and K^+ decreases (Akbarimoghaddam *et al.*, 2011).

2.3. Response of plants to salinity

Two types of stresses occur in plants when salinity is elevated above a threshold level of 40 mM NaCl in soil water. These stresses are referred as osmotic and ionic stresses which drastically reduce crop yield (Munns and Tester, 2008). Osmotic stress effects occur immediately after exposure that brings about stomatal closure and affect cell growth and metabolism, affect shoot growth rate, shoot dry matter, and total leaf area. Ionic stresses do not show immediate effects on exposure to elevated salt, but their effects are after 2-4 weeks (Bhojwani, 2009). Most commonly salt stress is caused by elevated level of Na^+ and Cl^- in solution. High salinity results in increase in hyper ionic and hyper osmotic effect. An immediate effect of salinity is stomatal closure. Plants regulate ion movement to protect the activity growing and metabolizing cells (Munns, 1993). One of the strategy plants have utilized to control salt flux to shoot is the entry of ions into the xylem. It has been observed that under salt stress, mature and old leaves accumulate large ions which

dehisce (Munns, 1993). As Na^+ competes for K^+ binding sites which are essential for cellular processes, its toxicity is considered as severe. Various enzymatic processes are disordered in cytoplasm due to high Na^+/K^+ ratios (Tester and Davenport, 2003). Chlorophyll content is reduced and photosynthesis is inhibited due to ionic stress, inducing leaf senescence and premature leaf death. Due to this photosynthetic capacity, biomass and yield is reduced (Tester and Davenport, 2003). Plant accumulates the Na^+ and Cl^- when their concentration is high in soil. The accumulation Na^+ is associated with reduced conductance of stomata and inhibition of photo system II and chlorophyll is affected directly due to accumulation of Cl in shoots (Tavakkoli *et al.*, 2011).

2.4. Effect of salt on growth

One of the initial effects of salt stress on plant is the reduction of growth rate. Due to salinity, suppression of growth occurs almost in all plants but their tolerance level varies widely among different plant species (Hasegawa *et al.*, 2000). The mechanism by which salinity affects growth of a plant depends on the time scale over which the plant is exposed to salt. The effects of salt stress on plants depend upon the environmental factors, genotype of plant and salt concentration as well as exposure to salt treatment. When duration of exposure to salt is in seconds or minutes, immediate reduction in leaf and root occurs which is sometimes partially recoverable. If this treatment is given in hours that result in Ca^{2+} deficiency, it shows marked effects on permanent reduction in root and leaf elongation. If stress is given upto few days increase in root: shoot ratio occurs and it also causes reduction in leaf emergence. If the duration is increased to weeks, reduced branches/tiller formation and death of mature leaves occurs. And if it is continued up to a month, immature death of plant occurs and flowering time is also altered (Alvarez, 2006). Due to elevation of salt concentration, leaf cells loose water, but this loss is transient and cells regain their volume and turgor within hours owing to osmotic adjustment, but cell elongation rates are reduced (Cramer, 2002; Gilliham and Tester, 2005). Overall it has been observed leaf size is decreased due to reduction in cell elongation and cell division. Also older leaves die but younger leaves continue to grow. It has been observed that depressive effect of salt was mainly shown by young leaves than in roots during initial stage of development (Thiam *et al.*, 2013). The reason is that salt

accumulation increases osmotic pressure, ionic toxicity and nutritional imbalance or transport of nutrients to the stem (Evelin *et al.*, 2009). But what factors down regulate leaf growth and shoot development under stress is not precisely known (Munns and Tester, 2008). The reduction in leaf development must be regulated by hormonal signals as reduced leaf growth rate is independent of carbohydrate supply and water status (Munns *et al.*, 2000). In comparison to shoot, root growth is usually less affected and recover after exposure to NaCl or other osmotic changes and it is also observed that roots recover fast from high NaCl concentration as high as 150 mM (Munns, 2002). When salt accumulates in cells, it cause their dehydration inhibiting various enzymes which are used in carbohydrate metabolism or photosynthetic processes (Munns and Tester, 2008).

2.5. Salt tolerance in halophytes

The halophytes posses special anatomical and morphological adaptations, or mechanisms of avoidance for high salt tolerance (Greenway and Munns, 1980). However, halophytes are rare among the 250,000 species of flowering plants (Flowers and Flowers, 2005). It is difficult to transfer the unique characteristics of halophytes to crop plants (Flowers, 2004). In previous studies, plants were categorized in to salt includers and salt excluders. In first category Na^+ is translocated to shoot and sequestered to vacuole. Salt excluders on the other hand adapt to saline stress by avoiding Na^+ uptake. In rice and wheat which are glycophytes, tolerance to salt is attained by keeping low Na^+ or high K^+/Na^+ ratio (Munns *et al.*, 2012).

2.6. Mechanism of salt tolerance at molecular level

The tolerance towards salt stress shown by plants at molecular level is basically characterized by regulation and activation of salt stress genes which are reported to be involved in signaling, transcriptional control, ion homeostasis, scavenging of toxic ions and free radicals and ultimately membrane and protein function (Wang *et al.*, 2003). However, the limited success of molecular approach in elucidating salt tolerance mechanism is primarily due to two factors. First, the approach is correlative and now it has been recognized that many salt responsive genes do not contribute in salt tolerance but their induction reflects salt damage. Second, molecular approach has identified these genes or gene products on the

basis of expression, but many genes which are important for salt tolerance may not be induced by salt stress (Ali *et al.*, 2012). When the plant is exposed to salt, the receptors present in the membrane (ion channels, histidine kinases) produce stress signal which inturn produces secondary signal molecules like ca^{2+}, ROS and abscisic acid. Such signal induces stress response genes, which helps in tolerance of plants towards stress (Tuteja, 2007). The three common mechanisms utilized by plants are osmotic adjustment, Na^+ exclusion from sensitive tissue and proper control of Na^+ uptake by roots, Na^+ compartmentation (Berman and Ahuja, 2008). These mechanisms are under adequate control of physiological, biochemical and signaling pathways (Zhu, 2001). In osmotic adjustment, synthesis of compatible solutes occurs in cytoplasm. These solutes scavenge ROS molecules (Hasegawa *et al.*, 2000). The compatible solutes act as low molecular weight chaperones by replacing water at the surface of proteins (Carillo et al., 2011). As high Na^+/K^+ ratio is toxic to plants, keeping the high K^+/Na^+ ratio is basic factor to cope with salt stress. In this regard several transporters and genes have been found which keep high K^+/Na^+ ratio (Rana Munns and Tester, 2008). Some of these include:

1. Na^+/H^+ antiporter of plasma membrane (Huang *et al.*, 2009). It is also called SOS1. During salt stress low Na^+ concentration in cytoplasm is maintained by three salt overly sensitive (SOS) proteins (SOS 1, SOS 2 and SOS 3) by regulatory role in the expression and activity of ion transporters.

2. Vacuolar Na^+/H^+ antiporters (NHXs) (Blumwald *et al.*, 2000; Hu and Schmidhalter, 2007) and energy suppliers of these NHXs (like H^+ pumps: HVA/68and HVP1) (Ligaba and Katsuhara, 2010). These antiporters maintain osmotic balance by sequestering Na^+ in vacuoles (Apse *et al.*, 1999). Similarly, Cl is likely transported into the vacuole by anion transporters such as CLC proteins (Bennett and Khush, 2003; Teakle and Tyerman, 2010).

3. High-and-low affinity K^+ transporters (HKT) (Cuin *et al.*, 2009; Shabala *et al.*, 2010). The HKT family consists of two classes which function either as specific Na^+ transporters or Na^+ and K^+ co-transporter (Hauser and Horie, 2010). HKT2;1 was shown to increase Na^+ uptake and higher Na^+ concentration in xylem sap (salt including behaviour) which is associated with increased salt tolerance (Mian *et*

al., 2011). Various scientists recommended that Na^+ elimination from the shoot is related with salt tolerance and that genes from the HKT1 subfamily such as HKT1;4 and HKT1;5 are involved (Barakat, 2011; James *et al.*, 2011).

Salt tolerance controlling genes are influenced by many environmental factors, expressed differentially and in different tissues during the life time of plants. This complexity makes salt tolerance difficult to breed. Breeding for salt tolerance is difficult as the trait is multigenic (Colmer *et al.*, 2006). Several molecular markers are now known which are reliable to select for salt tolerance (Collard and Mackill, 2008). Transgenic approaches are also being put forward to develop salt tolerant plants (Bajji *et al.*, 1998; Flowers, 2004).

2.7. Na^+ and Cl^- transport across the plasma membrane

In saline environment Na^+ and Cl^- transport across plasma membrane should be considered in two cellular contexts i.e after salt stress and after re-establishment of ionic homeostasis. Alteration of electrochemical gradient occurs immediately after salt stress. Inward movement of Na^+ dissipates the membrane potential facilitating the Cl^- down the chemical gradient (Czempinski *et al.*, 1999). Physiological data indicates that Na^+ competes with K^+ for intracellular influx because these cations are transported by common protein (Blumwald *et al.*, 2000). K^+ is an essential co-factor for many enzymes while as Na^+ is not. Plants have not evolved transport system that completely exclude Na^+ relative to K^+, this may be due to the fact that Na^+ is required as vacuolar osmolytes. Many K^+ transport system show some affinity for Na^+ (Blumwald et al., 2000). These include inward rectifying K^+ channels, Na^+-K^+ symporter, K^+ transporter (Fu and Luan, 1998), voltage dependant non selective outward rectifying cation channels that mediate Na^+ influx upon plasma membrane depolarization (Blumwald *et al.*, 2000) and voltage independent cation channels (Amtmann and Sanders, 1998).

2.8. Role of proline in enhancing salt tolerance

One of the most studied compatible solute which protect the plants from osmotic stress is proline (Ashraf and Foolad, 2007). It accumulates in larger amounts than any other amounts and osmotic potential of the cell is regulated by it. It is

generally observed that proline acts as a compatible solute in osmotic adjustment (Alfocea *et al.*, 1993; Larher *et al.*, 1993). One of the fast and efficient technique for evaluating salt tolerance in plants is to determine the rate of accumulation of proline (Thiam *et al.*, 2013). Proline protects plants by detoxifying radicals, stabilizing membrane structure and act as enzyme protectant (Hare and Cress, 1997). Proline accumulates in plants under stress, maintains osmotic balance, scavenges reactive oxygen species (RO) and stabilizes sub cellular structures. However, some authors have shown that exogenous application of proline cause damage to plants (Ashraf and Foolad, 2007). In cucumber, it has been found that salt tolerance is enhanced by exogenous foliar application of proline not endogenous proline accumulation (Huang Y *et al.*, 2009). Malonaldihyde is indicator of lipid peroxidation and show greater accumulation under salt stress. It has been found that exogenous proline reduces the amount of malonaldihyde in salt stressed plant (Huang Y *et al.*, 2009). In response to salt stress proline is produced to balance the water potential of the cytosol with the apoplast and vacuolar lumen (Szabados and Savoure, 2010). Under salinity stress proline helps in scavenging of free radicals in plants including wheat (Kahrizi *et al.*, 2012) and sorghum (de Lacerda *et al.*, 2005) and is storage of carbon and nitrogen.

2.9. Role of antioxidant enzymes in salt tolerance

Several cytotoxic reactive oxygen species (ROS) are continuously generated in cytoplasm, peroxisomes and mitochondria due to oxidative stress induced by salinity which cause damage to lipids, proteins and nucleic acids (Turkan and Demiral, 2009). Plants produce antioxidant enzymes such as Super Oxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Glutathene Reductase (GR). SOD increases with increase in salt stress but this increase varies according to genetic makeup. SOD, APX, Guiacol Peroxidase, Glutathene Reductase and Catalase are produced by plants to scavenge ROS (Ediga *et al.*, 2013). SOD acts as a first line of defense in plants to convert O_2^- to H_2O_2. Under oxidative stress GR regulates the reduction potential of cells. It protect the cells maintaining high reduced glutathione to oxidized glutathione GSH/GSSG ratio (Yannarelli *et al.*, 2007). SOD is the major enzyme present in aerobic organisms which dismutate the superoxide radical to hydrogen peroxide (H_2O_2) and oxygen (Zare and Pakniyat, 2012). Catalase is another anti oxidant enzyme which convert H_2O_2 to water. By doing this these

enzymes increase the stability of membranes and carbon dioxide (CO_2) fixation because several enzymes of the Calvin cycle within chloroplast are extremely sensitive to H_2O_2. There are several reports that anti oxidant enzymes can be utilized as growth regulators for salt resistance (Gunes *et al.*, 2007). It has also been observed that with increase in salinity especially at 150 mM and 200 mM of NaCl, there is progressive increase in APX, SOD, CAT (Barakat, 2011). A strong relation between antioxidant enzymes and salt stress was observed by (Sreenivasulu *et al.*, 2000). According to them, under different sodium chloride (NaCl) concentration, modulation of antioxidant component in salt sensitive or salt tolerant cultivar of foxtail millet (*Setaria italica*) was observed. They found that SOD and APX activity decreased severely in sensitive cultivars while in salt tolerant cultivar, these enzymes showed enhanced activity. From this experiment they concluded that oxidative stress tolerance induced by salt is conferred with the enhancement of activity of these antioxidant enzymes. APX is considered an important peroxidase enzyme which catalyze the reduction of H_2O_2 to water using the reducing power of ascorbate (Noctor and Foyer, 1998).

2.10. SOS pathways as cellular signaling under salt stress

Salt overlay sensitive (SOS) pathway that comprises of SOS3, SOS2 and SOS1 has been proposed to mediate cellular signaling under salt stress to maintain ion homeostasis (Ji *et al.*, 2013). It has been recognized as a key mechanism for Na^+ exclusion and ion homeostasis control at the cellular level (Zhu, 2003). Nutritional Na^+ uptake under non saline conditions is regulated by HKT family. The HKT transporters are classified as class 1 and class 2 transporters. Class 1 transporters are those which show preference to Na^+ only and class 2 show preference to Na^+- K^+ symport (Ali *et al.*, 2012). When Na^+ concentration outside the cell become high, three possible mechanisms have long been considered to participate in protection against high cytosolic Na^+. These are as:

1. Resistance towards Na^+ uptake.
2. Compartmentalization of Na^+ into vacuole and
3. Efflux of Na^+ out of cell.

Among these three, Na^+ exclusion is considered as best to maintain low concentration of cytosolic Na^+ (Ji *et al.*, 2013). Over the years a number of evidences have been gathered that after perception of salt stress, Ca^+ in root cells activate the SOS signal transduction cascade to protect the cells from damage (Chinnusamy *et al.*, 2005; Guo *et al.*, 2004). Increase in calcium is sensed by calcium binding protein encoded by SOS3 which inturn activates SOS2 (Hrabak *et al.*, 2003). Recently SOS3 like calcium binding protein 8 (SCaBP8) also known as Calcinurin B like CBL10) found in shoot of Arabidopsis is alternate regulator of SOS2 where SOS3 is found mainly in roots (Quan *et al.*, 2007). It has been found that loss of function in SCaBP8 resulted in more prominent increase in salt sensitivity of shoot compared to SOS3 (Quan *et al.*, 2007). Due to interaction of SOS3-SOS2 or SCaBP8-SOS2, recruitment of SOS2 occurs at plasma membrane which leads to activation of SOS1, a Na^+/H^+ antiporter that helps in extrusion of excessive Na^+ from the cytosol (Quan *et al.*, 2007; Quintero *et al.*, 2011). It is experimentally proved that SOS1 is used to transport Na^+ out of cells under salt stress by using yeast mutant strains (Shi *et al.*, 2002). Interestingly it has been found that the natural salt tolerant halophyte *Thellungiella salsugenea* (also known as *Thellungiella halophyla*) shows the activity of SOS1. Therefore we can say that SOS3/SCaBP8-SOS2-SOS signaling is best regulatory mechanism in Na^+ exclusion and cellular ion homeostasis. It is also necessary to mention here that SOS3-SOS2 complex is not solely responsible for activation of SOS1 (Shabala *et al.*, 2010). Actually when salt stress increases, Phospholipase D α (PLD α1) activation results in accumulation of Phosphatidic acid (PA) which inturn activates Mitogen Activated Protein Kinase 6 (MPK6) which phosphorylate SOS1 (Yu *et al.*, 2010). In *T. salugenea* a reverse genetic approach was used for confirmation with SOS1 transcript cause Na^+ accumulation in pericycle of RNAi suppressed transgenic lines and elevates the Na^+/K^+ ratio by 12 fold in the cells of root xylem parenchyma (Oh *et al.*, 2009). According to (Oh *et al.*, 2010), SOS1 plays an important role in Na^+ compartmentalization also in the vacuole and regulating the Na^+ loading in to the xylem.

CHAPTER III

MATERIALS AND METHODS

3. Experimental material used

The seeds of three *Petunia grandiflora* varieties viz, Violet Blue, Giant California and Nana Compecta used in this study were obtained from MS Plantsman Seeds Pvt. Limited, Patiala. Variety Violet Blue has purple flowers, Nana compecta has red flowers with varying intensity and Giant California mostly has white, creamy and light pink (Plate 1A, 1B and 1C) respectively. The experimental work was completed in two parts. Experiments in Part I related to selection of salt resistant seedlings after exposing seedlings to different concentrations of NaCl for different durations. The experiments in Part II aimed at standardization of a protocol to obtain tissue cultured plants of *Petunia grandiflora* as well as the studies on selection for salt tolerance under *in-vitro* conditions. The experimental details of these two parts are given in the following sections.

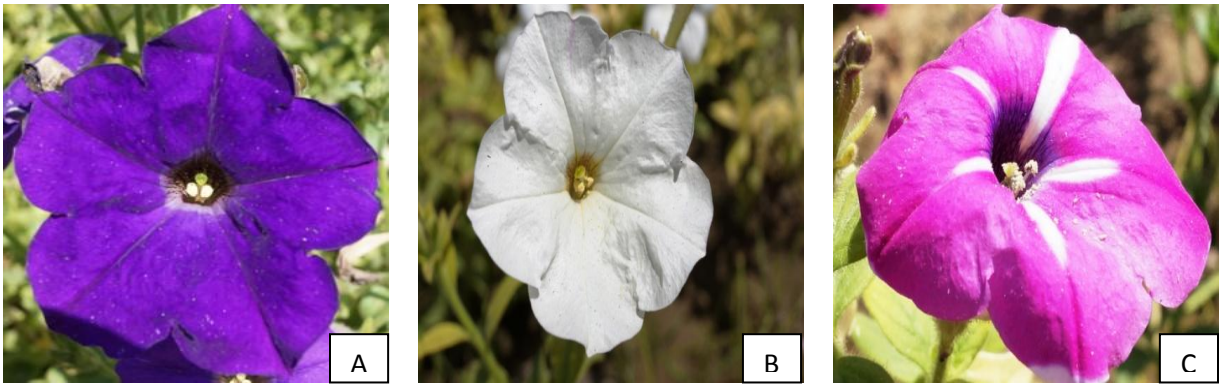


Plate 1. Flowers of three varieties Violet Blue (1A), Giant California (1B) and Nana Compecta (1C).

Part I

A. Selection of salt tolerant seedlings

3.1. Preparation of trays for seed sowing

Five hundred seeds were counted before sowing in plastic trays of size 14 cm X 22 cm. The trays were filled with sand and vermicompost in 1:1 ratio and irrigated. Muslin cloth was placed on wet sand-vermicompost mixture and the seeds were placed on muslin cloth to ensure that tiny seeds do not go deep in the sand which may decrease germination percentage. The muslin cloth used for placing the seeds was cut according to size of the tray and soaked in water for 2 hours before sowing. For sufficient germination, sand and vermicompost was mixed manually to make a fine homogenous mixture. During mixing materials like wood, polythene pieces, pebbles etc. were removed. One kg of sand and vermicompost mixture was put in each tray and 134 such trays were prepared to accommodate all treatments. After treatment period was over, a hole was cut in the bottom of each tray to drain out excess water.

3.2. Watering of seedlings

The seed germination commenced after three days of sowing that reached maximum 8 days after sowing. The trays were watered twice a day using (RO) water taking precaution that the trays are not waterlogged. Extra care was taken for irrigation for first 15 days when the seeds began to germinate as direct watering of seedlings would damage the tiny seedlings which acquired only 3 mm in size in 15 days. These seedlings were kept in plant tissue culture laboratory for one month at 25± 2^0C with 12 hours light and dark period. Later on, these seedlings were shifted to a growth house with temperature and humidity maintained at 25±2^0C and 50% respectively. The 45 day old seedlings of all the three varieties were given NaCl (Sodium chloride 100 mM, 200 mM, 300 mM, 400 mM, 500 mM and 600 mM) treatment in three replications as per details given in table 1. Untreated control was kept with all the three varieties and these trays were simply irrigated with RO water.

Table 1. Detail of the treatments given to seedlings of three varieties of *Petunia grandiflora*

Sr. No.	Variety	Treatment (mM)	Duration (hours)	No. of Replications
1.	**Violet Blue**	100	4, 6, 12 and 24	3
		200	4, 6, 12 and 24	3
		300	4, 6, 12 and 24	3
		400	4, 6, 12 and 24	3
		500	4, 6, 12 and 24	3
		600	4, 6, 12 and 24	3
		Control	4, 6, 12 and 24	3
2.	**Giant California**	100	4, 6, 12 and 24	3
		200	4, 6, 12 and 24	3
		300	4, 6, 12 and 24	3
		400	4, 6, 12 and 24	3
		500	4, 6, 12 and 24	3
		600	4, 6, 12 and 24	3
		Control	4, 6, 12 and 24	3
3.	**Nana Compecta**	100	4, 6, 12 and 24	3
		200	4, 6, 12 and 24	3
		300	4, 6, 12 and 24	3
		400	4, 6, 12 and 24	3
		500	4, 6, 12 and 24	3
		600	4, 6, 12 and 24	3
		Control	4, 6, 12 and 24	3

3.3. Transfer of seedlings to growth house and salt treatment

Before treatment, all the seedlings present in each replicate of three varieties of *P. grandiflora* were counted. Forty five day old seedlings of each of the three varieties were treated with different concentration of NaCl for four time durations as given in table 1. On the day of treatment different concentrations of salt solution were prepared by dissolving appropriate amount of NaCl in distilled water. Four hours after completion of treatment the salt solution was drained by making a hole in the bottom of each tray to make sure that no salt water remains inside the tray. To remove the salt from trays, the trays were drenched by passing distilled water three times through each tray.

Plate 2. Seedlings grown in growth house after treatment with salt

3.4. Shifting of treated seedlings to field

Twenty days after treatment, the seedlings were shifted to open experimental field plot. Before shifting, the field was prepared with the help of garden hoe to make sure that there are no hindrances in smooth growth of seedlings. This field plot was divided in plots of size 6 feet X 40 feet for growing the treated seedlings of three varieties. Fifteen gram (15g) of fertilizer Diammonium Phosphate (DAP) and 2 Kg of Farm Yard Manure (FYM) was applied in to each plot.

For shifting of seedlings to field plots these were uprooted gently by pouring water in trays without harming the seedlings. Survival of seedlings from each treatment and control was recorded for each variety. Growth parameters such as shoot length, and number of leaves per plant were counted for 30 randomly selected transplants 20 days of growth in the field. Plant to plant distance of six inches (15 cm) was maintained to allow good growth.

After one month of growth in the field 15 gram Diammonium Phosphate and 5 gram of urea was uniformly applied to each plot again. Weeds were removed from the field plots regularly. Healthy plants free from any disease were tagged and flowers were bagged to collect seeds for further testing. One month after fruit formation when fruits were almost ripe, the seeds were harvested and stored at 4^0C.

Part II

A. Selection for salt tolerance through *in-vitro* culture

3. A. Standardization of protocol to obtain tissue cultured plants

3.1.1. Raising of plants for tissue culture

Forty seeds of each of the three selected varieties, Violet Blue, Giant California and Nana Compecta were sown in plastic cups of size 7 cm X 8 cm filled with autoclaved sand kept in growth chamber maintained at 25± 2^0C with 12 hours light and dark periods. The cups were watered thrice a day using RO water. After 60 days of growth, the seedlings were shifted to earthen pots containing mixture of sand and vermicompost in equal proportion. The plants from each cup were carefully removed manually in a bucket full of water which made it to easier to separate intermingled seedlings. Only three plants of each variety were maintained in each pot and ten pots of each variety were kept as a source of material for healthy tissue to be used for tissue culture. Initially the plants were watered twice a day with RO water. When plants picked up growth, canal water was used for irrigation. The pH of canal water was 7.20 as an average of thirty days. The temperature of growth house was maintained at 25± 2^0C. Some plants showed virus infection and explants were taken from healthy plants for culturing experiments.

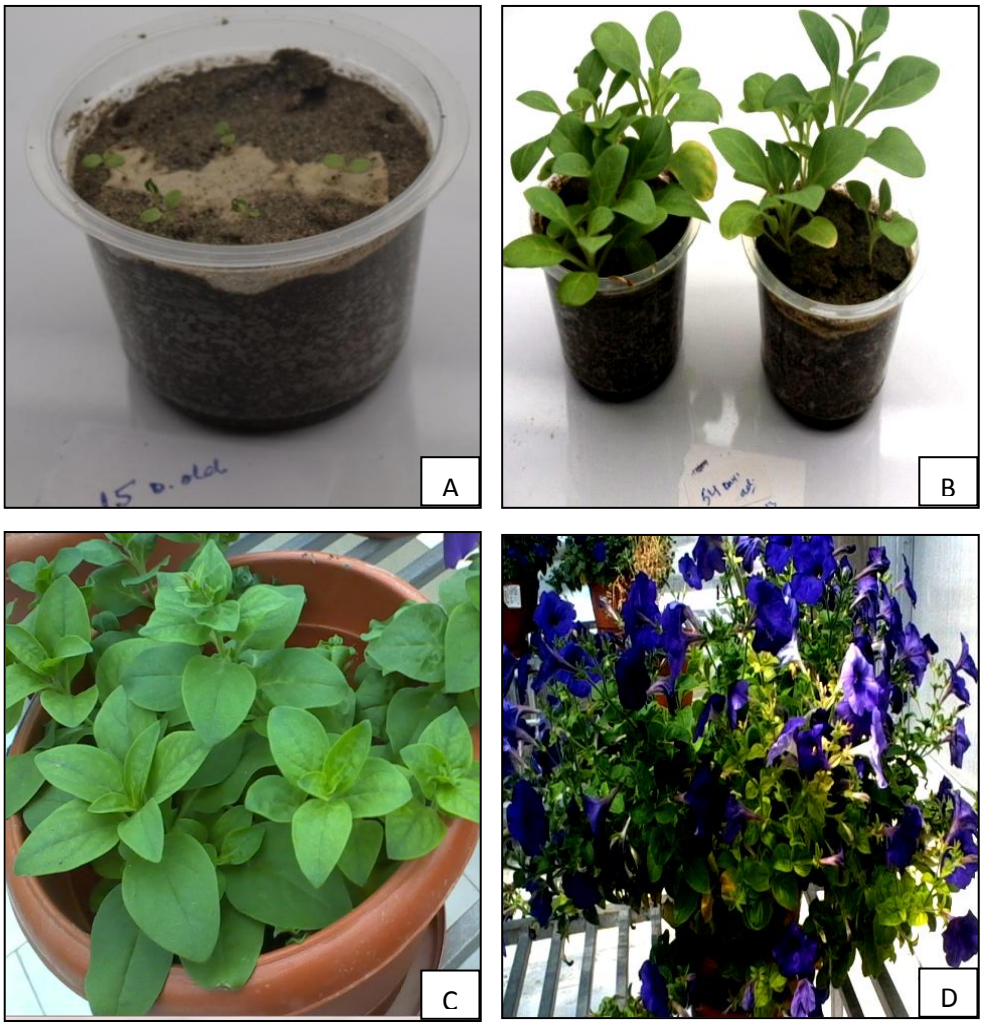


Plate 3. Plants grown for tissue culture.

3 A. 15 day old seedlings 3 B. 54 day old seedlings 3 C. 80 day old seedling 3 D. 120 days old plant

3.1.2. Sterilization of explant material

The explants used for callus induction were young healthy leaves collected from 90 day old plants retained in the growth house of the Central University of Punjab, Bathinda. The explants were plucked from plants and washed thoroughly under running tap water for four to five minutes to make sure that no inert material remains on the leaves. After washing, these were treated with 2% Polysorbate 80 (polyoxyethylene sorbitan mono oleate, Tween-80) obtained from Sisco Research Laboratories Pvt. Ltd (SRL), (v/v) till all foam disappeared. The explants were then immersed in 0.05% $HgCl_2$ for 5 minutes in a laminar air flow chamber with vigorous shaking to achieve surface sterilization. These were then washed with sterile distilled water inside the laminar airflow chamber three times for one minute each to remove the traces of $HgCl_2$. Explants of varieties Nana Compecta and Giant California had shown more susceptibility to fungi in preliminary experimentation. Therefore, the explants for these varieties were first washed by tap water for four minutes followed by rinsing in water containing 0.2% Bavistin (fungicide) for 10 minutes to prevent contamination. After that these were treated with 2% Polysorbate 80 (polyoxyethylene sorbitan mono oleate, Tween-80) of SRL (w/v), 0.05% $HgCl_2$ for 7 minutes in a laminar air flow chamber and washed with sterile distilled water as done for the first variety. The explants were ready for inoculation after this process. Inoculation of these sterilized explants needed aseptic conditions and sterilization of equipment which was done prior to inoculation by following the procedure given in section below.

3.1.3. Sterilization of equipment

All the glassware were soaked overnight in 0.05% washing solution (Teepol) followed by repeated washing in tap water to remove traces of washing solution. After washing these were dried in an oven at 60^0C and autoclaved at 121^0 C, under 15 lb pressure, for 20 minutes for sterilization. Forceps and scalpels were also washed and autoclaved similarly. The working area of laminar flow chamber was surface sterilized with rectified spirit (70% ethyl alcohol). The forceps and scalpels used for inoculation were kept ready in the laminar air flow chamber after autoclaving. The ultra violet light of laminar air flow chamber was switched on 20 minutes before inoculation of explants. During inoculation of explants the forceps and scalpels were dipped

regularly in rectified spirit, flamed, cooled and used for inoculation. Hands were sterilized with rectified spirit before inoculation.

3.1.4. Preparation of media

MS nutrient medium (Murashige and Skoog, 1962) obtained from Himedia, India (Appendix C) and also MS media (Murashige and Skoog, 1962; Appendix A) was prepared and used to raise the callus and shoot induction. These media were supplemented with different concentration of growth regulators for standardizing the induction of callus (Appendix B and Table 10). For preparation of media, Millipore water with 0.22 µm pore size and 3 step purification process was used so as to make it free of any contamination. After addition of all the media constituents, pH was adjusted to 5.8 using 1N NaOH and 1N HCL. Himedia Agar Agar 0.6% was used as gelling agent for callus inducing media and 3% sucrose was added in media. Media was boiled in microwave oven to melt the gelling agent completely. Then 25 ml of media was dispensed in culturing jars and they were autoclaved at 121^0C under 15 lb pressure for 15 minutes. All the plant growth regulators used in this experiment were added to media prior to autoclaving. The cultures for callus induction were maintained in a growth room with 16/8hr light and dark duration using (White Fluorescent light) supplemented with incandescent lamps which generated light intensity of 15000 lux.

3.1.5. Callus induction

For callus induction juvenile leaf discs of approximately 5 mm X 5 mm size with small holes made in leaf discs cut end surface placed in contact with culture medium were used (Appendix D). Different plant growth regulators (PGR) with different concentrations were tried for callus induction. First 2, 4-Dichlorophenoxy Acetic acid (2, 4-D) in concentration of 2 mg/l named as Media “A” (Table 10) was tested. Similarly 2, 4-D (2 mg/L) in combination with Kinetin (0.5 mg/L) named as media “B” (Table 10) was tested. Kinetin (2 mg/L) alone named as media ‘K’ was also tested for callus induction. The details and PGR concentrations tried for callus induction are given in (Table 10). The percent induction of callus was calculated by formula given below:

$$\text{Percent callus induction} = \frac{\text{Number of explants showing response}}{\text{Total number of explants inoculated}} \times 100$$

3.1.6. Shoot induction from leaves

Shoots were also directly induced from leaves without passing through callus phase (Appendix E). For standardization of this micro propagation protocol, MS Media with different hormonal concentrations were tried. To induce shoots, 2 mg/l Benzyl Amino Purine (BAP) and 0.1 mg/L Naphthalene Acetic Acid (NAA) were added to MS media. This media was designated as media "C" (Table 10). Other PGR with different concentrations were Kinetin 2 mg/L in combination with Indole Acetic Acid (IAA) 2 mg/L designated as media "D" (Table 10). Similarly MS media with kinetin 2 mg/L with Indole Acetic Acid 1.5 mg/L and Kinetin alone was also tested for inducing shoots directly from leaf explants. Shoot induction percentage was calculated by formula given below:

$$\text{Percent shoot induction} = \frac{\text{shoot formed in explants}}{\text{Total number of explants inoculated for shoot induction}} \times 100$$

3.1.7. Root induction

For inducing roots, selected shoots for inducing roots were excised from media under aseptic conditions, cleaned and placed on half strength MS media supplemented with 0.5 mg/l NAA and 0.1 mg/l IBA designated as media "S" (Table 17). BAP 2 mg/l and 0.1NAA mg/l which induce shoots were tested for root induction also. Also 2 mg/l Indole 3 Butyric Acid (IBA) was tested for root induction according to (Hegde, 2012). The 2 mg/l activated charcoal was used as an adsorbent during sub culturing of shoots.

3.1.8. Handling of micro propagated seedlings

When the shoots gained sufficient height and a root system, these were ready to be removed from *in-vitro* to *ex-vitro* condition. This period is crucial for survival of plants as growth conditions provided to these under *in-vitro* conditions induce abnormal morphology and change physiology of plants (Kumar and Rao, 2012). Therefore, before removing these following precautions were taken:

I. Aseptic conditions were maintained.
II. Adjustment of *ex-vitro* environment appropriate to growth of *P. grandiflora* was ensured.
III. Plants were washed properly with distilled water to remove agar.
IV. Pots filled with sand and vermicompost in equal proportion were kept ready for transplantation.

Further procedure is mentioned in section 3.1.9

3.1.9. Hardening of seedlings

Micro cuttings are very sensitive to changes in external physical environment and the success of their establishment entirely depends upon their acclimatization to environment. These are poorly adapted to growth house conditions where these are removed from culturing jars because of their poor mesophyll differentiation and weak vasculature of leaves, which makes these susceptible to transplantation shock. Therefore, to remove the *in-vitro* plants from culturing jars, these plants were gradually shifted to external environment. These were kept in tightly covered jars covered with polythene bags. Sprinkling of a fine mist of water in the polythene bags was done to maintain high humidity. The polythene bags were perforated gradually so that the plants get acclimatized to natural environment and the bags were finally removed after 10 days. After this these plantlets were shifted to pots containing sand and vermicompost in 1:1 ratio.

3.1.10. Transfer of plantlets to pots

Pots were prepared by properly mixing sand and vermicompost in equal proportion to make a homogenous mixture. These pots containing plants were kept in the growth chamber with temperature and humidity in the growth chamber maintained at 25 ± 2^{0}C and 50%, respectively. Artificial light having intensity of 15000 Lux produced by florescent tube lights and halogen bulbs for 16 hours followed by 8 hours of dark period was maintained. When plantlets appeared to be sturdy and self sustainable, these were shifted to open experimental plot.

B. Regeneration of salt tolerant seedlings from salt treated callus.

3.2.1. *In-vitro* NaCl treatment to callus

After 30 days of inoculation of explant, calli obtained from inoculated explants of variety Violet Blue were sub cultured on media 'C' (Table 10) for further proliferation to get sufficient mass of calli. Calli of variety Giant California were sub cultured on media 'L' (Table 10). After 25 days of sub culturing, healthy calli 2 mm to 4 mm in size were inoculated on media 'C' (Table 10) supplemented with different concentration of salt. For each treatment, eight culturing vessels were used and media without salt was kept as untreated control. The details of treatments given to calli are given in table 2. Calli from variety Nana Compecta were treated only upto 50 mM because calli for further treatment were not available. Two methods were used for selecting salt resistant calli. These methods were: (1) Direct treatment (2) stepwise treatment. In the Direct treatment method salt treatment was given to calli to find out the lethal salt concentration at which death of calli occurred. Based on this, only sub lethal treatments were tried for selection. The highest concentration at which calli survived and showed good growth was selected for further experimentation and regeneration of salt tolerant shoots. For treatment, regeneration media was supplemented with NaCl. Different salt concentrations used for direct treatment for variety Violet Blue are 50 mM, 100 mM,150 mM, 200 mM, 250 mM and 300 mM and for Giant California same as Violet Blue but only upto 200 mM. In variety Nana Compecta salt treatment was given only upto 50 mM. The observations were recorded after 25 and 52 days after I^{st} and 2^{nd} sub culturing, respectively.

In stepwise treatment method, first the calli were sub cultured on media 'B' for further proliferation. After getting sufficient mass of cells, approximately 3 mm to 4 mm size calli were transferred to media 'B". In the first sub culture, media "B" was supplemented with 25 mM salt concentration and calli were maintained for 21 days. After 21 days, healthy calli selected from 25 mM salt concentration were transferred to media 'C' with 50 mM salt concentration and again maintained for 21 days to select healthy calli. Similar procedure was followed for 75 mM and 150 mM salt concentrations. Since death of callus occurred in stepwise treatment at 150 mM salt concentration during fourth sub culture, this treatment was not tried. Calli survived at

75 mM salt concentration were selected and inoculated on to shoot regeneration media supplemented with 75 mM salt concentration to get the shoots. Sub culturing of salt treated calli and those without salt treatment was regularly performed after every 21 days. Characteristics of sub cultured calli on proliferation media supplemented with salt was recorded. The effect of different hormonal treatments on callus characteristics was also recorded.

Table 2. Details of salt treatments given to calli of three varieties of *P. grandiflora*

Sr. No.	Variety	NaCl treatment (mM)						
1	Violet Blue	50	100	150	200	250	300	0 (control)
2	Giant California	50		100		150	200	0 (control)
3	Nana Compecta	50						0 (control)

3.2.2. Regeneration of shoots from callus

For shoot organogenesis, calli of NaCl tolerated lines were inoculated on MS media containing 3% sucrose, 0.6% agar supplemented with different plant growth regulators (Appendix F and Table 16). The pH was adjusted at 5.8 prior to autoclaving. Calli from untreated controls were also cultured on MS medium having the same composition except NaCl. All the cultures were incubated at 25± 2^0C under a 16 hr photoperiod with a light intensity of 15000 lux and relative humidity maintained at 50-70%.

3.2.3. *In-vitro* NaCl treatment to shoots

After two sub cultures, fast growing and healthy shoots obtained from MS media containing BAP 2 mg/l and NAA 0.1 mg/l were selected for NaCl treatment. These shoots were excised and sub cultured on media ‘C” for three weeks for further growth. Subsequently, these were inoculated on media containing 50 mM, 100 mM, 150 mM, 200 mM and 250 mM NaCl. But in variety Violet Blue, shoots were also treated with 300 mM salt concentration. For each treatment ten culturing jars were

used and each jar contained one explant. The surviving shoots after 15 days growth in salt were again sub cultured on salt containing media. Finally the shoots which tolerated two sub cultures of salt stress were selected for root induction.

3.2.4. Statistical analysis

Statistical analysis were performed by Analysis of Variance (two way ANOVA) using sigma plot software version 11, using Tukey Test at significant level ($p<0.050$).

CHAPTER IV

RESULTS

4.1. Effect of *ex-vitro* salt treatment on seedling survival

The detailed observations on seedling survival rate of the three varieties after treatment with different concentrations of salt for different time durations is given in Appendices H, I and J. The mean survival rate based on appendices H, I and J is given in table 3. The mean survival rate of the three varieties Violet Blue, Giant California and Nana Compecta varied from 54.65% to 28.28%, 52.86% to 17.86% and 69.6% to 51.95%, respectively at salt concentration of 100 mM to 600 mM for 4 hours as compared to control where the mean survival percentages were 94.23%, 94.65% and 97.33%, respectively. Similarly the mean survival rate of the three varieties Violet Blue, Giant California and Nana Compecta varied from 51.13% to 29.15%, 52.9 to 29.82% and 70.21% to 52.65%, respectively at salt concentration of 100 mM to 600 mM for 6 hours as compared to control where the mean survival percentages were 94.58%, 96.69% and 95.92%, respectively. For 12 hours treatment, the mean survival rate of the three varieties Violet Blue, Giant California and Nana Compecta varied from 58.04% to 30.54%, 61.91% to 29% and 68.77% to 48.72%, respectively at salt concentration of 100 mM to 600 mM as compared to control where the mean survival percentages were 96.13%, 91.61% and 89.44%, respectively. For 24 hours treatment the mean survival rate of the three varieties Violet Blue, Giant California and Nana Compecta varied from 51.57% to 27.55%, 67.52% to 29.29% and 67.6% to 51.11%, respectively at salt concentration of 100 mM to 600 mM as compared to control where the mean survival percentages were 94.88%, 90.17% and 97.98%, respectively.

The mean survival rate of the three varieties Violet Blue, Giant California and Nana Compecta over all the salt concentration for different durations is given in table 4 was 40.51%, 35.00% and 59.31%, respectively for the three varieties for duration of 4 hours. For 6 hour treatments of salt the average survival percentage of three varieties Violet Blue, Giant California and Nana Compecta was 38.93%, 41.62% and 59.98%, respectively. For 12 hour salt treatments the average survival percentage of three varieties Violet Blue, Giant California and Nana Compecta was 50.42%, 48.23% and 63.25%, respectively. For 24 hours the average survival percentage over all the concentrations for varieties Violet Blue, Giant California and Nana Compecta was 38.76, 40.18 and 58.42%, respectively. These observations in table 4 suggest that

variety Nana Compecta is least affected with salt treatment as compared to other two varieties.

There was significant decrease ($p<0.05$) in survival percentage as the salt concentration was increased in all the three varieties as compared to untreated control.

Table 3. Average survival percentage of three varieties of *P. grandiflora* after salt treatment

Salt concentration	Variety	Duration of treatment							
		4 hr	S.D (σ)	6hr	S.D (σ)	12 hr	S.D (σ)	24 hr	S.D (σ)
100 mM	Violet Blue	54.65[a] ±5.391	9.33	51.13[a] ±1.603	2.77	58.04[a] ±3.545	6.13	51.57[a] ±8.434	14.60
	Giant California	52.86[a] ±7.922	13.72	52.90[a] ±5.024	8.70	61.91[a] ±9.300	16.12	67.52[a] ±8.723	15.10
	Nana Compecta	69.60[a] ±3.445	5.96	70.21[a] ±6.199	10.73	68.77[a] ±2.734	4.73	67.60[a] ±0.650	1.12
200 mM	Violet Blue	45.60[a] ±3.962	6.86	41.97[a] ±5.37	9.31	48.38[a] ±5.819	10.07	43.12[a] ±14.55	25.20
	Giant California	39.49[a] ±0.982	1.70	52.58[a] ±2.808	4.86	47.22[a] ±13.89	24.05	41.55[a] ±2.72	4.71
	Nana Compecta	65.11[a] ±3.597	6.23	65.69[a] ±1.462	2.53	63.48[a] ±1.609	2.78	63.09[a] ±0.913	1.58
300 mM	Violet Blue	41.87[a] ±5.972	10.34	39.15[a] ±4.278	7.41	41.89[a] ±7.485	12.96	40.21[a] ±9.43	16.34
	Giant California	37.07[a] ±1.227	2.12	46.13[a] ±17.005	29.45	39.36[a] ±1.270	2.20	41.40[a] ±3.677	6.36
	Nana Compecta	62.03[a] ±8.118	14.06	61.23[a] ±1.618	2.80	61.04[a] ±1.902	3.29	58.71[a] ±1.936	3.35
400 mM	Violet Blue	38.79[a] ±3.485	6.03	37.21[a] ±8.596	14.88	40.32v ±2.240	3.88	39.40[a] ±3.390	5.87
	Giant California	32.81[a] ±0.947	1.64	36.16[a] ±4.809	8.33	36.75[a] ±0.432	0.74	34.96[a] ±8.104	14.03
	Nana Compecta	54.06[a] ±6.158	10.66	56.32[a] ±4.32	7.48	58.12[a] ±3.107	5.38	56.30[a] ±6.062	10.49
500 mM	Violet Blue	33.87[a] ±8.510	14.74	34.95[a] ±1.473	2.55	37.67[a] ±12.837	22.23	30.74[a] ±0.707	1.22
	Giant California	29.89[a] ±3.620	6.26	32.15[a] ±0.880	1.52	31.75[a] ±1.959	3.39	29.96[a] ±1.192	2.06

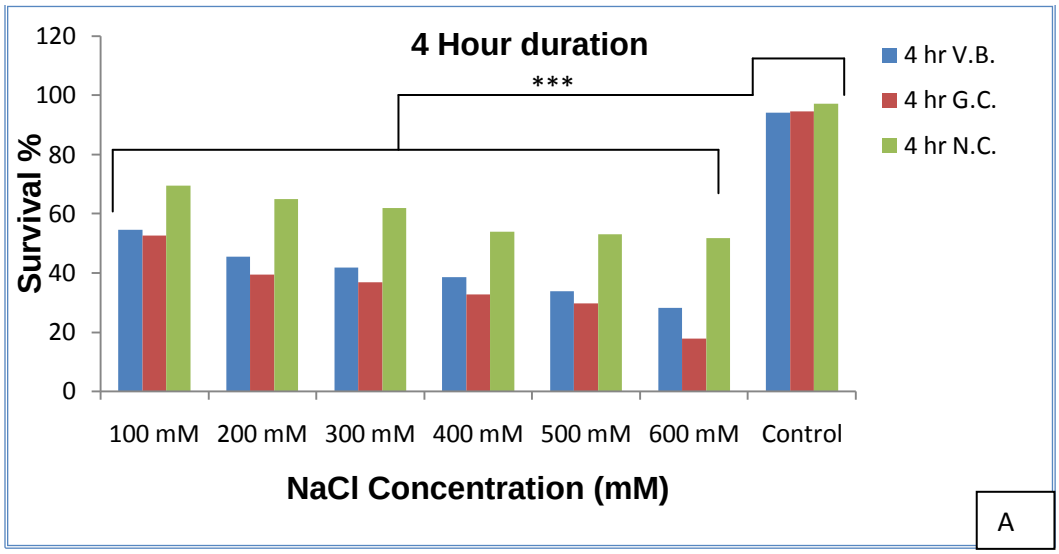
Table 3 contd…

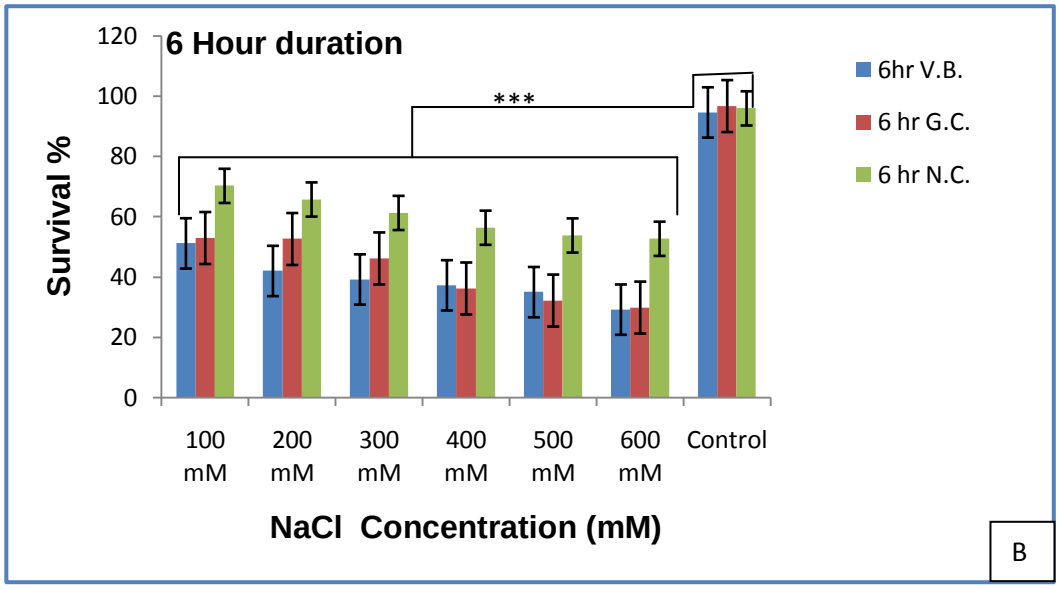
	Nana Compecta	53.13 [a] ±8.884	15.38	53.75 [a] ±5.384	9.32	53.18 [a] ±1.756	3.04	53.70 [a] ±6.333	10.97
600 mM	Violet Blue	28.28 [a] ±8.338	14.44	29.15 [a] ±3.152	5.45	30.54 [a] ±0.454	0.78	27.55 [a] ±7.027	12.17
	Giant California	17.86 [a] ±7.671	13.28	29.82 [a] ±2.707	4.68	29.00 [a] ±0.754	1.36	29.29 [a] ±0.269	0.46
	Nana Compecta	51.95 [a] ±6.604	11.43	52.65 [a] ±1.905	3.30	48.72 [a] ±1.133	1.96	51.11 [a] ±7.561	13.09
Control	Violet Blue	94.23 ±1.137	1.96	94.58 [a] ±0.478	0.82	96.13 [a] ±0.665	1.15	94.88 [a] ±0.731	1.26
	Giant California	94.65 ±1.615	2.79	96.69 ±0.407	0.70	91.61 ±4.174	7.22	90.17 ±3.638	6.30
	Nana Compecta	97.33 ±0.577	1.00	95.92 ±0.801	1.38	89.44 ±2.440	4.22	97.98 ±0.740	1.28

Values are means of three replicates [a]*P<0.001,* (±) = Standard Error of Mean (SEM)

Table 4. Average survival percentage of three varieties of *P. grandiflora* over salt concentrations for four durations of treatment

Variety	Duration of treatment (Average of 100 mM, 200 mM, 300 mM, 400 mM, 500mM and 600 mM NaCl Treatment			
	4 hr	6 hr	12 hr	24 hr
Violet Blue	40.51	38.93	50.42	38.765
Giant California	35.00	41.62	48.23	40.78
Nana Compecta	59.31	59.98	63.25	58.42
Control Violet Blue	94.23	94.58	96.13	94.88
Control Giant California	94.65	96.69	91.61	90.17
Control Nana Compecta	97.33	95.92	89.44	97.98





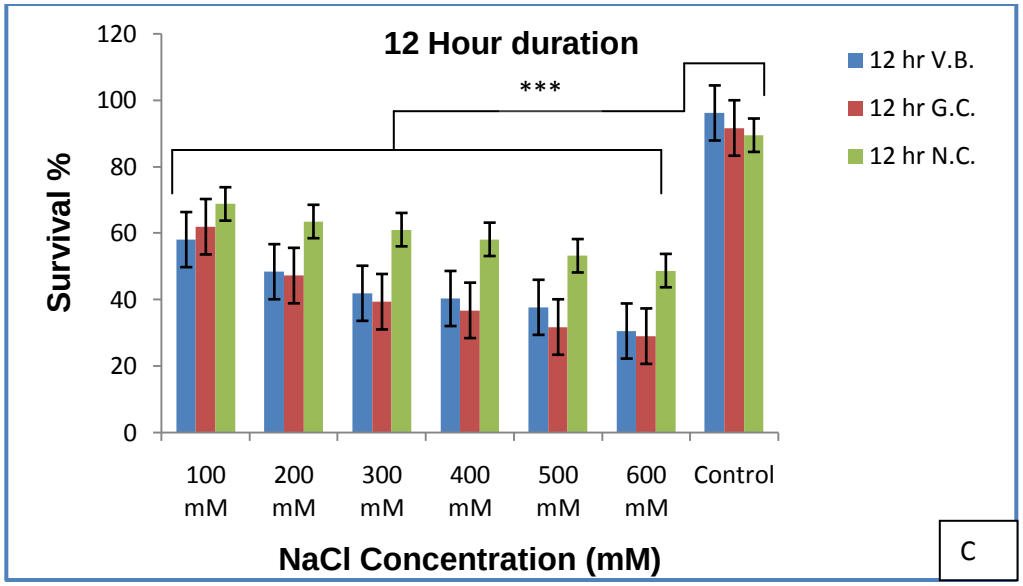
Values are the means of three replicates per treatment ***p<0.001

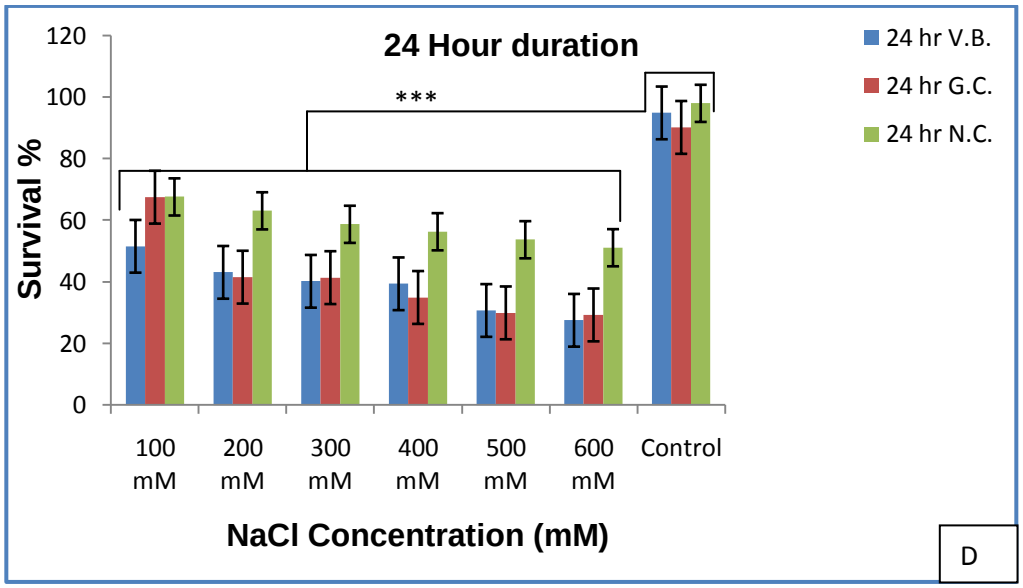
Figure 1. Average survival percentage of three varieties of *P. grandiflora* treated with different concentrations of NaCl for different time durations (A andB).

V.B. = Violet Blue

G.C. = Giant California

N.C. = Nana Compecta





Values are the means of three replicates per treatment ***p<0.001

Figure 2. Average survival percentage of three varieties of *P. grandiflora* treated with different concentrations of NaCl for different time durations (C and D).

V.B. = Violet Blue

G.C. = Giant California

N.C. = Nana Compecta

4.2. Effect of *ex-vitro* treatment of salt concentrations on seedlings.

The seeds of three varieties of *P. grandiflora* which were sown in trays started germinating after 3-4 days. The untreated control seedlings of varieties Violet Blue, Giant California and Nana Compecta exhibited normal growth and these were green and healthy (Plates 4A, 4B and 4C, respectively). The seedlings however, showed varying response to salt treatment. The leaves of seedlings after salt treatment, became pale yellow (necrotic) and started to dry and finally death of seedlings occurred. In untreated control, these continued to grow vigorously with lush green broad leaves and maintained a healthy stem. These also attained sufficient height in shorter duration as compared to salt treated seedlings.

Chlorosis of leaves was more pronounced in seedlings treated with 600 mM salt for 24 hours (Plate 5C). Variety Giant California was observed to the most affected variety after 24 hours salt treatment (Plate 6C). Variety Nana Compecta exhibited better growth as compared to other two varieties (Plate 7).

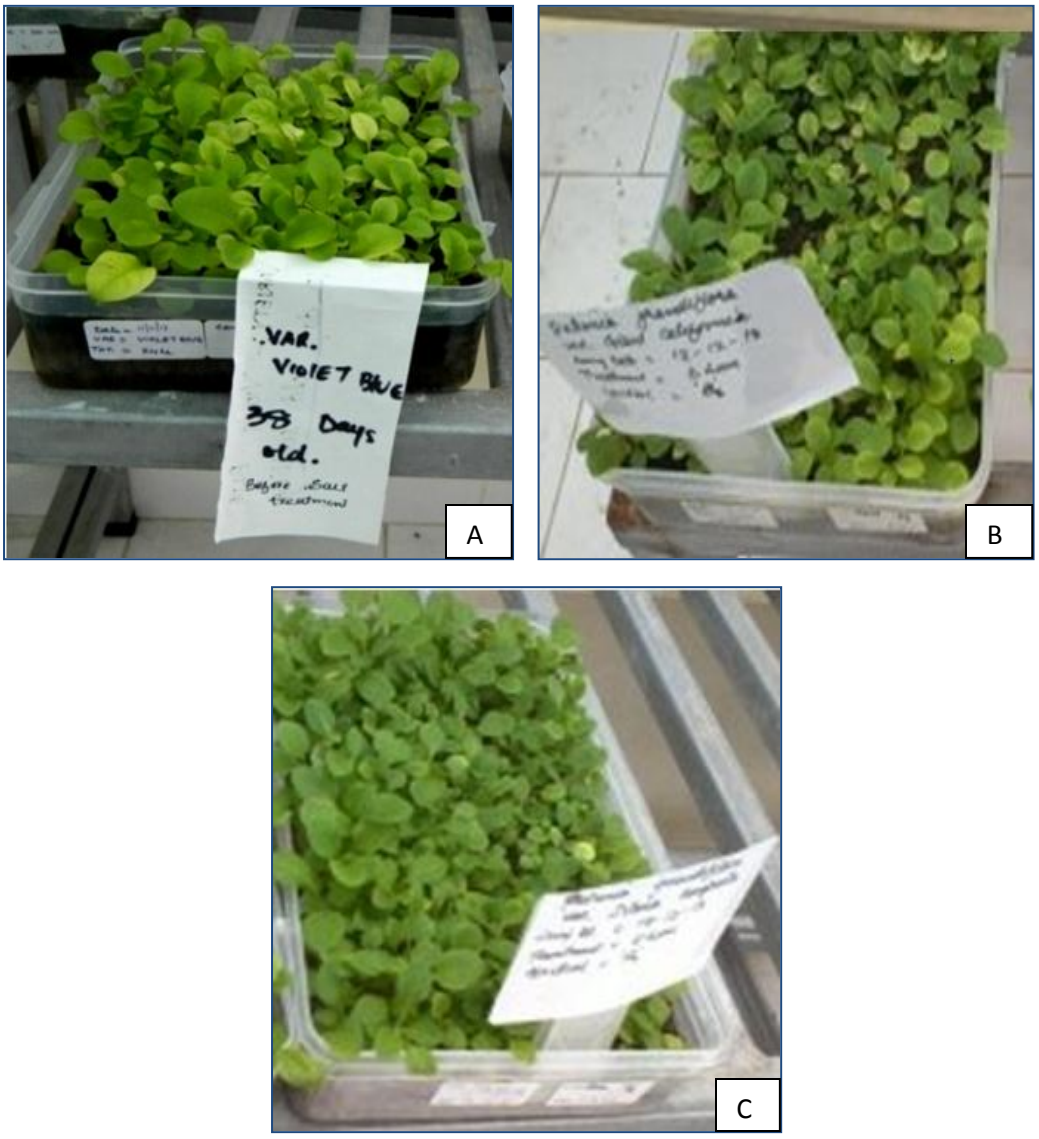


Plate 4. 38 days seedlings of three varieties of *P. grandiflora* before salt treatment. Variety Violet Blue (4A), variety Giant California (4B), variety Nana Compecta (4C).

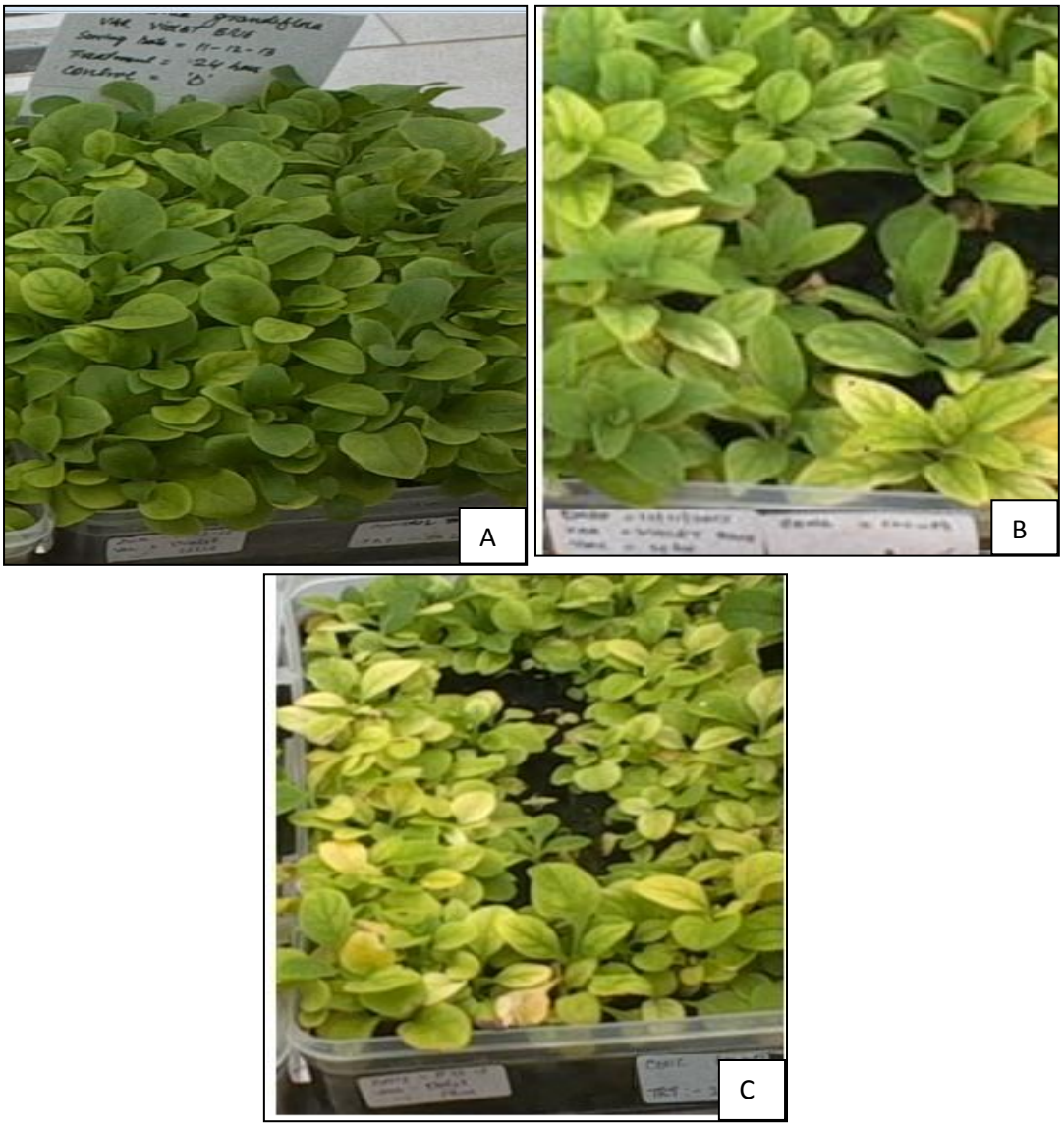


Plate 5. Seedlings of variety Violet Blue 15 days after salt treatment showing chlorosis.

Control (5A), seedlings treated with 100 mM salt (5B), seedlings treated with 600 mM salt (5C).

Plate 6. Seedlings of variety Giant California 15 days after salt treatment showing chlorosis.

Control (6A), seedlings treated with100 mM salt (6B), seedlings treated with 600 mM salt (6C).

Plate 7. Seedlings of variety Nana Compecta 15 days after salt treatment.
Control (7A), seedlings treated with100 mM salt (7B), seedlings treated with 600 mM salt (7C).

4.3. Effect of salt treatment on growth of *Petunia grandiflora*

An important growth parameter shoot length was recorded 20 days after salt treatment. The detailed shoot length of all replicates of varieties Violet Blue, Giant California and Nana Compecta after salt treatment is given in Appendices K, L, and M, respectively.

The mean shoot length over the treatments based on appendices k, L and M is given in table 5. The mean shoot length of the three varieties Violet Blue, Giant California and Nana Compecta over replications varied from 2.95 cm to 2 cm, 2 cm to 1.79 cm and 4.22 cm to 2.32 cm, respectively at salt concentration of 400 mM to 600 mM for 4 hours as compared to control where the mean shoot length of the varieties was 10.49 cm, 11.9 cm and 6.34 cm respectively. Similarly the mean shoot length of the three varieties Violet Blue, Giant California and Nana Compecta varied from 2.45 cm to 1.92 cm, 1.91 cm to 1.73 cm and 3.51 cm to 2.05 cm, respectively at salt concentration of 400 mM to 600 mM for 6 hours as compared to control where the mean shoot length was 10.66 cm, 11.63 cm and 6.22 cm respectively. For treatment duration of 12 hours the mean shoot length of varieties Violet Blue, Giant California and Nana Compecta varied from 2.68 cm to 2.24 cm, 1.85 cm to 1.64 cm and 3.65 cm to 2.31 cm, respectively at salt concentration of 400 mM to 600 mM as compared to control where the mean shoot length was 9.52 cm, 12.53 cm and 6.41 cm, respectively. For treatment duration of 24 hours, the mean shoot length of the three varieties Violet Blue, Giant California and Nana Compecta varied from 2.63 cm to 2.26 cm, 2.04 cm to 1.71 cm and 3.65 cm to 2.06 cm, respectively, at salt concentration of 400 mM to 600 mM as compared to control where the mean shoot length was 10.69 cm, 12.1 cm and 6.26 cm, respectively.

The average shoot length of three varieties Violet Blue, Giant California and Nana Compecta over the treatments for different durations is given in table 6. The average shoot length for the three varieties Violet Blue, Giant California and Nana Compecta tested over different concentrations of salt was 2.26 cm, 1.90 cm and 3.18 cm, respectively for 4 hours duration. The average shoot length of three varieties Violet Blue, Giant California and Nana Compecta was 2.26 cm, 1.84 cm and 2.65 cm, respectively for 6 hour treatment duration. For 12 hour treatment duration, the

average shoot length of three varieties Violet Blue, Giant California and Nana Compecta was 2.44 cm, 1.77 cm and 2.8 cm, respectively. For treatment duration of 24 hours the average shoot length over all the salt concentrations for varieties Violet Blue, Giant California and Nana Compecta was 2.43 cm, 1.82 cm and 2.69 cm, respectively. It is evident from these results that the shoot length of variety Nana Compecta was least affected with salt treatment as compared to other two varieties. Therefore, in terms of shoot length, variety Giant California is the most susceptible variety to salt stress of the three varieties tested. The results given here clearly indicate that shoot length decreases with increase in salt concentration in all the three varieties but the response of three varieties to salt stress is different.

Table 5. Average shoot length (cm) of three varieties of *P. grandiflora* after treatment over four durations

Salt concentration	Treatment time and variety											
	4 Hour			6 Hour			12 Hour			24 Hour		
	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta
400 mM	2.95[a] ± 0.094	2.00[a] ± 0.127	4.22[a] ± 0.089	2.45[a] ± 0.087	1.91[a] ± .111	3.51[a] ±0.113	2.68[a] ± 0.150	1.85[a] ± 0.058	3.65[a] ±0.107	2.63[a] ±0.11	2.04[a] ±0.04	3.65[a] ±0.04
S.D (σ)	0.52	0.69	0.49	0.48	0.60	0.61	0.81	0.32	0.58	0.63	0.25	0.68
500 mM	2.28[a] ± 0.095	1.9[a] ± 1.43	3.01[a] ±132	2.4 ± 0.059	1.88[a] ± .114	2.39[a] ±0.085	2.41[a] ± 0.073	1.81[a] ± 0.134	2.44[a] ±0.102	2.39[a] ±0.08	1.72[a] ±0.13	2.35[a] ±0.13
S.D (σ)	0.52	0.78	0.72	0.32	0.62	0.46	0.40	0.73	0.56	0.47	0.73	0.75
600 mM	2[a] ± 0.074	1.79[a] ± 0.088	2.32[a] ± 0.080	1.92[a] ± 0.085	1.73[a] ± 0.093	2.05[a] ±0.064	2.24[a] ± 0.061	1.64[a] ± 0.108	2.31[a] ± 0.094	2.26[a] ±0.06	1.71[a] ±0.07	2.06[a] ±0.08
S.D (σ)	0.41	0.48	0.44	0.46	0.51	0.35	0.33	0.59	0.51	0.33	0.39	0.47
Control	10.49[a] ± 0.11	11.9[a] ± 0.34	6.34[a] ± 0.26	10.66[a] ± 0.142	11.63[a] ±0.242	6.22[a] ±0.142	9.52[a] ± 0.096	12.53[a] ± 0.310	6.41[a] ± 0.230	10.69[a] ±0.18	12.1[a] ±0.23	6.26[a] ±0.26
S.D(σ)	0.63	1.88	1.44	0.77	1.32	0.78	0.53	1.26	1.69	1.01	1.29	1.45

Values are means of three replicates with ten plants per replicate [a]*P<0.001,* ± = Standard Error of Mean (SEM)

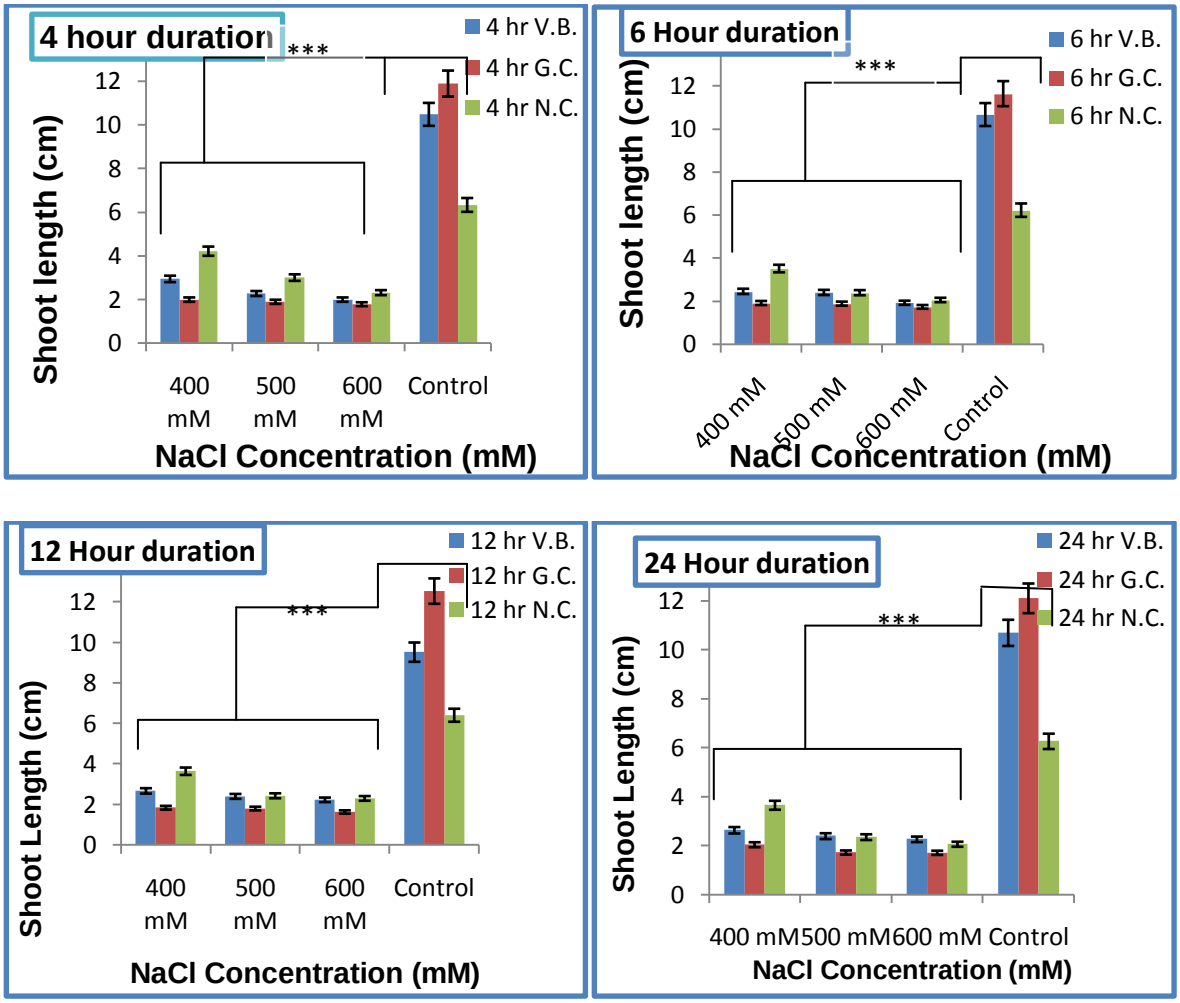


Figure 3. Average shoot length of three varieties of *P. grandiflora* treated with different concentrations of NaCl over different durations

V.B. = Violet Blue

G.C. = Giant California

N.C. = Nana Compecta

Table 6. Average shoot length (cm) of three varieties of *P. grandiflora* after salt treatment over four durations

Varieties	Duration of treatment (Average of 400 mM, 500 mm and 600 mM NaCl concentration)					
	4 hr	6 hr	12 hr	24 hr	Average	Reduction in shoot length (%)
Violet Blue	2.41	2.26	2.44	2.43	2 .39	23.07
Giant California	1.9	1.84	1.77	1.82	1.83	15.22
Nana Compecta	3.18	2.65	2.8	2.69	2.83	44.87
Violet Blue (control)	10.49	10.66	9.52	10.69	10.34	-
Giant California (control)	11.9	11.63	12.53	12.1	12.04	-
Nana Compecta (control)	6.34	6.22	6.41	6.26	6.31	-

4.4. Effect of salt on leaf number

The number of leaves of the three varieties of *P. grandiflora* were counted 20 days after salt treatment. The detailed information on replicates of three varieties is given in Appendices N, O and P of Nana Compecta, Violet Blue and Giant California respectively.

The mean number of leaves based on appendices N, O and P is given in table 7. The mean leaf number of the three varieties Nana compecta, Violet Blue and Giant California over replications varied from 7.5 to 6.37, 7.8 to 7.07 and 8.29 to 5.73, respectively at salt concentration of 400 mM to 600 mM for 4 hours as compared to control where the mean number of leaves of the varieties was 10.53, 11.13 and 10.83, respectively. Similarly the mean number of leaves of the three varieties Nana compecta, Violet Blue and Giant California varied from 8.8 to 6.97, 8.3 to 7.27 and 8.3 to 5.37, respectively at salt concentration of 400 mM to 600 mM for 6 hours as compared to control where the mean number of leaves was 10, 11.33 and 10.9 respectively. For treatment duration of 12 hours, the mean number of leaves varieties Nana compecta, Violet Blue and Giant California varied from 8.53 to 6.67, 8.5 to 7.17 and 7.93 to 5.53, respectively at salt concentration of 400 mM to 600 mM as compared to control where the mean number of leaves was 10.8, 11.57 and 10.83, respectively. For treatment duration of 24 hours, the mean number of leaves of the three varieties Nana compecta, Violet Blue and Giant California varied from 8.3 to 6.47, 8.73 to 6.5 and 8.13 to 5.5, respectively at salt concentration of 400 mM to 600 mM as compared to control where the mean number of leaves was 10.9, 11.36 and 10.87, respectively.

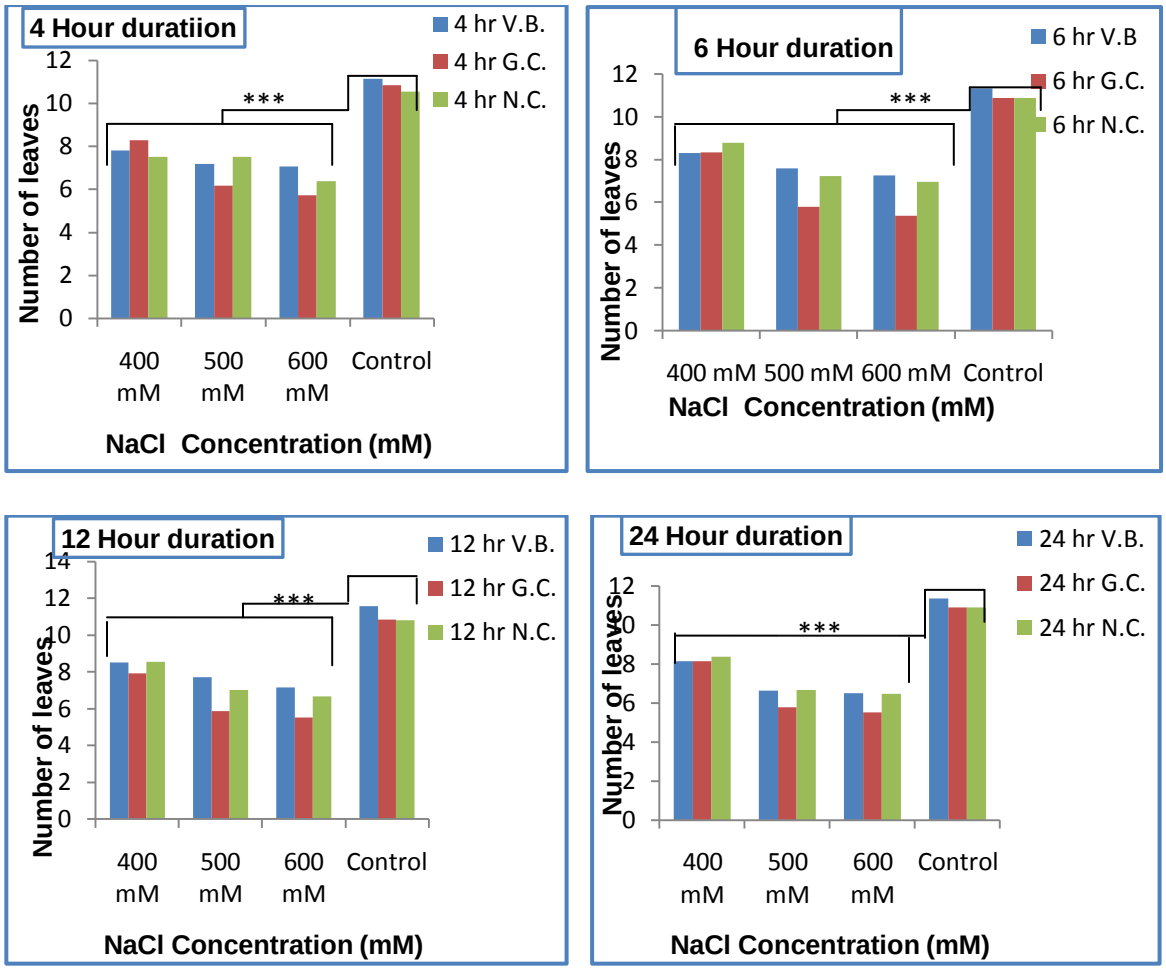
The average number of leaves of three varieties Nana compecta, Violet Blue and Giant California over treatments for different durations is given in table 8. The average number of leaves for the three varieties Nana Compecta, Violet Blue, and Giant California was 7.12, 7.35 and 6.73, respectively for 4 hours duration. The average number of leaves of three varieties Nana compecta, Violet Blue and Giant California over the concentrations was 7.67, 7.72 and 6.50, respectively. For 12 hour treatment duration, the average number of leaves of three varieties Nana compecta, Violet Blue and Giant California was 7.41, 7.79 and 6.44, respectively. For treatment

duration of 24 hours, the average number of leaves over all the salt concentrations for varieties Nana compecta, Violet Blue and Giant California was 7.17, 7.09 and 6.47, respectively. It is evident from these results that variety Giant California was most affected in terms of the number of leaves as compared to other two varieties over all the salt concentrations. The results given here clearly indicate that leaf number decreases with increase in salt concentration in all the three varieties.

Table 7. Average number of leaves 20 days after treatment of three varieties of *P. grandiflora* with different concentrations of NaCl over different durations

Salt concentration	Treatment time and varieties											
	4 Hour			6 Hour			12 Hour			24 Hour		
	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California
400 mM	7.50[a] ±0.321	7.80[a] ±0.388	8.29[a] ±0.359	8.80[a] ±0.301	8.30[a] ±0.407	8.33[a] ±0.435	8.53[a] ±0.208	8.50[a] ±0.295	7.93[a] ±0.383	8.30[a] ±0.256	8.13[a] ±0.287	8.13[a] ±0.367
S.D (σ)	1.75	1.96	1.96	1.64	2.23	2.38	1.13	1.61	2.10	1.40	1.57	2.01
500 mM	7.50[a] ±0.224	7.17[a] ±0.167	6.17[a] ±0.240	7.23[a] ±0.207	7.60[a] ±0.256	5.80[a] ±0.217	7.03[a] ±0.227	7.70[a] ±0.226	5.87[a] ±0.224	6.67[a] ±0.227	6.63[a] ±0.237	5.77[a] ±0.223
S.D (σ)	1.22	0.91	1.31	1.13	1.40	1.18	1.24	1.23	1.22	1.24	1.29	1.22
600 mM	6.37[a] ±0.200	7.07[a] ±0.185	5.73[a] ±0.249	6.97[a] ±0.227	7.27[a] ±0.197	5.37[a] ±0.176	6.67[a] ±0.251	7.17[a] ±0.250	5.53[a] ±0.229	6.47[a] ±0.202	6.5[a] ±0.287	5.5[a] ±0.229
S.D (σ)	1.09	1.36	1.01	1.24	1.08	0.96	1.37	1.36	1.25	1.10	1.57	1.25
Control	10.53 ±0.300	11.13 ± 0.270	10.83 ±0.328	10. ±0.385	11.33 ±0.350	10.9 ±0.360	10.8[a] ±0.301	11.57 ±0.327	10.83[a] ±0.365	10.90 ±0.277	11.36 ±0.338	10.87 ±0.377
S.D (σ)	1.79	1.47	1.64	2.10	1.91	1.97	1.64	1.79	2.00	2.06	1.85	1.51

Values are means of three replicates with ten plants per replicate [a]*P<0.001,* ± = Standard Error of Mean (SEM)



Values are means of three replicates with ten plants per replicates ***p<0.001

Figure 3. Average number of leaves of replicas of three varieties of *P. grandiflora* treated with NaCl for different durations

V.B. = Violet Blue

G.C. = Giant California

N.C. = Nana compecta

Table 8. Average Number of leaves of three varieties of *P. grandiflora* after salt treatment over four durations

Violet Blue	Duration of treatment (Average of 400 mM, 500 mm and 600 mM NaCl treatment)					
	4 hr	**6 hr**	**12 hr**	**24 hr**	**Average**	**Reduction in leaf number (%)**
	11.13	11.33	11.57	11.36	7.49	65.98
Giant California	6.73	6.50	6.44	6.47	6.54	60.19
Nana Compecta	7.12	7.67	7.41	7.17	7.34	68.10
Violet Blue (control)	10.53	10.90	10.80	10.90	11.35	-
Giant California (control)	10.83	10.90	10.83	10.87	10.86	-
Nana Compecta (control)	7.35	7.72	7.79	7.09	10.78	-

4.5. Delay to 50% flowering

Days to 50% flowering for all the three varieties was increased after salt treatment as compared to untreated controls (Table 9). Flowering occurred after 70 days of salt treatment in variety Nana Compecta, after 80 days in variety Violet Blue and after 92 days in variety Giant California. Whereas 50% plants from Varieties Nana Compecta, Violet Blue and Giant California started flowering in 70, 80 and 92 days, respectively. Salt treatment increased days to 50% flowering from 66 to 80.66 over the varieties as compared to control.

Table 9. Effect of salt treatment on days to 50% flowering of three varieties of *P. grandiflora*

Variety	Days to 50% flowering in control	Number of days taken to reach 50% flowering
Violet Blue	66	80
Giant California	68	92
Nana compecta	64	70

4.6. Observation on plants after shifting to field plots

Plants were shifted to field plots 20 days after salt treatment. Even after shifting of treated plants to field plots their leaves continued to become chlorotic and later, some plants died. Plants were weak with chlorotic leaves for about one month, after which these started to branch. As multiple thick branches emerged from main stem, most plants became greenish and exhibited normal growth. These continued to bloom for three to four months after initiation of flowering. Flowers of variety Violet Blue were mostly purple but some flowers with light purple color and some with dark velvety blue color were also seen in this variety. The reason may be heterogeneity in seeds. In some flowers the placement of stigma above the stamens and in some below the stamens was observed. In other two varieties also similar observation with respect to placement of stigma was recorded. Of the nearly 10,000 thousand plants grown in the field, twenty plants of each variety showing vigorous growth were identified selected for seed collection.

Part II

4.7. Standardization of protocol for callus induction

Fifteen media designated as Media 'A' to Media 'M' were tested with different concentrations and components (Table 10) for callus induction in all the three varieties. The three varieties showed different response to callus induction in different media supplemented with plant growth regulators. Four of the media namely media 'C' 'D', 'M' and 'N' did not induce callus but the remaining eleven were useful in inducing callus, although the size of callus induced by different media was highly variable. Media which induced more than 3 cm of callus in 30 days were selected for further work. In this experiment media 'B' was selected for callus induction in Violet Blue and media "L" for varieties Giant California and Nana compecta. When media "A" was tested for callus induction in Violet Blue, it initiates callus after 24 days and percentage of callus formation was 45% with insufficient proliferation. Media "B" produced highest percentage of calli (90%) in Violet Blue but in Giant California and Nana compecta, it failed to induce callus. Media "C" induced direct shoots from leaves of all the three varieties. Media "D" tested for all the three varieties produced no result. Media "E" produced 20% and 30% callus in varieties Violet Blue and Giant California and callus initiation was observed after 26 and 19 days, respectively. Media "F" tested in Giant California initiated callus after 23 days and the callus formation was 27%. Similarly, Media "G" tested in variety Nana Compecta initiated callus after 17 days with only 17% of callus formation. Media "H" induced callus after 19 days in varieties Giant California and Nana Compecta but percentage of callus formation was 60% and 35%, respectively. Media "I" induced callus after 17 and 15 days in Giant California and Nana compecta and percentage of callus obtained was 60% in both the varieties. Similarly callus initiation was observed after 14 days and 12 days in Giant California and Nana Compecta, respectively, when tested with media "J" and 65% callus formation was observed in both varieties with this media. Media "L" induced callus after 13 and 11 days in Giant California and Nana Compecta, respectively, and percentage of callus obtained was 70% and 65% in Giant California and Nana Compecta, respectively. This media was used for further work on these two varieties as it yielded good amount of callus. Media "C", "D", "M" and "N" tested for all

the three varieties produced no result. Initially, small greenish white calli developed on the cut ends after inoculation which subsequently covered the entire surface of the explant. The calli formed were granular, soft and yellow from variety Violet Blue and green, compact and hard in Giant California and Nana Compecta. It was observed that in all the three varieties, sufficient mass of callus was formed after these were sub cultured on their respective media.

Table 10. Effect of supplementation of MS media with different concentrations of Plant Growth Regulators (PGR) on callus induction from *in-vitro* leaf explants of three varieties of *P. grandiflora*

Media Designation	Plant growth regulator treatment mg/l	% callus formation			Days to callus initiation			Morphology of callus			Degree of callus formation		
		Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta
A	2 mg/l 2, 4 –D	45	Not tested	Not tested	24	Not tested	Not tested	White hard	Not tested	Not tested	+	Not tested	Not tested
B	**2, 4- D 2 mg/l and kinetin 0.5 mg/l kinetin**	**90**	**No result**	**No result**	**14**	**No result**	**No result**	**No result**	**No result**	**No result**	**+++**	**No result**	**No result**
C	BAP 2 mg/l and NAA 0.1 mg/l	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result
D	Kinetin 2 mg/l and IAA 2 mg/l	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result	No result
E	Kinetin 2 mg/l and IAA 1.5 mg/l	20	30	Not tested	26	19	Not tested	Light green compact hard	Light green hard	Not tested	+	+	Not tested
F	2,4-D 2 mg/l and 1 mg/l kinetin	Not tested	27	18	Not tested	23	16	Not tested	Green compac t	Green hard	Not tested	+	+

Table 10 continued……

G	2,4-D 2 mg/l and kinetin 1.5 mg/l	Not tested	Not tested	15	Not tested	Not tested	17	Not tested	Not tested	GCH	Not tested	Not tested	+
H	2,4-D 1 mg/l and kinetin 1 mg/l	Not tested	60	35	Not tested	19	19	Not tested	White yellow hard	White Compact hard	Not tested	++	+
I	2,4-D 1.5 mg/l and 1 mg/l Kinetin	56	60	60	23	17	15	Friable yellow	Friable yellow	Friable yellow	++	++	++
J	BAP 0.5 mg/l and 0.5 mg/l NAA	45	65	65	24	11	12	Green compact hard	Green compac t hard	Green compact hard	+	++	++
L	**BAP 1.0 mg/l and 1.0 mg/l NAA**	**Not tested**	**70**	**68**	**22**	**13**	**11**	Green compact hard	Green compac t hard	Green compact hard	**Not tested**	**+++**	**++**
M	2,4-D 2 mg/l and 1 mg/l BAP	Not tested	No result	No result	Not tested	No result	No result	Not tested	No result	No result	Not tested	No result	No result
N	2,4-D 2 mg/l and 0.5 mg/l BAP	Not tested	No result	No result	Not tested	No result	No result	Not tested	No result	No result	Not tested	No result	No result

Degree of callus formation: + = Poor callusing, ++ = Moderate callusing, +++ = Good callusing

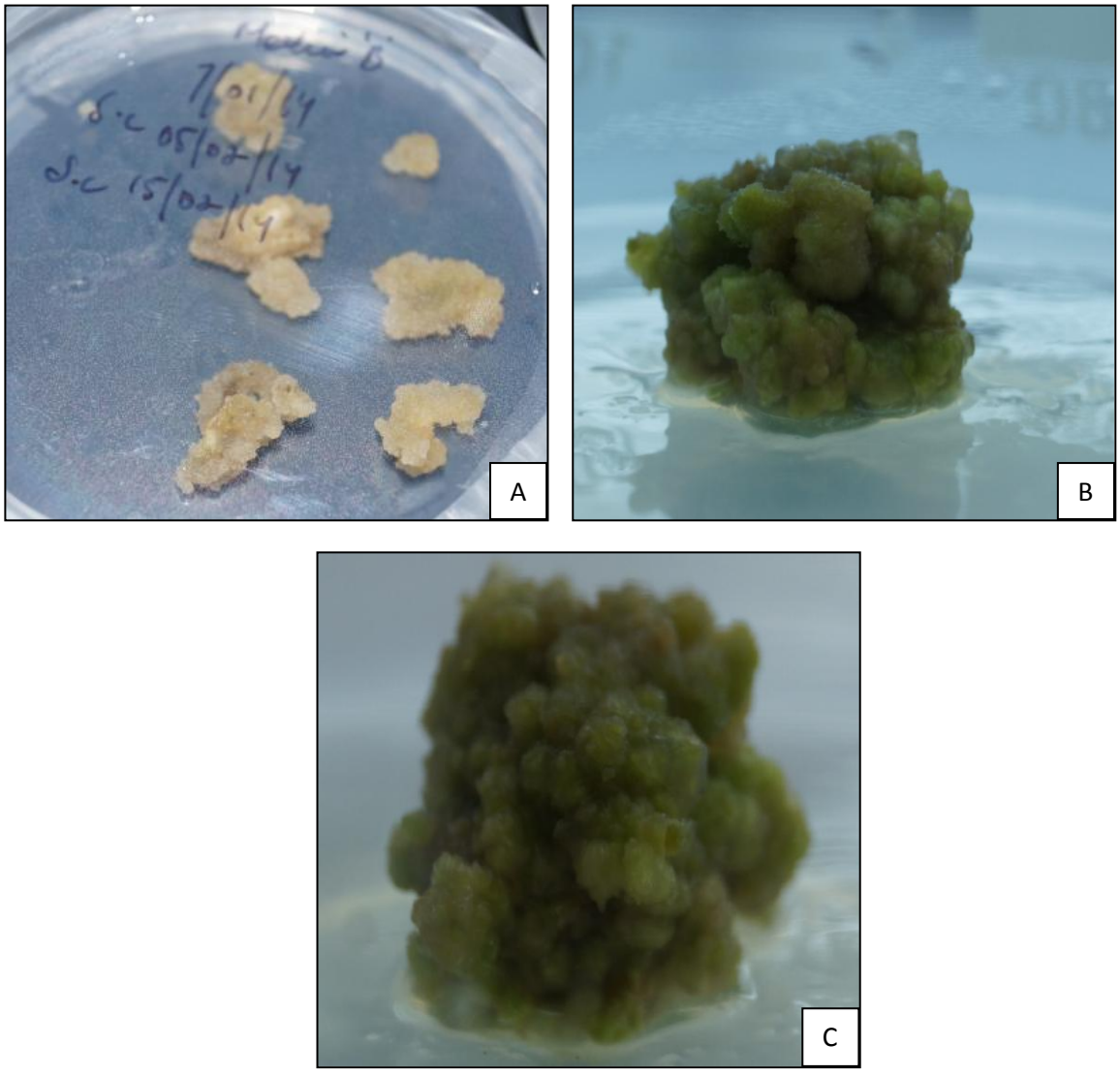


Plate 8. Proliferation of callus of three varieties of *P. grandiflora*

8 A. Sub culturing twice after 14 days interval on media containing 2, 4-D 2 mg/L and Kinetin 0.5 mg/L. 8 B. Proliferation of callus after sub culturing twice after 14 days in variety Giant California 8 C. Nana Compecta on media containing 1 mg/l both BAP and NAA.

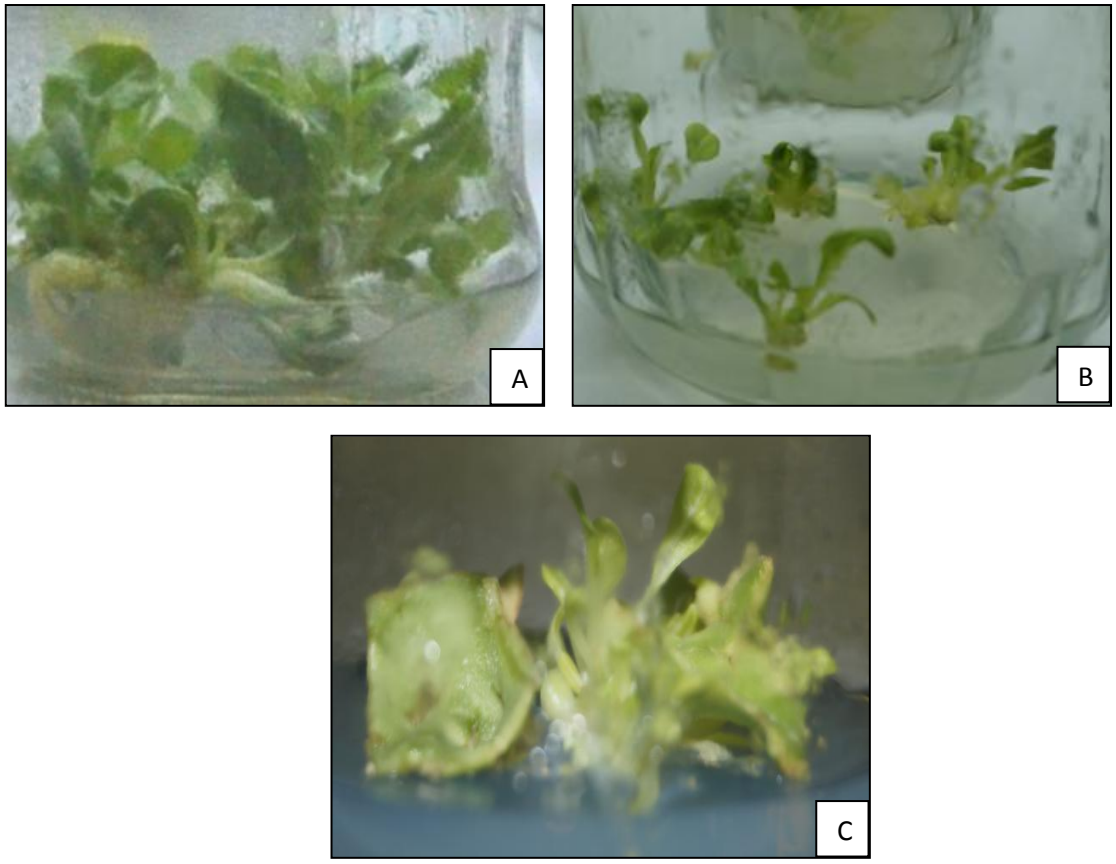


Plate 9. Direct shoot induction from leaves of three varieties of *P. grandiflora*
9 A and 9 B. on media containing 2 mg/l BAP and 0.1 mg/l NAA and 9 C on media containing kinetin.

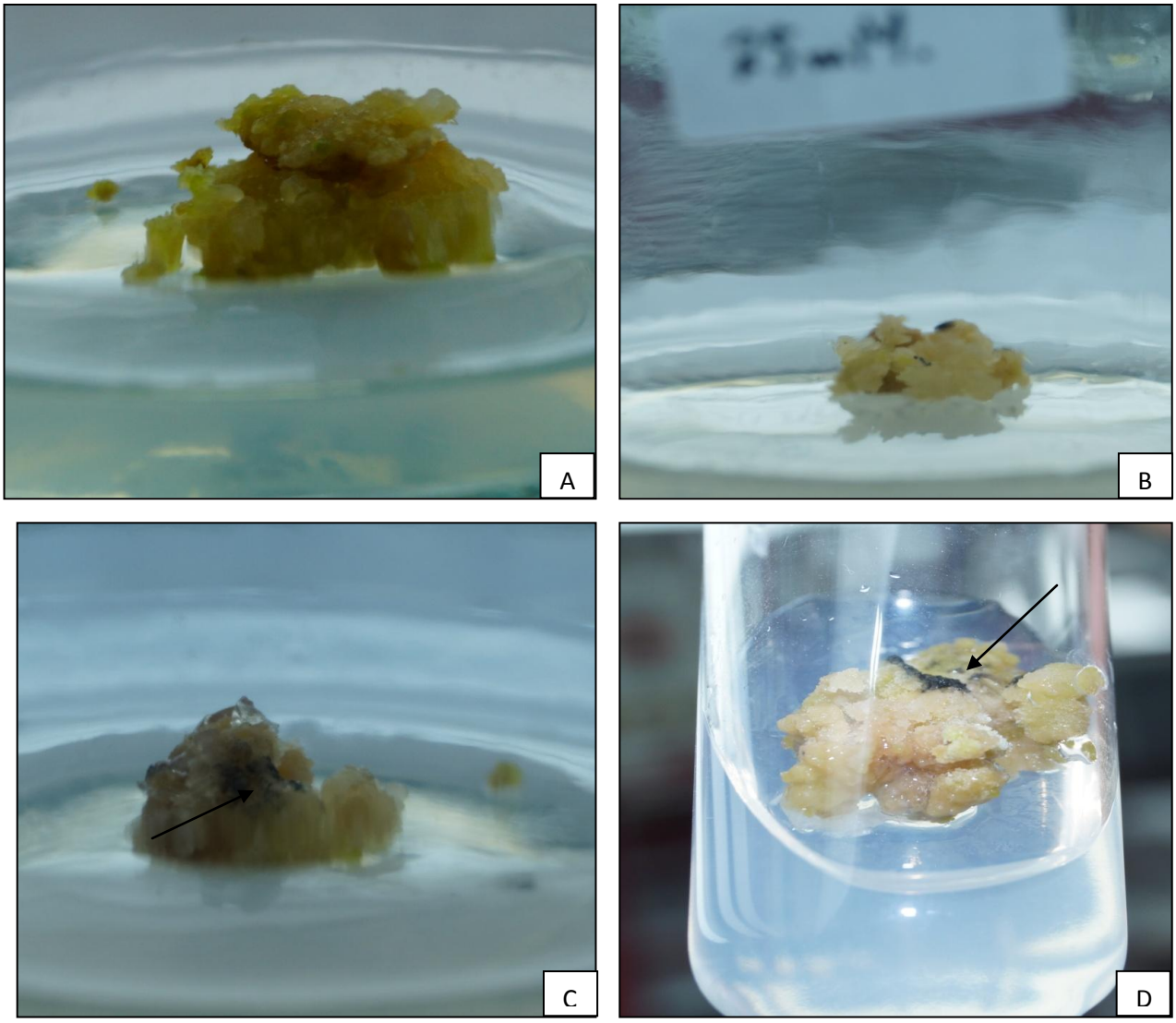
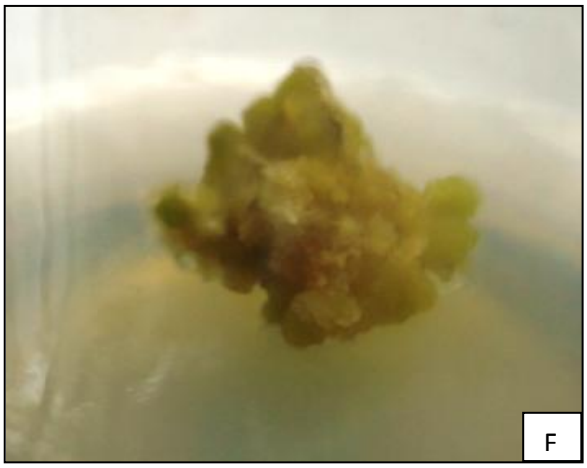


Plate.10. Stepwise adaptation by sequential treatment of callus from low to high concentration of salt.

10 A. Control, 10 B. Callus treated with 25 mM NaCl, 10 C. Callus treated with 50 mM NaCl (arrow showing necrosis), 10 D. Callus treated with 75 mM (arrow showing necrosis)



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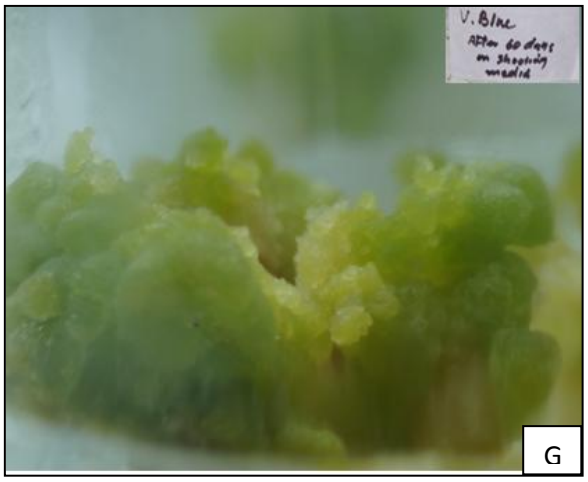

Plate.11. Stepwise adaptation by sequential treatment of callus from low to high concentration of salt

11 E. Callus treated with 150 mM (Burning and death of callus) and 11 F and 11 G. Photograph of selected callus on regeneration media treated with 75 mM NaCl taken after 45 days and 60 days, respectively.

4.8. Effect of salinity on callus growth in stepwise selection procedure

The proliferation response in terms of callus growth is difficult to express qualitatively. Therefore, its narration is based on increase or decrease in growth of callus based on visual observation only. In stepwise method, a slight decline in growth was observed in calli inoculated on media with 25 mM salt concentration as compared to control. In control, almost calli showed better growth but slight necrosis was observed in some culture vessels. As the calli selected from 25 mM were transferred to vessels containing 50 mM salt, necrosis of calli increased (Plate 10C). Slowest growth of calli was observed at 75 mM. Further, when these calli were transferred to 150 mM salt concentration during fourth sub culture, death of callus occurred (Plate 11E). Therefore calli obtained after 75 mM salt treatment were shifted to shoot inducing media for shoot regeneration. These selected calli showed better proliferation and turned green (Plate 11G). It was observed that increase in salt concentration decreased the growth of callus till tolerance level of callus reached, beyond which callus death occurred.

4.9. Effect of salinity on callus growth in direct selection method

In direct selection procedure, calli showed normal growth similar to that of control (Plate 12B) after 50 mM salt treatment for 25 days. Further increase in salt concentration, reduced the callus size. Growth reduction and necrosis occurred after treatment with 200 mM and 250 mM NaCl (Plate 13E and 13F). Further sub culturing increased necrosis. However, after these treatments, calli tolerant to salt showing healthy growth were selected. These were further cultured on regeneration media for shoot regeneration. Same results were obtained in calli of variety Giant California where salt inhibited growth at increased concentrations. In Giant California, good growth of callus was observed up to 100 mM, but at 150 mM concentration the growth of callus started to decline and necrosis occurred in all the vessels. At 200 mM, all calli become brown and their death occurred (14E). In comparison, calli of variety Violet Blue showed complete death at 300 mM (Plate 13G) concentration, but necrosis in this variety reached to maximum at 200 mM to 250 mM concentration. However, some calli after 200 mM treatment and all calli after 250 mM salt treatment

obtained through direct selection did not sustain good growth and died when maintained on salt supplemented media beyond 52 days.

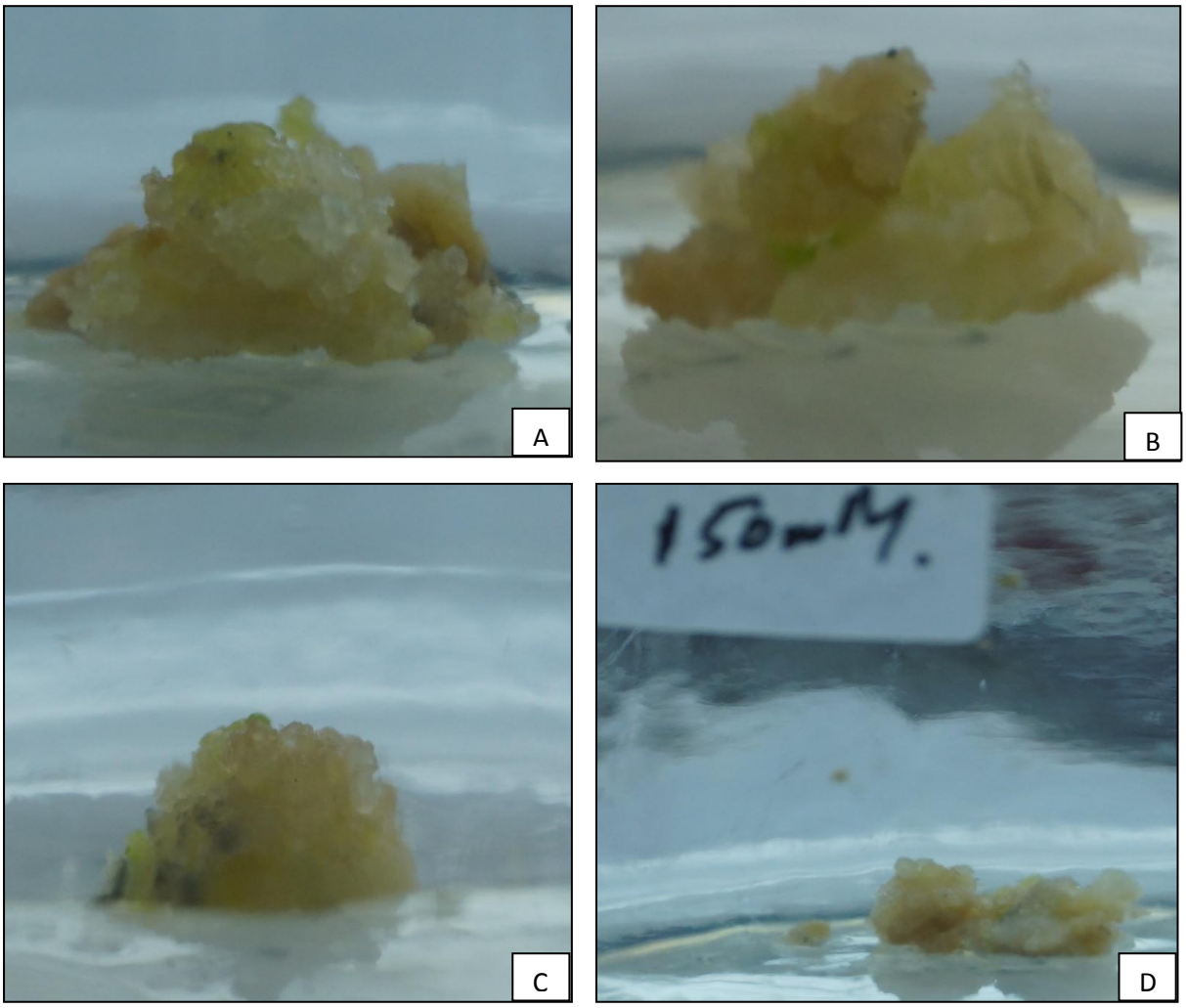

Plate 12. Direct treatment of calli of *P. grandiflora* variety Violet Blue with different salt concentrations

12 A. Control, 12 B. Callus on 50 mM salt, 12 C. Callus on 100 mM salt, 12 D. Callus on 150 mM salt

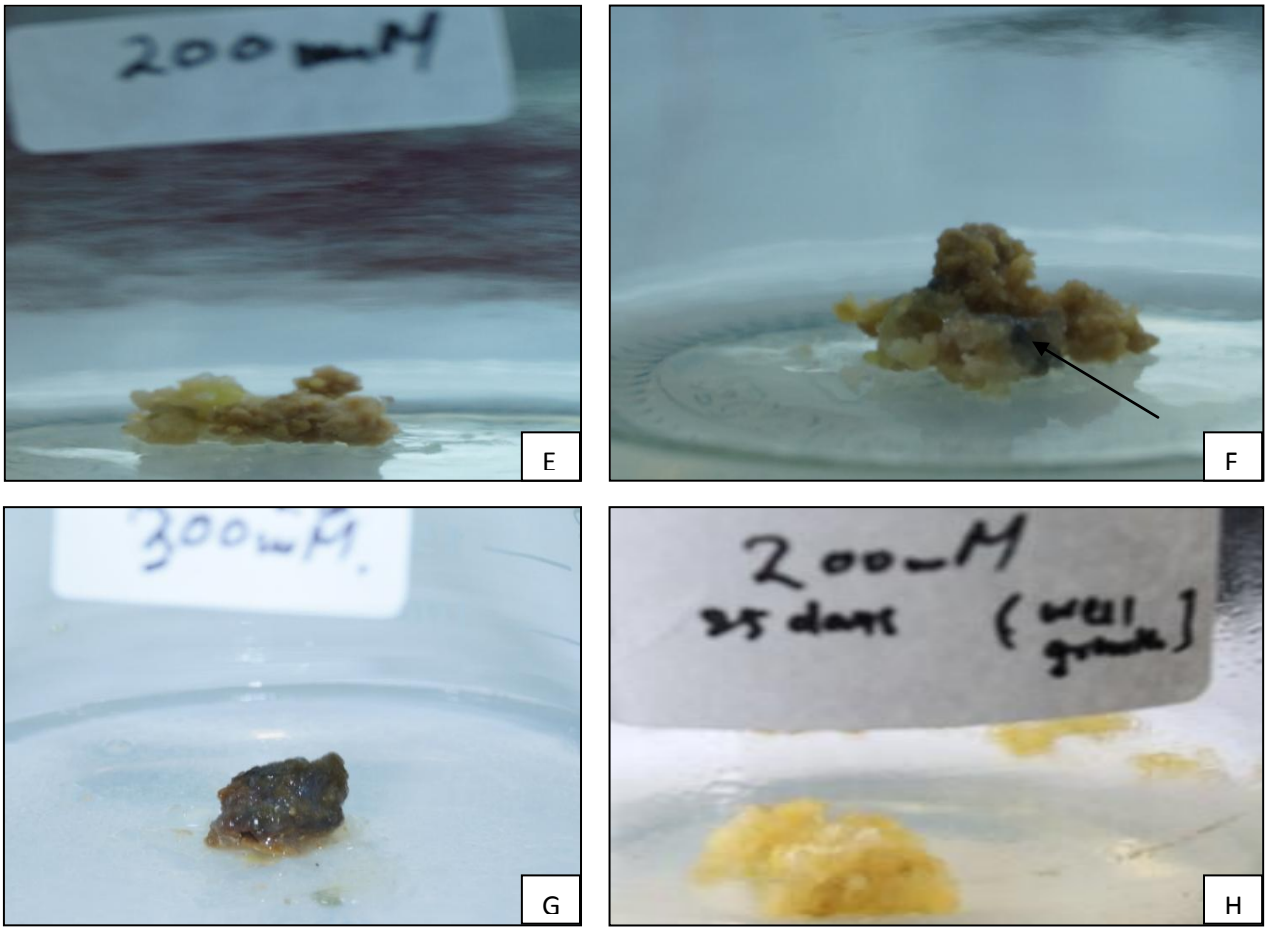

Plate 13. Direct treatment of calli of *P. grandiflora* variety Violet Blue on different salt concentrations

13 E. Callus on 200 mM salt, 13 F. Callus on 250 mM salt, 13 G. Callus on 300 mM salt, (complete death of callus) 13 H. Selected calli showing good growth at 200 mM salt concentration among 8 replicates.

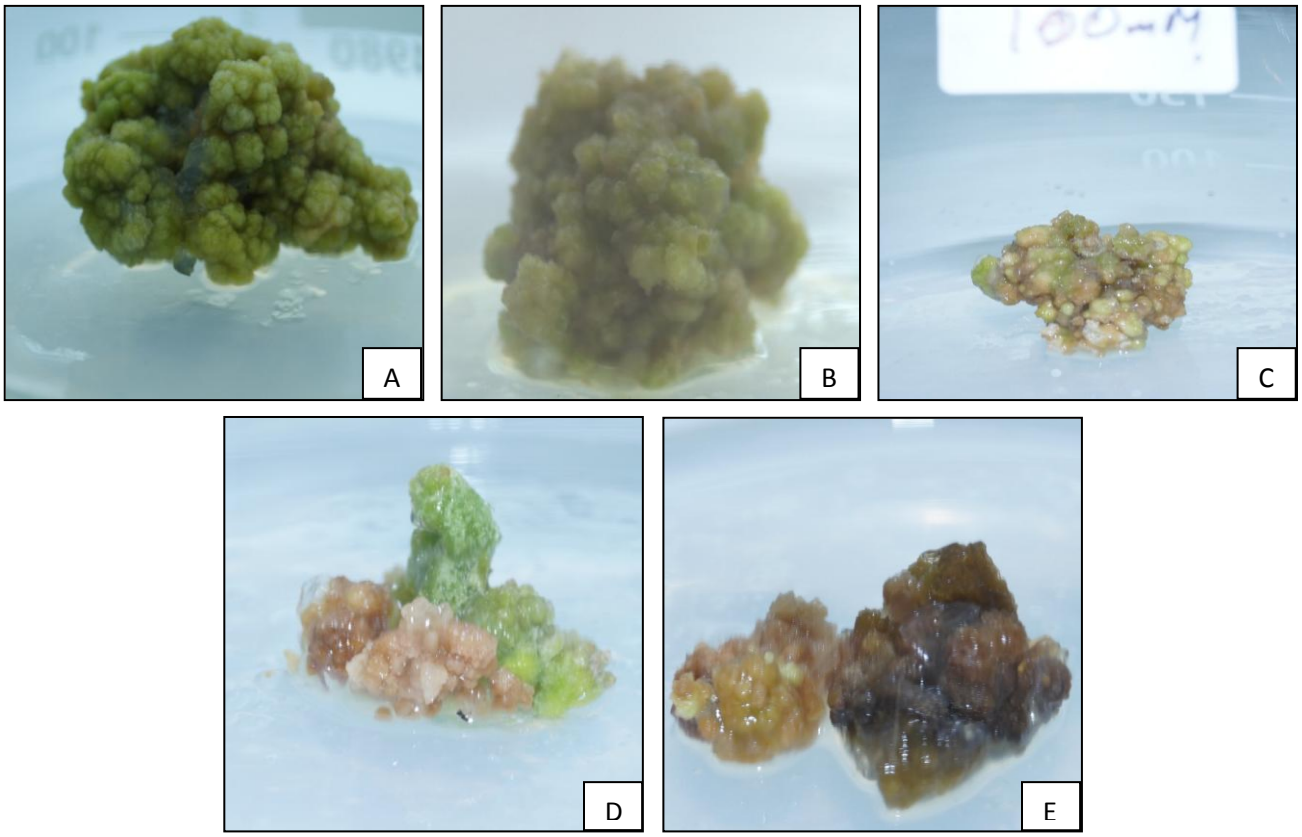

Plate 14. Direct treatment of Calli of *P. grandiflora* variety Giant California with different salt concentrations

14 A. Control, 14 B. Callus on 50 mM salt, 14 C. Callus on 100 mM salt, 14 D. Callus on 150 mM salt, 14 E. Callus on 200 mM salt (death of callus).

Table 11. Characteristics of callus of variety Violet Blue before and after NaCl treatment

Status of callus		
NaCl concentration	**Before NaCl treatment at 25 days after sub culturing for proliferation**	**After NaCl treatment at 25 days on media containing salt**
	Characteristics	
0 mM	Granular yellow and soft	Granular yellow and soft
50 mM	Granular yellow and soft	Granular yellow and soft, little necrosis
100 mM	Granular yellow and soft	Friable, brownish granular and necrosis increased
150 mM	Granular yellow and soft	Brownish yellow with necrotic spots
200 mM	Granular yellow and soft	Brownish yellow, granular with necrotic spots
250 mM	Granular yellow and soft	Blackish brown, friable and necrotic
300 mM	Granular yellow and soft	Blackish brown

Table 12. Characteristics of callus of *P. grandiflora* of variety Giant California before and after NaCl treatment

Status of callus		
NaCl concentration	**Before NaCl treatment at 25 days after sub culturing for proliferation**	**After NaCl treatment at 25 days on media containing salt**
	Characteristics	
0 mM	Greenish granular, compact and hard	Greenish granular, compact and hard
50 mM	Greenish granular, compact and hard	Greenish granular, compact and hard
100 mM	Greenish granular, compact and hard	Greenish yellow and granular, compact and hard
150 mM	Greenish granular, compact and hard	Greenish yellow, compact with necrotic spots and soft
200 mM	Greenish granular, compact and hard	Blackish brown, friable and necrotic

4.10. Characteristics of callus before and after salt treatment

Characteristics of callus of varieties Violet Blue and Giant California, changed with addition of salt in the medium over successive sub cultures. The characteristics of calli of varieties Violet Blue and Giant California are given in tables 11 and 12, respectively. It was observed that the calli without the addition of salt in medium at 25 days of growth were granular yellow and soft for variety Violet Blue. When shifted to media supplemented with salt (50 mM) slight necrosis occurred. With 100 mM and 200 mM salt, the calli became brownish in color, the necrosis increased and calli became soft. At 250 mM blackish brown calli of variety Violet Blue was observed. At 300 mM salt concentration the calli became blackish brown and death occurred (Plate 13G). In variety Giant California the callus was green, compact and hard before NaCl treatment. As salt was added to the media, variation in morphology and color

occurred. Up to 50 mM, callus was similar to the control but at 100 mM it became greenish yellow. At 150 mM, necrotic spots appeared and the calli were soft in nature. Calli became blackish with necrosis all over at 200 mM salt concentration (Plate 14 E).

4.11. Effect of hormonal treatments on callus characteristics

Different characteristics of calli were observed in all the three varieties at different hormonal concentration (table 13). As on media 'I' variety Violet Blue was whitish yellow (Plate 15B) and varieties Giant California and Nana Compecta was friable yellow. But on media 'B' variety Violet Blue was yellow granular and slight whitish. On media 'L' varieties Nana Compecta and Giant California was greenish compact hard and granular and variety Violet Blue was yellow green and whitish granular (Plate 15 D,E and F respectively).

Table 13. Effect of different hormonal treatments on callus characteristics of three varieties of *P. grandiflora*

Sr. No.	Plant growth regulator mg/l	Media designation in study	Varieties		
			Violet Blue	Giant California	Nana Compecta
1	2, 4-D 2 mg/l and kinetin 0.5 mg/l	B	Yellow whitish granular	No result	No result
2	1.5 mg/l 2,4-D and 1 mg/l Kinetin	I	Whitish yellow friable	Yellow friable	Yellow friable
3	1 mg/l BAP and 1 mg/l NAA	L	Yellow green and whitish granular	Greenish compact hard, granular	Greenish compact hard and slight granular

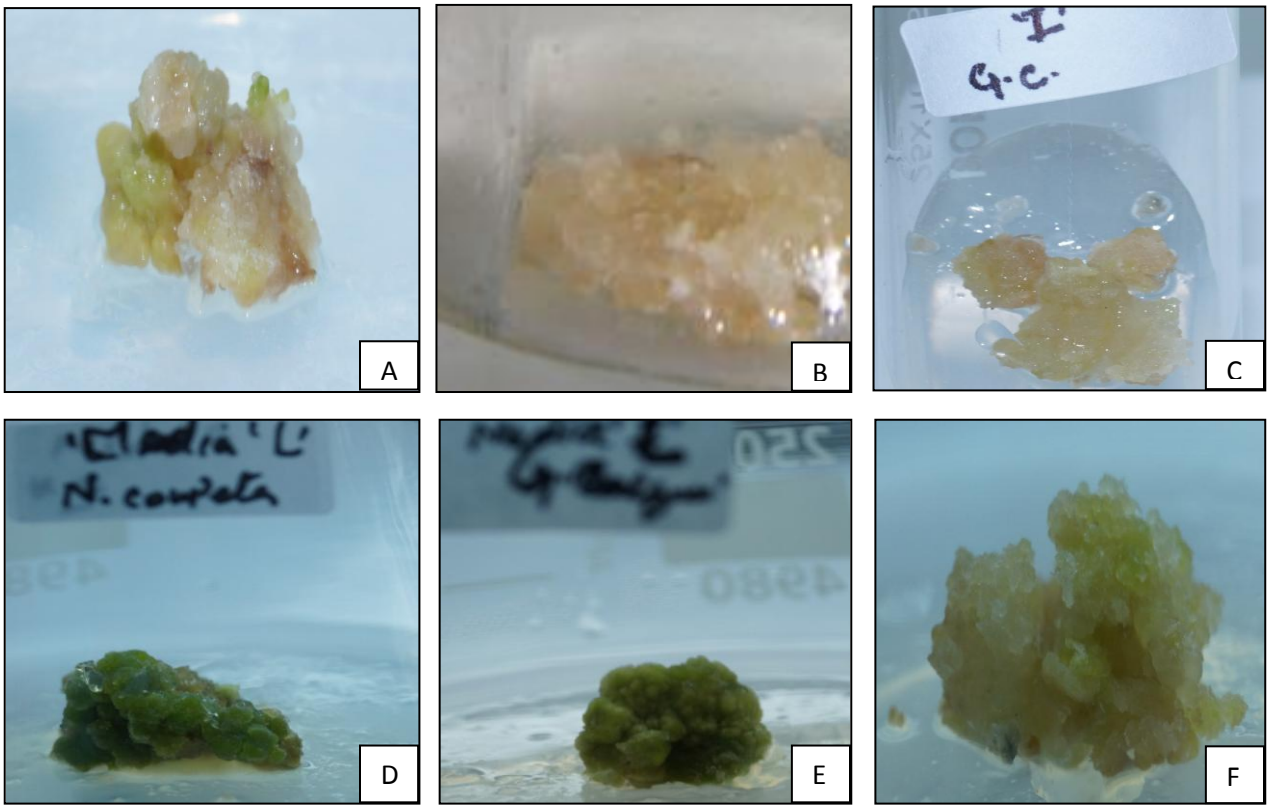

Plate 15. Characteristics of calli after different PGR treatments.

15 A. Violet Blue on media ‘B’ (yellow whitish granular), 15 B and 15 C. Violet Blue (friable yellow whitish) and Giant California (friable yellow) on media ‘I’, 15 D, 15 E and 15 F**.** Nana Compecta, Giant California and Violet Blue (green yellowish and whitish granular) on media ‘L’, respectively.

4.12. Effect of Plant Growth Regulators on shoot induction from leaves

Kinetin 2 mg/l alone and BAP 2 mg/l with NAA 0.1 mg/l (Media “C”) induced shoots direct from the leaves (Table 14). Media “C” induced shoots 14 days after and media “K” induced shoots 25 days after inoculation. Formation of roots was also observed one month after by repeated sub culturing of explants of *P. grandiflora* in all the three varieties. Among the three varieties, shoot emergence was first noticed in variety Nana Compecta 15 days after inoculation as compared to variety Violet Blue where the shoots appeared after 17 days. In variety Giant California shoots appeared after 19 days. Highest percentage of shoots was observed in variety Nana Compecta (90%), followed by Violet Blue (86.27%) and in Giant California (77.77%) (Table 15) cultured on media containing BAP 2 mg/l and 0.1 mg/l NAA. The other media tested also produced shoots but the percentage was less. Media “I” induced shoots in variety Nana Compecta (42.85%), in variety Violet Blue (38%) and in Giant California (42.85%). Media “J” induced shoots in variety Nana Compecta (41.37%) and in Giant California (32.14%). Similarly media “L” induced shoots in variety Nana Compecta (39.28%), in variety Violet Blue (28.57) and in Giant California (35.71%). The shoots were sub cultured for further proliferation on media “C” (Table 10 and Plate 16) as these media gave better percentage shoot formation as compared to other media tested by me.

4.13. Effect of salt on *in-vitro* shoots

After 3 weeks of sub culturing, shoots were excised and these were subjected to treatments with different concentrations of salt. The salt concentrations used were 50 mM, 100 mM, 150 mM, 200 mM and 250 mM. After eight days of inoculation on media with salt, it was observed that with increase in salt concentration the margins of leaves started burning and subsequently entire leaf became yellow and finally it died. In variety Violet Blue control plants showed the healthy growth with root formation. Up to a concentration of 100 mM salt, pants were growing efficiently but the growth was less compare to control plants. At 150 mM salt concentration, margins of leaves

started burning and the leaves subsequently wilted (Plate 17D). The burning of leaves was less as compared to the explants at 200 mM and 250 mM salt concentration (Plate 17E and 17F). Shoots of variety Violet Blue at 300 mM salt concentration also showed complete burning of leaves and these shoots wilted 30 days after inoculation on this salt treated media (Plate 17G). Both stem and leaves of plantlets at high salinity under *in-vitro* conditions were yellowish in color with shoot tip dying with stunted growth, poor root development and burning on further growth. During sub culturing of the plantlets of all the three varieties, root formation was observed mostly in control as well as in 50 mM and at 100 mM salt concentrations. But at 50 mM and 100 mM salt concentrations, root formation was comparatively lesser as compared to control. Several of these shoots survived when cultured on same media with highest concentration of salt and produced healthy roots.

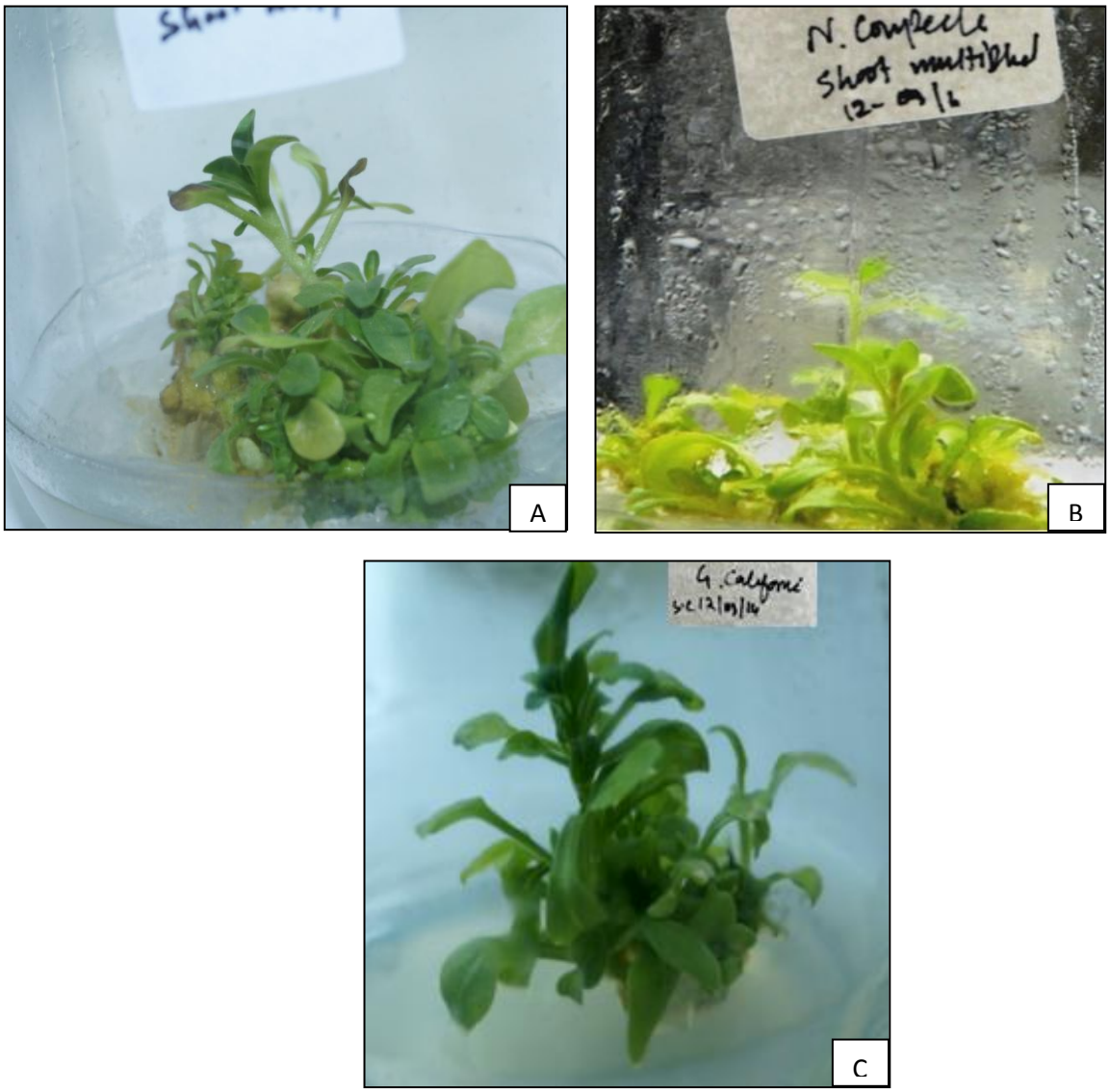

Plate 16. Shoots of three varieties of *P. grandiflora* sub-cultured on shoot multiplication media

16 A. Shoot multiplication in Violet Blue, 16 B. Shoot multiplication in Nana Compecta, 16 D. Shoot multiplication in variety Giant California.

Table 14. Effect of Plant Growth Regulators (PGR) in MS media on *in-vitro* leaf explants of three varieties of *P. grandiflora*

Plant growth regulators tested	Days to shoot initiation			% shoot formation		
	Varieties					
	Violet Blue	Giant California	Nana Compecta	Violet Blue	Giant California	Nana Compecta
BAP 2 mg/l and 0.1 mg/l NAA	17	19	14	86.27	77.77	86.27
Kinetin 2 mg/l	25	28	29	81	65	64
BAP 0.5 mg/l and 0.5 mg/l NAA	27	22	19	45	43	52
1.5 mg/l 2,4-D and1 mg/l Kinetin	38	29	28	38	44	42.85
1 mg/l BAP and 1 mg/l NAA	Not tested	32	35	Not tested	32.14	41.37
BAP 1.0 mg/l and 1.0 mg/l NAA	41	29	31	28.57	35.71	39.28

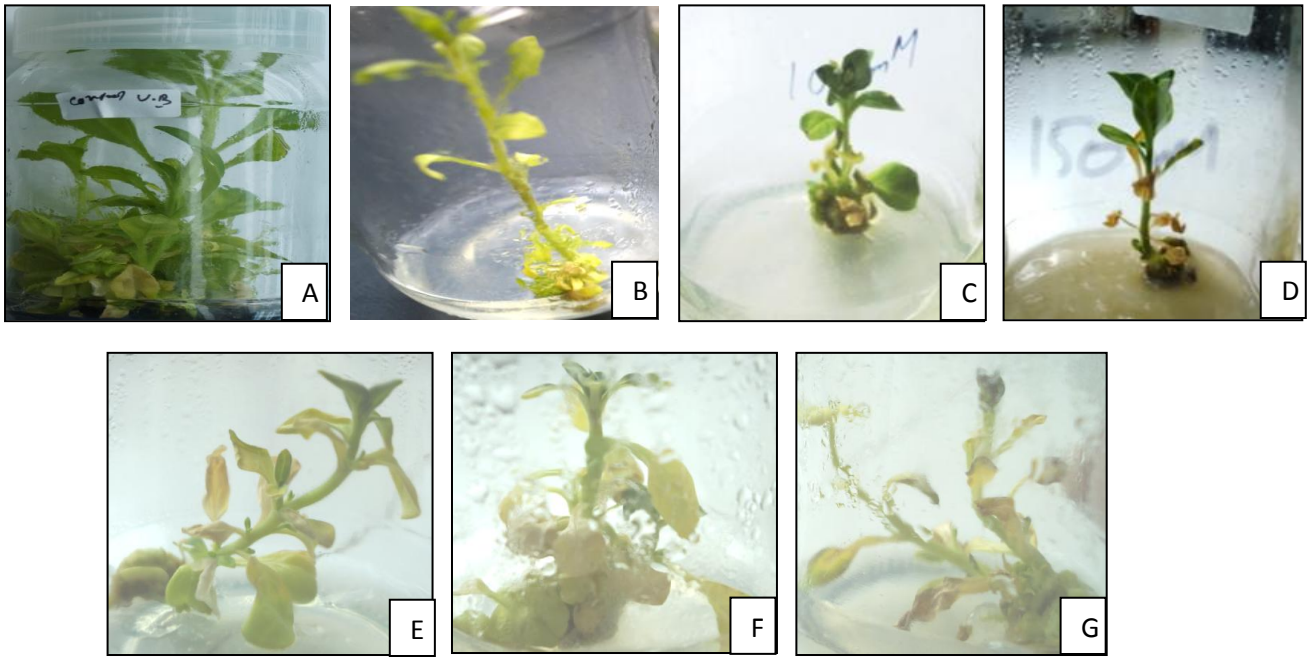

Plate 17. Shoots of *P. grandiflora* variety Violet Blue 14 days after treatment on different salt concentrations.

17 A. Control, 17 B. 50 mM, 17 C. 100 mM, 17 D. 150 mM, 17 E. 200 mM,17 F. 250 mM and 17 G. 300 mM (complete burning of shoot).

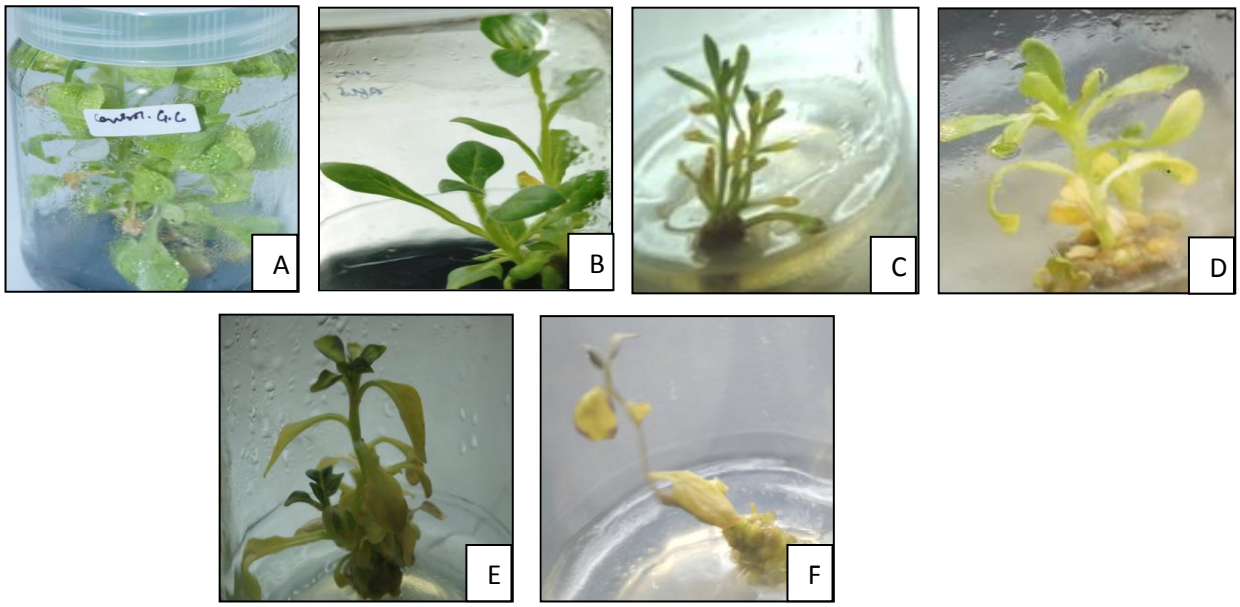

Plate 18. Shoots of *P. grandiflora* variety Giant California 14 days after treatment on different salt concentrations.

18 A. Control, 18 B. 50 mM, 18 C. 100 mM, 18 D.150 mM,18 E. 200 mM and 18 F. 250 mM (complete burning of *in-vitro* shoot).

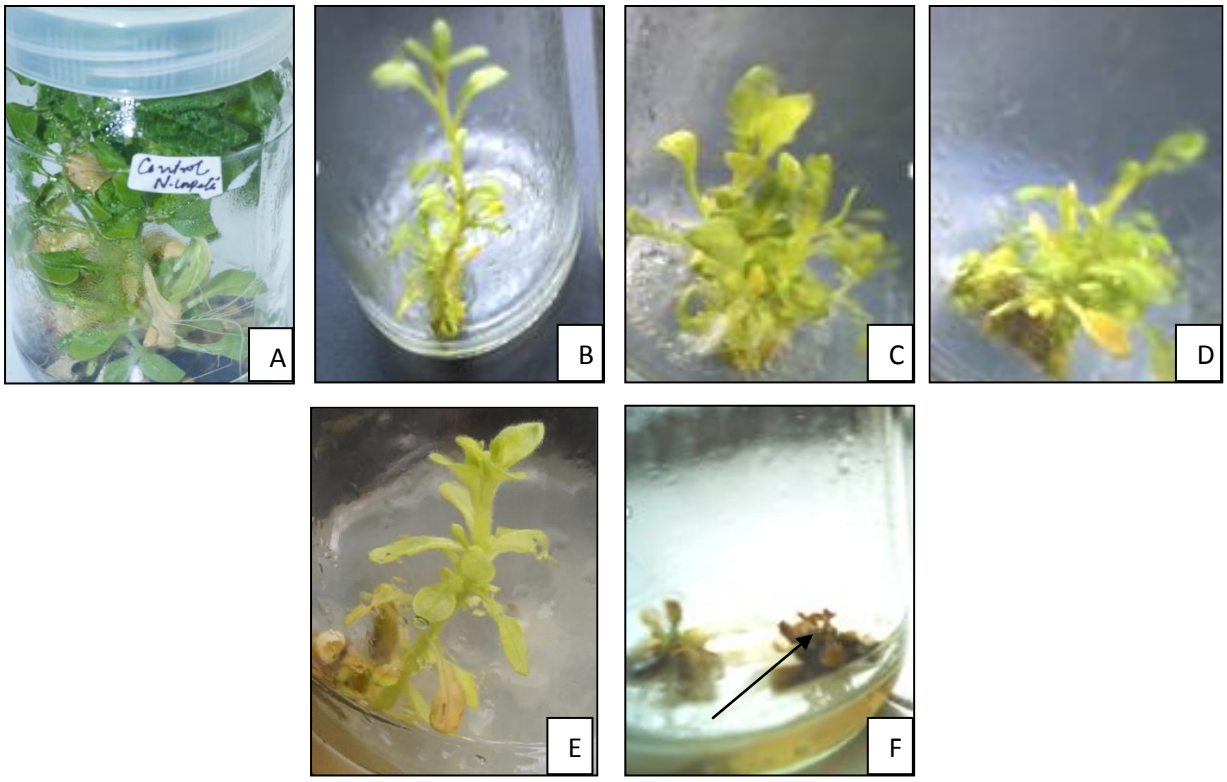

Plate 19. Shoots of *P. grandiflora* variety Nana Compecta 14 days after treatment on different salt concentrations.

19 A. Control, 19 B. 50 mM, 19 C.100 mM, 19 D.150 mM, 19 E. 200 mM, 19 F. 250 mM (Arrow showing complete burning of *in-vitro* shoot).

The observations of effect of different salt concentrations on survival of *in-vitro* shoots of the three varieties of *P. grandiflora* are given in (Table 15). Shoots of variety Giant California treated with 50 mM, 100 mM, 150 mM, 200 mM and 250 mM salt, showed complete death at 250 mM (Plate 18F). At 50 mM concentration explant shoots were similar to control plants but secretion of phenolic compounds was observed. At 100 mM and 150 mM leaves started burning which increased with increase in phenolic compound secretion. This resulted in inhibition of growth and also death of some explants. Variety Nana compecta shoots treated up to 50 mM salt concentration during first 14 days of inoculation, showed good growth but with increase in salt concentration from 100 mM to 250 mM, leaves started burning and become yellow (Plate 19E and 19F). As they were sub cultured, shoots treated with 100 mM and 150 mM showed good growth but growth was less as comparable to control. Further increase in salt concentration beyond 150 mM inhibited growth, yellowing of leaves occurred and finally the explants died. Comparing the survival percentage of all the three varieties, Violet Blue at 200 mM showed 57.5% survival of explants, while varieties Giant California and Nana Compecta showed only 10% survival (Table 15). Shoots of Violet Blue were less susceptible to salts as compared to the other two varieties. At 250 mM the survival percentage of both the varieties Nana Compecta and Giant California was 5% but the survival percentage for Violet Blue was 48.33%. These results clearly show that salt treatment has significant effect on shoot survival but this was variable among the varieties.

In-vitro explants of three varieties of *P. grandiflora* were grown for two sub cultures under continuous salt stress. For varieties Nana Compecta and Violet Blue 100% survival was observed in control. With 50 mM salt concentration, the effect of salt was less pronounced in all the three varieties but survival percentage of explants decreased with increase in salinity as shown in table 15. The lowest survival percentage of 10% was observed in variety Violet Blue at 300 mM concentration. Similarly 5% survival was observed both for varieties Giant California and Nana Compecta at 250 mM salt concentration. These results clearly show that with increase in salinity there is a decrease in survival percentage. A comparison of survival percentage of the three varieties in salt stress suggests (Table 16) that *in-*

vitro shoots of Violet Blue are more tolerant to salt stress as compared to varieties Giant California and Nana compecta. A comparison of the three varieties at 250 mM concentration indicate that survival percentage of Violet Blue was 48.34% and only 5% in both the varieties Giant California and Nana Compecta. At 150 mM concentration, 32.5%, 48.33% and 65% survival was observed in varieties Giant California, Nana compecta and Violet Blue, respectively. The effect of salinity in relation to the number of sub cultures indicates that the lowest survival percentage was observed in first sub culture (54.29%) in variety Violet Blue, 51.67%, in Giant California and 53.33% in Nana compecta (Table 15) which gradually increased with sub culturing in all the three varieties except in Giant California where it shows decreased percentage 43.98% (Table 15). In second subculture, the survival percentage was 68.57% and 56.35% in varieties Violet Blue and Nana Compecta, respectively (Table 15). It indicates that variety Giant California is more sensitive to salt stress as compared to other two varieties.

Surviving shoots from all the three varieties were selected and further cultured for 3 weeks at salt concentrations (mentioned below) and latter transferred to rooting media with same salt concentration. Variety Nana compecta showed good growth as compared to Giant California. Few explants started to show burning of leaves at 100 mM and 150 mM. At 200 mM and 250 mM burning spread fast towards leaf base and phenolic secretion from explants was also observed (Plate 19E and 19F). These were sub cultured on salt containing media for three weeks and surviving shoots at 100 mM of varieties Giant California and Nana Compecta and 250 mM concentration of variety Violet Blue were selected and sub cultured for rooting.100 mM salt concentration was highest concentration for Nana compecta and Giant California and 250 mM for variety Violet Blue for further sub culturing for root induction.

Table 15. Effect of salt on survival percentage of explants (*in-vitro* shoots) of three varieties of *P. grandiflora* after sub culturing

Varieties									
Violet Blue				Giant California			Nana Compecta		
Treatment Mm	Subculture I %	Subculture II %	Mean	Subculture I %	Subculture II %	Mean B	Subculture I %	Subculture II %	Mean
0	100	100	**100**	90.00	100	**95.00**	100	100	**100**
50	80.00	100	**90**	90.00	88.88	**89.44**	90.00	100	**95.00**
100	60.00	83.33	**71.67**	60.00	50.00	**55.00**	70.00	71.42	**70.71**
150	50.00	80.00	**65.00**	40.00	25.00	**32.50**	30.00	66.66	**48.33**
200	40.00	75.00	**57.50**	20.00	0.00	**10.00**	20.00	0.00	**10.00**
250	30.00	66.66	**48.33**	10.00	0.00	**5.00**	10.00	0.00	**5.00**
300	20.00	0.00	**10.00**	Not tested	Not tested	**Not tested**	Not tested	Not tested	**Not tested**
Mean	**54.29**	**68.57**	**61.43**	**51.67**	**43.98**	**47.82**	**53.33**	**56.35**	**54.84**

4.14. Regeneration of shoots from salt treated callus

Nine media were tested for regeneration of shoots from callus but only media “L” and media “T” produced results and other media namely “C, U, V, W, X, Y,Z” does not produce results (Table 16). Both calli obtained from salt containing media and untreated calli were inoculated. The callus of different varieties showed different response to different regeneration media. The callus of Violet Blue started shoot initiation after 28 days of inoculation on regeneration media “T” (Table 16). But shoot initiation in this variety was observed in calli without salt media after 22 days. The calli of Violet Blue selected from 75 mM salt treatment under stepwise method was

inoculated for 70 days on media “C”. The callus proliferated sufficiently and become green but shoot regeneration was not initiated. However, regeneration of shoot was observed in calli of Violet Blue in direct treatment method treated with 50 mM salt concentration (Plate 20 B). Callus of varieties Giant California and Nana compecta initiated shoot formation 45 and 38 days after respectively, on media “L” (Table 16 and Plate 20C and 20D). These shoots were regenerated from callus treated with 50 mM salt in media “L. No result was observed from other media which were tested because after 50 days of inoculation, these cultures died.

4.15. Induction of roots

Three media were tested for induction of roots and among these media “R” did not produce any result for varieties Giant California and Nana Compecta (Table 17). When the shoots attained height of 3 cm to 4 cm these were sub cultured for inducing roots. Half strength MS media supplemented with different PGRs were tested for inducing roots (Table 17). IBA with 1 mg/l concentration initiated root induction after 14 days. BAP (2 mg/l and NAA 0.1 mg/l) was also tested and the root formation was observed after 25 to 32 days of repeated subculture of shoots (Plate 21C). On this media, root formation occurred in Variety Nana compecta after two subcultures followed by Giant California. Another media tested was 0.5 mg/l NAA and 0.1 mg/l IBA designated as media “S” in this study. It was observed that among the PGRs, media “S” induced roots in 8 days in Violet Blue (Plate 21B). Salt treatment inhibited root formation and no root formation occurred in shoots grown in media supplemented with above 100 mM salt concentration.

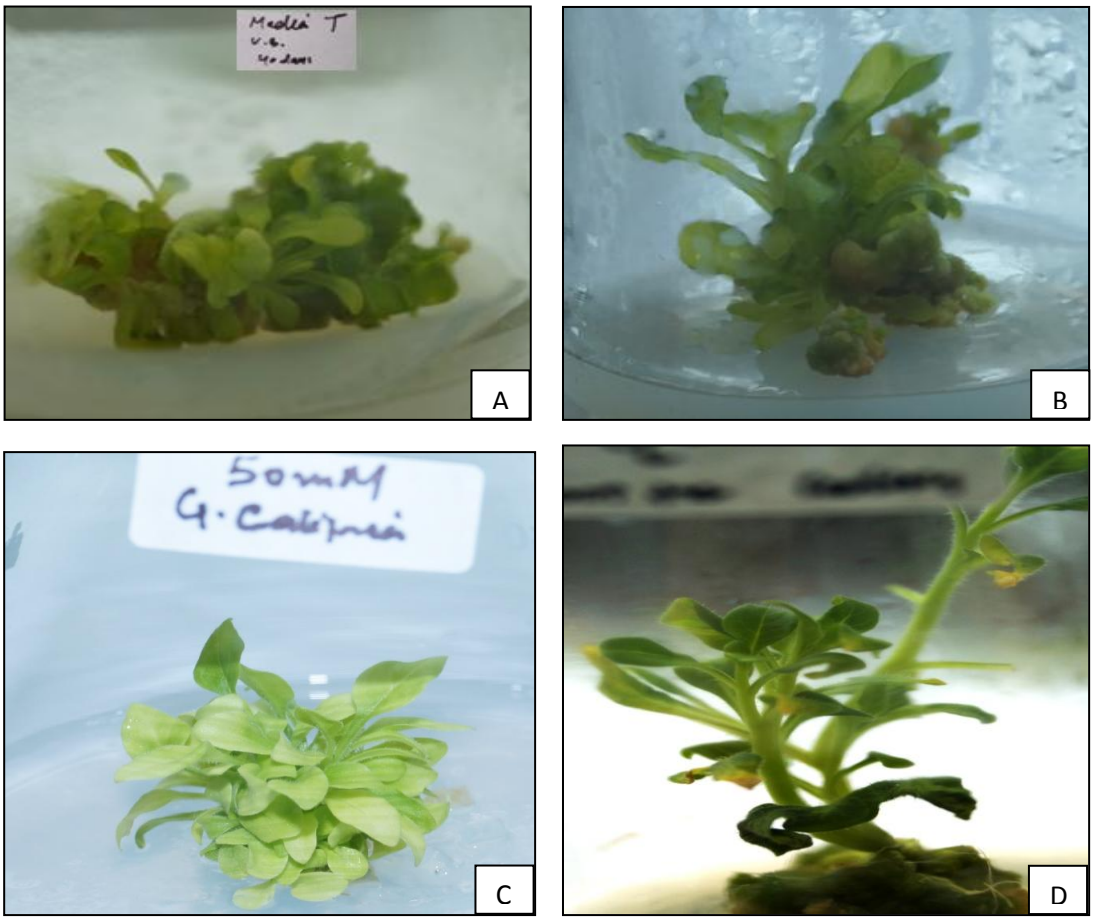

Plate 20. Shoot regeneration from callus of three varieties of *P. grandiflora.*
20 A and 20 B. Variety Violet Blue on media “T” supplemented with 50 mM salt concentration 40 and 50 days old respectively. 20 C and 20 D. Salt tolerant shoots of Giant California and Nana compecta on media “L” containing 50 mM concentration.

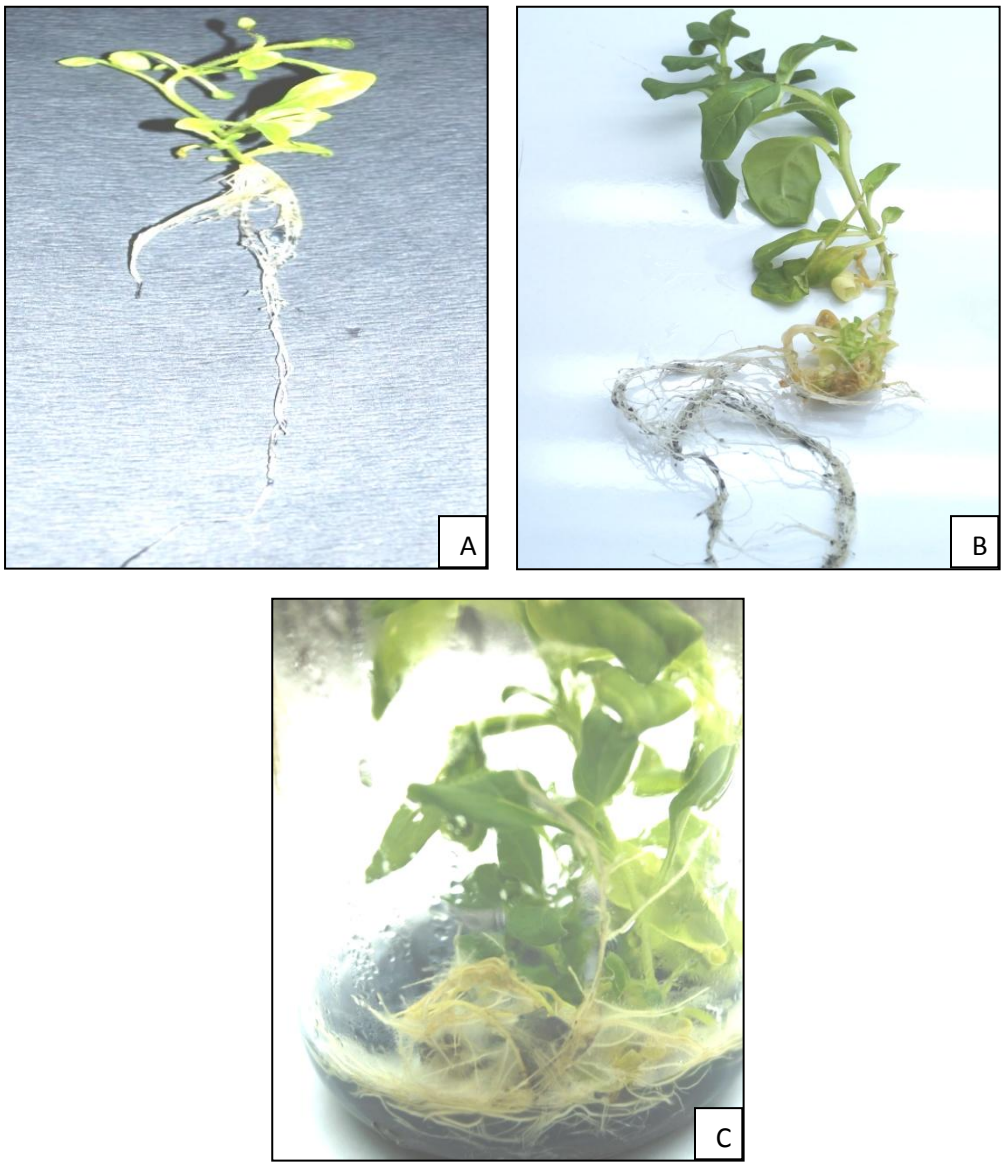

Plate 21. Shoots of varieties Giant California, Violet Blue and Nana Compecta on rooting media.

Giant California (21A), Violet Blue (21B) and Nana Compecta (Plate 21C) showing root induction on media (0.5 NAA and 0.1 mg/l IBA, media S).

Table 16. Effect of different Plant Growth Regulators (PGR) to induce shoots in treated callus of three varieties of *P. grandiflora*

Sr. No.	Media Designation	Variety	Composition mg/l	Response	Days to shoot initiation
1	**C**	Violet Blue	**2 mg/l BAP + 0.1NAA**	Callus became green only	Not determined
2	U	Violet Blue	Kinetin 3 mg/L + NAA 2 mg/l	No result	Not determined
3	V	Violet Blue	BAP 3 mg/l +1 mg/l NAA	No result	Not determined
4	W	Violet Blue	BAP 2.5 mg/l + Kinetin 0.5 mg/l	No result	Not determined
5	X	All three	2 mg/l BAP +1 mg/l NAA	No result	Not determined
6	Y	All three	2 mg/l BAP + 0.5 mg/l Kinetin + 0.1 mg/l NAA	No result	Not determined
7	**T**	**Violet Blue**	**2 mg/L BAP + 0.5 mg/l IAA**	**Shoot formation**	**28**
8	Z	Violet Blue	2.5 mg/l BAP + 1 mg/l IAA + kinetin 1mg/l	No result	Not determined
9	**L**	**Giant California and Nana Compecta**	**BAP 1.0 mg/l and 1.0 mg/l NAA**	**Shoot formation**	**Giant California: 45** **Nana Compecta: 38**

Table 17. Effect of different Plant Growth Regulators (PGR) in root induction in in-vitro shoots of three varieties of *P. grandiflora*

Sr. No.	Media Designation	Media Composition mg/l	Response	Varieties and days to root initiation		
				Violet Blue	Giant California	Nana Compecta
1	R	2 mg/l IBA	Rooting initiated	14	No result	No result
2	C	2 mg/l BAP and 0.1NAA	Rooting initiated	32	26	25
3	S	0.5 NAA and 0.1mg/l IBA	Rooting initiated	8	23	22

4.16. Hardening of rooted plant

When rooted shoots cultured *In-vitro* are transplanted to natural conditions, these can desiccate or wilt rapidly as a result of sudden change in environment, poor mesophyll differentiation and weak vasculature in leaves of plants produced under *in-vitro* conditions. Therefore, substantial precautions were taken to acclimatize them to natural conditions. Hence the environment *ex-vitro* was adjusted to accommodate transplants from culture by gradually weaning them towards ambient relative humidity, increasing light and temperature. When the plantlets attained their height up to 8-10 cms and had good root system, these were taken out from the culturing jars, washed properly to remove traces of agar and put on the cotton in a tray for four days. The tray was covered with polythene bag and plants were sprayed with water for 4 days (Plate 20A and 20B). Polythene bags were later perforated gradually to prevent sudden shock to plants and finally removed to pots after 10 days (Plate 20D).

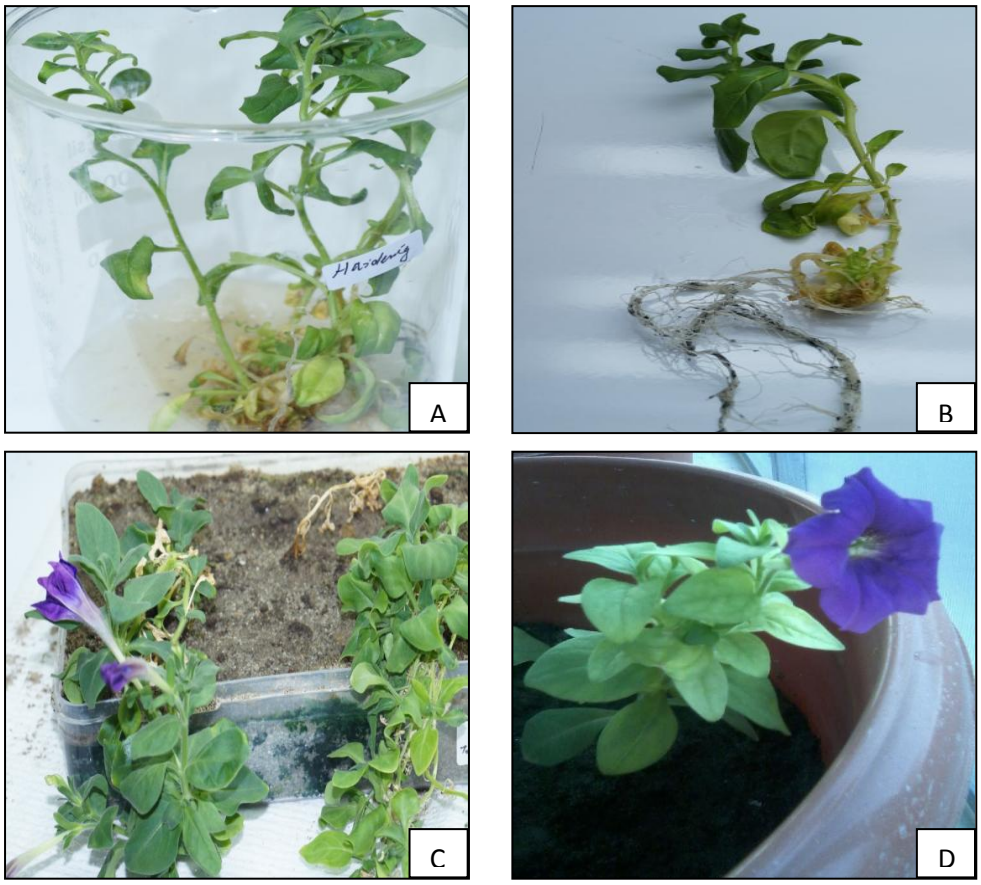

Plate 20. *In-vitro* plants shifted to trays and pots for hardening.

20 A and 20 B polythene cover removed from plants and ready to shift to growth chamber. 20 C plants in growth chamber. 20 D plants in glass house under natural condition.

CHAPTER V

DISCUSSION

Saline soils inhibit plant growth as osmotic potential of soil is enhanced due to excess salt concentration which restricts the water uptake by plants. Soil salinity induces abnormal morphological, physiological and biochemical changes that lead to reduction in growth and high seedling mortality. Due to high salt accumulation in leaves that exceeds the capacity of salt compartmentation in the vacuoles, raises salt level in cytoplasm to toxic levels (Munns, 2002). Thus excess of salinity reduces the plant growth affecting economic yield (Ando *et al.*, 2005). Desalination of the soil is an expensive process to achieve healthy growth of plants, therefore, to growing salt tolerant cultivars seems to be the only solution to increase production under conditions of soil salinity. Screening of plants successfully growing in saline soils is one of the methods to develop salt tolerant varieties. Using this strategy, the physiological, morphological, biochemical and enzymatic responses have been investigated at different developmental stages and reported in several crops (Ali *et al.*, 2002; Kamal *et al.*, 2003). Plant tissue culture facilitates propagation of plants under salt stress on large scale and further selection for salt tolerance. Therefore, the development of improved stress tolerant cultivars appears to be promising by using this technique. It is with this objective that selections for salt tolerance of three varieties of *P. grandiflora* has been attempted both by direct selection under salt stress as well as under *in-vitro* conditions using tissue culture techniques. The results obtained have been discussed in the following pages.

Effect of salt on survival of seedlings

The results indicate that increase in salt concentration in soil decreased survival of seedlings of all the three varieties of *P. grandiflora* but the response varied according to the varieties. This is in agreement with the results obtained in five medicinal plants *Lepidium sativum L., Linum usitatissimum L., Nigella sativa L., Planta goovata Forssk and Trigonella foenum-graecum L*. (Muhammad and Hussain, 2010). Among the three varieties tested at highest concentration of salt (600 mM), lowest survival of seedlings was observed in variety Giant California followed by the variety Violet Blue while the variety Nana Compecta showed maximum survival of seedlings. The reason for decrease in survival and growth of plants as salt

concentration may be due to salt accumulation in soil preventing roots to extract water from it and further salt accumulation within the plants might have inhibited many biological and physiological processes such as nutrient uptake and assimilation etc (Munns and Tester, 2008). Since variety Nana Compecta was affected the least, it is relatively tolerant and variety Giant California that was affected the most to salt stress is sensitive.

Deleterious effect of salinity on plant growth

Exposure of plants to different concentration of salt for different time durations, resulted in wilting of plants next day but some of them recovered after few days. Adaptation of plant to stressful conditions is a slow and gradual process and plants show slow growth in response to stress, which actually is a strategy of plants to reduce the deleterious effects of salt stress. Such adaptation not only save the energy which is utilized for growth but also prevent the heritable damage (May *et al.*, 1998). One of the initial effects of salt stress on plants is that growth rate is reduced. The ultimate effect of salt stress on plants depends upon the concentration and exposure time. It has been observed in the present study increase in salt concentration and exposure time reduced shoot growth which was partially recoverable.

After salt treatment most visual effect observed on salt treated seedlings was that leaves became pale yellow, dry and finally death of plants occurred while the plants in control continued to grow and attain good height, broad and green leaves. In all three varieties chlorosis was observed in leaves of salt treated seedlings but maximum chlorosis was observed at 600 mM salt treatment during 24 hrs. Among all the three varieties, leaves of variety Giant California showed maximum damage whereas variety Nana Compecta depicted less damage throughout the experiment. There are earlier reports that salt treatment increases Na^+ concentration in phloem where as K^+ decreases and in xylem sap Na^+ increases, and Na^+ accumulates in mesophyll cells, that leads to necrosis of leaf (Boughanmi *et al.*, 2003). However, no attempt to identify the basis of decline in growth and other visible effects was attempted. Soil salinity also delayed flowering initiation in all the three selected varieties as compared to control seedlings. Flowering delay has been also reported

in *Iris hexagona* (Van Zandt and Mopper, 2002), in Arabidopsis by (Ryu *et al.*, 2014). Flowering delay due to salt treatment is reported to disrupt GA signaling that promotes LFY expression and flowering (Achard *et al.*, 2006).

The growth of shoot length decreased with increase in salt concentration in all the varieties tested. This is in agreement with observations made on the effect of salinity on *Paulownia* clones grown *ex-vitro* (Miladinova *et al.*, 2013), in *Tagets* (Zapryanova and Atanassova, 2009), in *vicia faba* (Qados, 2011) and in (*Beta vulgaris*), cabbage (*Brassica oleracea* capitata L.), amaranth (*Amaranthus paniculatus*), in *Sorghum bicolor* cultivars (Chauhan *et al.*, 2012) and in *Brassica compestris* (Jamil *et al.*, 2006). A connection between increase in salt and decrease in shoot length has been reported for *Brassica chinensis* Memon *et al.*, (2010) also in *Suaeda salsa* plant height, number of branches, length of shoot and diameter of shoot were significantly affected by salt stress which was due to increased content of Na^+ and Cl^- (Guan *et al.*, 2011). It has also been observed that salinity stress decreased shoot and leaf number (Muhammad and Hussain, 2010).

Elevation in salt concentration reduced the number of leaves in all the three varieties. This is in agreement with the observations made by Jamil *et al.*, 2006. The reduction in number of leaves was more in variety Giant California. The chlorosis followed by necrosis observed in leaves of salt treated plants which may be due to leaf injury and death caused by accumulation of salt in the leaves that exceeds the capacity of salt compartmentation in the vacuoles, which finally result in building up of salt in cytoplasm to toxic level (Roohi *et al.*, 2011). Among salt treated seedlings maximum adverse effect was observed in variety Giant California and minimum in variety Nana compecta.

In-vitro selection for salt tolerance

In-vitro technique offers the opportunity to select and regenerate plants with desirable characteristics including tolerance to stress. Use of callus seems to play an important role to select stable variants showing improved salt tolerance (Rai *et al.*, 2011). In the present study, stepwise increase of NaCl concentration from 25 mM to cytotoxic level of 150 mM was found to be better than direct transfer of calli to

highest salt concentration of 300 mM. The suitability of stepwise selection over direct method is evidenced by good proliferation growth of callus seen in the present study. It has been reported earlier that proline level was higher in stepwise method than direct method. Proline is a compatible organic solute widely used as the basis of *in-vitro* selection of stress tolerant plants (Rai *et al.*, 2011). In direct treatment method, callus was directly given salt concentration of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM. It was observed that callus growth decreased with increase in salt concentration and at 300 mM salt concentration, calli of variety Violet Blue died whereas callus of the variety Giant California died at even a lower concentration of 200 mM. Similar results are reported in *Pisum sativum* where increased NaCl concentration lead to decreased callus growth followed by complete inhibition in growth beyond tolerant level (Sayed and Sayed, 2011), in rice (Rudra *et al.*, 2013) and in tomato (Mahdi and Idris, 2013). Inhibition of growth due to salinity may be due to restriction of water uptake and mineral nutrients from culturing medium (Nuran Cicek and Cakirlar, 2002).

In addition to inhibiting the callus growth, calli characteristics was also affected by salt treatment. The callus of variety Violet Blue, before salt treatment, was yellow, granular and soft but after salt treatment it became brown, blackish and necrotic spots appeared on callus. While in variety Giant California, callus was greenish compact hard but after treatment callus became yellow brown with necrotic spots. This is in agreement with reports in sugarcane callus by Gandonou *et al.*, (2005) and (Munir and Aftab, 2013).

Maximum callus induction and proliferation was observed in MS media supplemented with high auxin and low cytokinins ratio. The hormonal combination of 2, 4-D and kinetin was observed to be most appropriate for induction of good callus percentage (90%) in variety Violet Blue. Such results have also been reported in *Aequilaria malacensis* by Moitreyee Saikia *et al.*, (2013). For varieties Giant California and Nana Compecta equal ratio of auxins and cytokinins produced good proliferation and healthy callus with callus induction percentage 70% and 68% respectively. The callus texture was also influenced by varying hormonal concentration in different

varieties. Yellow granular and friable calli were obtained in variety Violet Blue when auxins were more than cytokinins. Similarly, green compact hard and granular calli were obtained in varieties Giant California and Nana Compecta when BAP and NAA were used in equal proportion. Jamshidnia and Tabatabaei, (2013) also reported in *Petunia* that high BAP and low NAA produce compact calli. For shoot induction directly from leaf explants of all the varieties, high cytokinins (BAP 2 mg/l) and low auxin (NAA 0.1 mg/l) was appropriate. These results are also supported by Hegde, (2012) in *Petunia hybrida* using similar concentration of hormones.

Regeneration of plants from calli of untreated controls of all three varieties was higher. Regeneration of shoot was observed in 50 mM salt concentration in all three varieties but it was less than that observed in control. The regeneration potential of calli without salt treatment was better as compared to salt treated calli reported by other workers also (Sajid, 2010). Although the callus selected from 75 mM salt concentration became green in variety Violet Blue and bud like structures were observed but regeneration of shoots was inhibited. However, regeneration occur in calli treated with 50 mM salt. Like present work in rice also maximum regeneration was reported at 50 mM (Priya *et al.*, 2013). The regeneration potential decreased in rice with increase in salt concentration (Zinnah *et al.*, 2013). MS media supplemented with 0.5 NAA and 0.1 mg/l IBA was used for root induction as it initiates roots quickly. Among the three varieties tested in present study variety Violet Blue showed quick response to this concentration. The untreated shoots produced good amount of rooting as compared to salt treated shoots. Root formation was inhibited at salt concentration above 100 mM. Such results were reported earlier in *Tanacetum cinerariaefolium* also by Abdi *et al.*, (2011). Similar results were obtained when salinity was increased in cucumber micro shoots above 50 mM (Alrahman *et al.*, 2010).

Survival of *in-vitro* shoots during stress

Several parameters have been considered to assess the salt tolerance of plants but the growth and survival rate represent the most common parameter to be used for assessing salt tolerance (Niknam and McComb, 2000). *In-vitro* explants of

three varieties of *P. grandiflora* were grown for two sub-cultures under continuous salt stress from control to 250 mM in two varieties Nana compecta and Giant California, but upto 300 mM salt concentration in variety Violet Blue. In all three varieties of *P. grandiflora* upto salt concentration of 50 mM little effect of salt concentration was observed but survival of explants sharply decreased with further increase in salinity. The lowest survival of shoots (10%) was observed in variety Violet Blue at 300 mM concentration while it was 5% at 250 mM salt concentration in varieties Giant California and Nana Compecta. It clearly shows that as the salinity increased, the survival percentage decreased but it varied according to the variety used. These results are in agreement with Sayed and Gabr, (2013). Lowest survival percentage was observed in first sub-culture which gradually increased with second sub-cultures in all three varieties except at the lethal concentration beyond which they died. This suggests that sub-culturing helps in attaining tolerance to explants shoots. However, in variety Giant California, from onwards, survival percentage decreased during 2nd sub-culture also after 100 mM as compared to varieties Violet Blue and Nana compecta. Enhancement of tolerance with sub-culturing was reported by Sayed and Gabr, (2013). In this respect, Chelli-Chaabouni *et al.*, (2010) cultured two pistachio root stocks (*Pistacia vera* L. and *P. atlantica* Desf) *in-vitro* and reported that the higher salt tolerance of *P. atlantica* observed seems to be correlated with a higher survival rate. During salt treatment to *in-vitro* shoots, stem and leaves of plantlets at high salinity under *in-vitro* conditions became yellowish in color with shoot tip dying, stunted growth, poor root development and burning on maturing. Similar response was observed in potato by Sudhersan *et al.*, (2012). In the present study secretion of some phenolic compound was observed in the *in-vitro* shoots treated with different salt concentrations as compared to the controls. Abu-Qauod, (2012) reported that at moderate salt stress phenolic compounds increase due to disturbance in secondary metabolic pathways and these phenolic contents are directly linked with antioxidant activity but at extremely high concentration of salt, phenolic content decreased. Also release of phenolics may be due to damage of cells because of mechanical injury (Akbarimoghaddam *et al.*, 2011).

CHAPTER VI

SUMMARY

Petunia plants are most popular bedding plants of the world due to their versatility, variety and range of color. Besides being an ornamental plant, it has antimicrobial and insecticidal properties, and also possesses phytoremediation potential. Over the last few decades, the monetary value of ornamentals has increased manifold and the market for ornamentals has steadily increased in a developing country like India but the main hurdle to increase the cultivation of *Petunia* is soil salinity in Bathinda region. Salinity adversely induces abnormal morphological, physiological and biochemical changes that leads to reduction in growth of plants and high seedling mortality which results in low market value. Thus it is imperative to develop varieties which can grow in saline soils. Therefore, attempts have been made to select salt tolerance among the three varieties of *P. grandiflora* namely Violet Blue, Giant California and Nana Compecta both under *ex-vitro* and *in-vitro* conditions

For *ex-vitro* salt treatments, seeds of all the three varieties were sown in plastic trays filled with 1:1 proportion of vermicompost and sand and were watered regularly with RO water. When seedlings became forty five days old, all the seedlings of three varieties were treated with 100 mM, 200 mM, 300 mM, 400 mM, 500 mM and 600 mM salt for time duration of 4 hr, 6 hr, 12 hr and 24 hrs. Survival percentage was recorded 20 days after salt treatment which decreased with increase in salt concentration in all the three varieties. However, less survival percentage was observed in variety Giant California at 600 mM salt treatment and highest survival percentage was found in Variety Nana Compecta. Shoot length and number of leaves was also recorded in all the three varieties. Increase in salt concentration also reduced the shoot length and number of leaves in all the three varieties. However variety Nana Compecta was affected least and variety Giant California was found to be sensitive to salt stress.

The most pronounced effect of salt treatment was chlorosis and necrosis of leaves, and finally death of plants. Whereas the control plants continued to attain good height, large and green leaves and thick stems. Among the three varieties, maximum chlorosis was observed in leaves of salt treated seedlings of variety Giant California at 600 mM salt concentration for 24 hrs. Variety Nana Compecta was

affected least. When the salt treated seedlings were shifted to field, the chlorosis of leaves vanished and these started to increase in height, leaves became green and sprouting of multiple branches from main stem occurred. In all these varieties flowering was delayed due to salt treatment. Days to 50% flowering in all the three selected varieties was reduced as compared to untreated controls. Flowering occurred 70 days after salt treatment in variety Nana Compecta, 80 days after in variety Violet Blue and 92 days after salt treatment in variety Giant California.

Part II

For *in-vitro* culture, seeds were sown in glass cups filled with autoclaved sand. These cups were watered regularly by RO water and maintained in glass house at 25±^{0}C to get the explants for inoculation. Leaves were used as explants for direct shoot induction and callus induction. Fifteen media were tested for callus induction in leaf explants of varieties Violet Blue, Giant California and Nana Compecta. Four failed to produce results. Among the rest eleven media, only those were selected which induce more than 3 cm of callus within 30 days. Media “B” which produced maximum percentage of callus (90%) in variety Violet Blue and media “L” producing maximum calli percentage in varieties Giant California (70%) and Nana Compecta (68%) were used for further work. For selecting salt tolerant callus, stepwise and direct methods of treatment were used. In stepwise method callus was treated in sequential order from low concentration to higher concentration, and the surviving calli were sub-cultured to next higher salt concentration. Callus growth was inhibited and necrotic spots appeared in callus when shifted from low to high concentration. At 150 mM salt concentration all calli died. Therefore, calli showing good growth at sub lethal concentration of 75 mM were selected and inoculated on shoot regeneration media. Such calli became green and showed good proliferation after 60 days.

In direct treatment method, callus was directly given high salt concentration treatments viz 50 mM, 100 mM, 150 mM, 200 mM , 250 mM and 300 mM for 25 days. Callus growth decreased with increase in salt concentration and at 300 mM salt concentration, calli of variety Violet Blue died whereas the calli of variety Giant California died at 200 mM salt concentration. The selected calli of variety Violet

Blue after 200 mM salt concentration sustained good growth in the first three sub-cultures but after that calli died. Therefore, calli were selected at 150 mM in variety Giant California, 50 mM in variety Nana Compecta and from 200 mM salt treated calli of variety Violet Blue for shoot regeneration. However, in variety Nana Compecta, calli were treated upto 50 mM salt treatment due to non availability of calli. Also beyond 50 mM shoot regeneration was inhibited in all the three varieties.

Besides inhibiting growth of calli, characteristic of calli from all the three varieties were also affected by salt treatment. The callus from variety Violet Blue was yellow, granular and soft before salt treatment. But after salt treatment, calli turned brown, blackish and developed necrotic spots. The callus of variety Giant California was greenish and compact hard but after salt treatment callus became yellow brown with necrotic spots. Hormonal concentration also affected characteristics of calli. As in case of variety Violet Blue, media “B” produced yellow granular callus while media ‘I’ produced whitish yellow callus. The callus of variety Giant California on media ‘I” was yellow and friable and on media “L” it was greenish compact hard and granular. Similar response of calli was observed in the variety Nana Compecta. However, the callus of variety Violet Blue on media ‘L” was yellow whitish and granular with slight greenish spots.

For inducing direct shoots from leaves different media were tested but media “C” produced good percentage of shoots in all the three varieties of *P. grandiflora* and it was used for further work. Further, the regenerated shoots were grown in salt containing media for selecting salt tolerant shoots. It was observed that increase in salt concentration led to burning and drying of leaves of *in-vitro* shoots. The shoots of variety Violet Blue tolerated up to 250 mM salt. Whereas the shoots of the varieties Giant California and Nana compecta tolerate up to 150 mM salt concentration only. Shoots of variety Giant California were more sensitive and died after third sub-culture.

To regenerate shoots from callus, different regeneration media were tested. Among these, media “T” induced shoots in variety Violet Blue and media “L” induced shoots in varieties Giant California and Nana Compecta. It was observed that

shoot formation does not occur in calli treated with concentration of salt beyond 50 mM, however, calli selected from 75 mM salt concentration of variety Violet Blue became green only on media “C” after 60 days. But shoot regeneration from 50 mM salt concentration was observed in this variety. In varieties Giant California and Nana Compecta, regeneration of shoots was obtained from 50 mM salt treated calli on media “L”. For inducing roots, media “C” and media “S” were effective in initiating roots in all the three varieties. Media “S” induced roots 7 days after in variety Violet Blue. It was also observed that after sub-culturing of shoots, root formation occurred on media “C” and even shoots treated with 50 mM salt produced roots.

It is concluded that variety Giant California is salt sensitive and variety Nana Compecta is relatively tolerant to salt. However all the varieties tolerated the salt concentration up to certain level both as whole plant and as well as at cellular level which gives hope that the progeny of salt tolerant seedlings identified during present study will be able to grow in salt.

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APPENDICES

Appendix A

Composition of Murashigue and Skoog (1962) media

Stock no.	Strength of stock	Constituent salts	Quantity(g)	Use of stock(ml/l	Actual amount in the culture medium(mg/l
			2 Litre		
1	20X	NH_4NO_3	66.0	50	1650
		KNO_3	76.0		1900
		KH_2PO_4	6.800		170
		H_3BO_3	0.248		6.2
		$MnSO_4.7H_2O$	0.892		22.3
		$ZnSO_4.7H_20$	0.344		8.6
		KI	0.033		0.825
		$^{*}CuSO_2.5H_2O$	1.0ml		0.025
		$^{**}CoCl_2.6H_2O$	1.0ml		0.250
		$^{***}Na_2MoO_4.2H_2O$	1.0ml		0.250
			½ litre		
2	50X	$CaCl_2.2H_2O$	11.0	20	440
3	50X	$MgSO_4.7H_2O$	9.250	20	370
			1 litre		
4	100X	Na_2EDTA	3.728	10	37.28
		$FeSO_4.7H_2O$	2.780		27.8
			1 litre		
5	100X	Thiamine HCL	0.010	10	0.1
		Nicotinic acid	0.050		0.5
		Pyridoxine HCL	0.050		0.5
		Glycine	0.050		2.0
		$^{\#}$Myoinositol	0.200		100
		$^{\#}$sucrose			30g/l

Dissolve 100mg $CuSO_2.5H_2O$ in 100ml distilled water.

** Dissolve 100mg $CoCl_2.6H_2O$ in 100ml distilled water.

***Dissolve 1g $Na_2MoO_4.2H_2O$ in 100ml distilled water.

Add 1ml concentrated HCL in stock solution no.1 to avoid precipitation.

Heat slightly stock solution no.4 to avoid precipitation.

Myoinositol and sucrose are added in solid form while preparing the medium.

Appendix B

Preparation of Stock Solutions of Plant Growth Regulators

For preparing the stock solutions of PGR, distilled water was used. The hormones were weighed and first dissolve in 1N NaOH and 1N HCl. For stock solution 1 mg/ml concentration was used. Except 2, 4-D, which was dissolved in absolute ethanol. Once dissolved, the final volume was made up with distilled water in an appropriate volumetric flask and stored at 4°C in refrigerator till use. If precipitation occurs in the stock solution, discard the stock solution. Usually it is better to use fresh solutions of hormones.

Appendix C

Murashige and Skoog Medium PT021 w/ CaCl2 and Vitamins

S.NO	Composition	mg/l
1	Potassium nitrate	1900.00
2	Ammonium nitrate	1650.00
3	Calcium chloride.$2H_2O$	440.00
4	Magnesium sulphate	180.69
5	Potassium phosphate monobasic	170.00
6	Manganese sulphate.H_2O	16.90
7	Boric acid	6.20
8	Potassium iodide	0.83
9	Molybdic acid (sodium salt).$2H_2O$	0.25
10	Zinc sulphate.$7H_2O$	8.60
11	Copper sulphate.$5H_2O$	0.025
12	Cobalt chloride.$6H_2O$	0.025
13	Ferrous sulphate.$7H_2O$	27.80
14	EDTA disodium salt.$2H_2O$	37.30
15	Myo - Inositol	100.00
16	Thiamine hydrochloride	0.10
17	Pyridoxine hydrochloride	0.50
18	Nicotinic acid (Free acid)	0.50
19	Glycine (Free base)	2.00

Suspend 4.41 grams of dehydrated medium in 600ml of distilled water and rinse media with small quantity of distilled water to remove traces of powder. Apply constant gentle stirring to the solution till the powder dissolves completely. Add desired heat stable supplements prior to autoclaving. Adjust the medium to the desired pH using 1N HCl/NaOH. Make up the final volume to 1000 ml with distilled water.

Appendix D

Protocol for callus induction in variety Violet Blue

1. Leaves plucked from plants were washed for 4-5 minutes under running tap water.
2. Then treated with 2% polysorbate 80 till foam disappears.
3. Then treated these leaves with 0.05 $HgCl_2$ for five minutes in Laminar air flow chamber and again wash with distilled water three times with one minute interval to remove traces of $HgCl_2$.
4. After sterilization, cut the 5 mm X 5 mm size leaves and put on the MS media containing 2, 4-D 2 mg/l and 0.5 mg/l kinetin.
5. Incubate them under 16/8 hour light and dark period respectively.

Protocol for callus induction in variety Giant California and Nana Compecta

1. Leaves plucked from plants were washed for 4-5 minutes under running tap water.
2. Immerse these leaves in 2% w/v Bavistin (fungicide) for 10 minutes.
3. Then treated with 2% polysorbate 80 till foam disappears.
4. Then treated these leaves with 0.05 $HgCl_2$ for seven minutes in Laminar air flow chamber and again wash with distilled water three times with one minute interval to remove traces of $HgCl_2$.
5. After sterilization, cut the 5 mm X 5 mm size leaves and put on the MS media containing 1 mg/l BAP and 1 mg/l NAA.
6. Incubate them under 16/8 hour light and dark period respectively.

Appendix E

Protocol for direct induction of shoots from the three varieties of *Petunia grandiflora*

1. Leaves plucked from plants were washed for 4-5 minutes under running tap water.
2. Washed leaves were treated with 2% polysorbate 80 till foam disappears.
3. Then treated these leaves with 0.05 $HgCl_2$ for five minutes in Laminar air flow chamber and again wash with distilled water three times with one minute interval to remove traces of $HgCl_2$.But leaves of variety Nana compecta and Giant California 2% w/v Bavistin (fungicide) for 10 minutes and 0.05 $HgCl_2$for seven minutes.
4. After sterilization, cut the 5 mm X 5 mm size leaves and put on the MS media containing 2 mg/l BAP and 0.1 mg/l NAA.
5. Incubate them under 16/8 hour light and dark period respectively.

Appendix F

Protocol for regeneration of shoots from callus of three varieties of *Petunia grandiflora*

1. Prepare the M/S media (Appendix I), to this media add 2 mg/l BAP and 0.5 mg/l IAA for variety Violet Blue and for variety Giant California and Nana Compecta 1 mg/l BAP and 1 mg/l NAA.
2. Adjust the pH of media 5.8 by using 1N NaOH and 1N HCl.
3. Add 3% sucrose and 0.6 % agar agar to media.
4. Autoclave the media at 121^0C under 15 lb pressure for 15 minutes and keep it overnight in laminar air flow chamber.
5. Now excise the 2 mm X 4 mm size callus and inoculate on prepared media and incubate under 16/8 hr light and dark period respectively.

Appendix G

Protocol for rooting in the three varieties of *Petunia grandiflora*

1. Prepare the MS media (Appendix A), to this media add 0.5 NAA and 0.1 mg/l IBA. Another hormonal combination used was 2 mg/l BAP and 0.1 mg/l NAA.
2. Adjust the pH of media 5.8 by using 1N NaOH and 1N HCl.
3. Add 3% sucrose and 0.6 % agar agar to media.
4. Autoclave the media at 121^0 C under 15 lb pressure for 15 minutes and keep it overnight in laminar air flow chamber.
5. Now remove the shoots from culturing jars and wash under tap water to remove traces of agar and MS media.
6. Inoculate these shoots on rooting media and incubate them under16/8 hr light and dark period respectively.

Appendix H

Survival percentage of variety Violet Blue after salt treatment

Concentration	Replication	Duration of treatment			
		4hr	6hr	12hr	24hr
100 mM	R1	50.77	50	51.16	43.75
	R2	47.89	49.09	62.96	68.42
	R3	65.31	54.29	60	42.53
	Mean	**54.65**	**51.13**	**58.04**	**51.57**
200 mM	R1	39.47	42.31	43.14	72.22
	R2	44.3	32.5	60	28.57
	R3	53.01	51.11	42	28.57
	Mean	**45.6**	**41.97**	**48.38**	**43.12**
300 mM	R1	40.98	30.84	34.38	33.65
	R2	32	41.54	34.43	58.82
	R3	52.63	45.07	56.86	28.16
	Mean	**41.87**	**39.15**	**41.89**	**40.21**
400 mM	R1	43.48	54.38	44.8	35.45
	R2	40.91	27.87	38.13	36.61
	R3	31.98	29.38	38.03	46.15
	Mean	**38.89**	**37.21**	**40.32**	**39.4**
500 mm	R1	27.21	32.11	50	29.41
	R2	50.75	35.71	51	31.82
	R3	23.61	37.04	12	30.99
	Mean	**33.87**	**34.95**	**36.66**	**30.54**
600 mM	R1	36.36	22.86	31.43	13.7
	R2	36.88	32.65	29.92	32.45
	R3	11.61	31.94	30.29	36.52
	Mean	**28.28**	**29.05**	**30.54**	**27.55**
Control	R1	96.08	94.74	94.85	93.75
	R2	92.16	95.31	97.09	96.25
	R3	94.45	93.68	96.44	94.65
	Mean	**94.23**	**94.58**	**96.13**	**94.88**

Appendix I

Survival percentage of variety Giant California after salt treatment

Concentration	Replication	Duration of treatment			
		4hr	6hr	12hr	24hr
100 mM	**R1**	37.04	42.86	80.00	57.14
	R2	60.00	57.50	49.06	84.85
	R3	61.54	58.33	56.67	60.56
	Mean	**52.86**	**52.90**	**61.91**	**67.52**
200 mM	**R1**	39.51	55.22	33.33	38.18
	R2	37.78	46.97	33.33	46.94
	R3	41.18	55.56	75.00	39.53
	Mean	**39.49**	**52.58**	**47.22**	**41.55**
300 mM	**R1**	38.78	26.56	37.14	35.29
	R2	34.69	80.00	41.54	48.00
	R3	37.74	31.82	39.39	40.91
	Mean	**37.07**	**46.13**	**39.36**	**41.4**
400 mM	**R1**	31.15	45.71	37.61	51.16
	R2	32.86	32.32	36.27	26.32
	R3	34.43	30.43	36.36	27.41
	Mean	**32.81**	**36.16**	**36.75**	**34.96**
500 mM	**R1**	25.00	33.33	33.66	30.00
	R2	27.72	30.43	33.77	32.00
	R3	36.96	32.69	27.84	27.87
	Mean	**29.89**	**32.15**	**31.75**	**29.96**
600 mM	**R1**	29.55	31.62	27.50	29.73
	R2	3.41	24.49	29.90	29.33
	R3	20.62	33.33	29.59	28.80
	Mean	**17.86**	**29.82**	**29.00**	**29.29**
Control	R1	96.00	96.67	83.33	91.43
	R2	96.51	97.41	94.83	95.74
	R3	91.43	96.00	96.67	83.33
	Mean	**94.65**	**96.69**	**91.61**	**90.17**

Appendix J

Survival percentage of variety Nana Compecta after salt treatment

Concentration	Replication	Duration of treatment			
		4hr	6hr	12hr	24hr
100 mM	**R1**	66.67	74.60	68.85	68.85
	R2	65.67	57.97	73.47	66.67
	R3	76.47	78.05	64.00	67.27
	Mean	**69.60**	**70.21**	**68.77**	**67.60**
200 mM	**R1**	60.61	68.52	61.54	61.70
	R2	62.50	63.64	66.67	64.81
	R3	72.22	64.91	62.22	62.75
	Mean	**65.11**	**65.69**	**63.48**	**63.09**
300 mM	**R1**	45.83	60.00	58.70	54.90
	R2	69.09	59.26	59.62	61.22
	R3	71.15	64.44	64.81	60.00
	Mean	**62.02**	**61.23**	**61.04**	**58.71**
400 mM	**R1**	42.07	48.00	61.02	50.00
	R2	62.50	62.50	51.91	68.42
	R3	57.60	58.46	61.43	50.48
	Mean	**54.06**	**56.32**	**58.12**	**56.30**
500 mM	**R1**	60.98	57.33	53.49	41.11
	R2	35.40	43.17	50.00	61.18
	R3	63.01	60.76	56.06	58.82
	Mean	**53.13**	**53.75**	**53.18**	**53.70**
600 mM	**R1**	58.42	52.94	48.00	58.06
	R2	38.75	55.79	50.94	36.00
	R3	58.70	49.21	47.22	59.26
	Mean	**51.95**	**52.65**	**48.72**	**51.11**
Control	**R1**	96.84	96.67	94.32	98.93
	R2	98.48	96.77	87	96.52
	R3	96.67	94.32	87	98.48
	Mean	**97.33**	**95.92**	**89.44**	**97.98**

Appendix K

Shoot length (cm) of *Petunia grandiflora* variety Violet Blue after 20 days of salt treatment

Concentration	Replication	Duration of treatment			
		4hr	**6hr**	**12hr**	**24hr**
400 mM	R1	2.73	2.48	1.85	2.3
	R2	2.95	2.45	3.07	2.43
	R3	3.17	2.43	3.12	3.16
Mean		**2.95**	**2.45**	**2.68**	**2.63**
500 mM	R1	2.01	2.43	2.45	2.55
	R2	2.02	2.40	2.49	2.38
	R3	2.82	2.36	2.28	2.24
Mean		**2.28**	**2.40**	**2.41**	**2.39**
600 mM	R1	2.13	2.09	2.32	2.24
	R2	1.72	1.73	2.23	2.29
	R3	2.15	1.95	2.16	2.26
Mean		**2.00**	**1.92**	**2.24**	**2.26**
Control	R1	10.61	10.63	9.56	10.91
	R2	10.44	10.48	9.51	10.57
	R3	10.41	10.88	9.48	10.58
Mean		**10.49**	**10.66**	**9.52**	**10.69**

Appendix L

Shoot length (cm) of *Petunia grandiflora* variety Giant California after 20 days of salt treatment

Concentration	Replication	Duration of treatment			
		4hr	**6hr**	**12hr**	**24hr**
400 mM	R1	1.91	1.57	1.80	2.15
	R2	2.33	1.73	1.80	1.95
	R3	1.75	2.42	1.96	2.03
Mean		**2.00**	**1.91**	**1.85**	**2.04**
500 mM	R1	1.58	1.90	1.88	1.82
	R2	1.78	1.79	1.41	1.36
	R3	2.33	1.96	2.14	1.97
Mean		**1.90**	**1.88**	**1.81**	**1.72**
600 mM	R1	2.17	2.07	1.61	1.83
	R2	1.52	1.83	1.36	1.86
	R3	1.68	1.30	1.96	1.44
Mean		**1.79**	**1.73**	**1.64**	**1.71**
Control	R1	12.30	12.30	13.30	12.30
	R2	12.00	11.30	12.30	12.10
	R3	11.40	11.30	12.00	11.90
Mean		**11.90**	**11.63**	**12.53**	**12.10**

Appendix M

Shoot length (cm) of *Petunia grandiflora* variety Nana Compecta after 20 days of salt treatment

Concentration	Replication	Duration of treatment			
		4hr	6hr	12hr	24hr
400 mM	R1	3.93	3.52	3.91	3.58
	R2	4.34	3.88	3.63	3.89
	R3	4.39	3.13	3.41	3.47
Mean		**4.22**	**3.51**	**3.65**	**3.65**
500 mM	R1	2.52	2.30	2.46	2.35
	R2	2.72	2.11	2.21	2.47
	R3	3.78	2.76	2.64	2.24
Mean		**3.01**	**2.39**	**2.44**	**2.35**
600 mM	R1	2.28	2.11	2.27	1.94
	R2	2.32	1.90	2.14	2.29
	R3	2.37	2.13	2.52	1.96
Mean		**2.32**	**2.05**	**2.31**	**2.06**
Control	R1	6.56	6.06	6.31	6.32
	R2	6.32	6.32	6.54	6.33
	R3	6.13	6.28	6.39	6.12
Mean		**6.34**	**6.22**	**6.41**	**6.26**

Appendix N

Number of leaves of *Petunia grandiflora* variety Nana Compecta after 20 days of salt treatment

Concentration	Duration of treatment				
	Replication	4hr	6hr	12hr	24hr
400 mM	R1	6.80	8.90	7.60	8.40
	R2	7.10	9.60	8.60	7.10
	R3	8.60	7.90	9.40	9.60
Mean		**7.50**	**8.80**	**8.53**	**8.37**
500 mM	R1	7.30	7.40	8.00	7.90
	R2	7.80	7.40	6.60	6.30
	R3	7.40	6.90	6.50	5.80
Mean		**7.50**	**7.23**	**7.03**	**6.67**
600 mM	R1	6.30	6.90	6.80	6.50
	R2	6.40	7.00	7.20	6.10
	R3	6.40	7.00	6.00	6.80
Mean		**6.37**	**6.97**	**6.67**	**6.47**
Control	R1	10.60	10.90	10.90	11.10
	R2	10.50	10.80	10.60	11.00
	R3	10.50	11.00	10.90	10.60
Mean		**10.53**	**10.90**	**10.80**	**10.90**

Appendix O

Number of leaves *Petunia grandiflora* variety Violet Blue after 20 days of salt treatment

Concentration	Replication	Duration of treatment			
		4hr	**6hr**	**12hr**	**24hr**
400 mM	R1	6.60	8.80	7.70	7.40
	R2	8.40	9.40	9.20	7.80
	R3	8.40	6.70	8.60	9.20
Mean		**7.80**	**8.30**	**8.50**	**8.13**
500 mM	R1	7.50	7.40	7.70	6.30
	R2	7.10	6.70	7.30	6.80
	R3	6.90	8.70	8.10	6.80
Mean		**7.17**	**7.60**	**7.70**	**6.63**
600 mM	R1	7.10	7.00	7.00	7.60
	R2	7.50	7.10	7.30	5.30
	R3	6.60	7.70	7.20	6.60
Mean		**7.07**	**7.27**	**7.17**	**6.50**
Control	R1	11.20	11.20	11.90	12.00
	R2	11.20	11.60	11.30	11.20
	R3	11.00	11.20	11.00	11.50
Mean		**11.13**	**11.33**	**11.57**	**11.36**

Appendix P

Number of leaves of *Petunia grandiflora* variety Giant California after 20 days of salt treatment

Concentration	Replication	Duration of treatment			
		4hr	6hr	12hr	24hr
400 mM	R1	8.00	8.10	8.30	7.70
	R2	7.90	7.90	7.30	8.00
	R3	8.97	9.00	8.20	8.70
Mean		**8.29**	**8.33**	**7.93**	**8.13**
500 mM	R1	6.90	6.30	6.50	7.00
	R2	6.10	5.40	5.30	5.20
	R3	5.50	5.70	5.80	5.10
Mean		**6.17**	**5.80**	**5.87**	**5.77**
600 mM	R1	6.00	5.50	4.50	6.00
	R2	5.80	5.30	6.60	5.90
	R3	5.40	5.30	5.50	4.60
Mean		**5.73**	**5.37**	**5.53**	**5.50**
Control	R1	11.10	10.60	10.40	11.00
	R2	11.10	11.70	11.20	10.60
	R3	10.30	10.40	10.90	11.00
Mean		**10.83**	**10.90**	**10.83**	**10.87**