Physiological and Biochemical Studies of NaCl-Salinity Stress in Crop Plants

Chapter 1

INTRODUCTION
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Every change of an environmental factor influences plant growth and development. Every deviation of a factor from its optimum is not necessarily a stress for a flexible plant acclimatized to its environment. Stress begins with a constraint (biotic or abiotic) (table 1) or with highly unpredictable fluctuations imposed on the regular metabolic pattern that cause bodily tension. Stress, in the sense of a stressor or stress inducer (Cassells and Curry 2001), is an unusual factor or a usual factor of the biotic and abiotic environment modified in such a way (excess or deficit) that it has the capability of causing bodily injury, disease, or aberrant physiology. The disease is considered as a condition of the living (animal or plant) body or one of its parts that impairs the performance of a vital function(s), as a response to environmental factors or inherent genetic defects. Stress, in the sense of the physiological state, is the condition caused by factors that tend to alter an equilibrium (Nilsen and Orcutt 1996).

Table 1. Some of the sources of the environmental stress in plants (reproduced from Nilsen and Orcutt 1996).

<table>
<thead>
<tr>
<th>Physical</th>
<th>Chemical</th>
<th>Biotic</th>
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<tr>
<td>Drought</td>
<td>Air pollution</td>
<td>Competition</td>
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<td>Temperature</td>
<td>Heavy metals</td>
<td>Allelopathy</td>
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<td>Radiation</td>
<td>Pesticides</td>
<td>Herbivory</td>
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<td>Flooding</td>
<td>Toxins</td>
<td>Diseases</td>
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<td>Wind</td>
<td>Soil pH</td>
<td>Pathogenic fungi</td>
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<td>Magnetic field</td>
<td>Salinity</td>
<td>Viruses, Bacteria</td>
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Though the term ‘stress’ has been defined exactly in mechanics, in case of biology it has been given widely different meanings. Probably due to an extension of the physical meaning, many of these definitions converge in attributing “stress” to any environmental factor “unfavorable” for the living organism under consideration (Levitt 1980, 1982). In accordance, the ability of the organism to survive the unfavorable factor has been called “stress resistance”. Because, plants are confined to
the place where they grow, they have a limited capacity to avoid unpredictable unfavorable changes in their environment (confrontation with extremes of temperature, excess of salts, water shortage, insufficient or excessive light or mineral nutrients, attack by pathogenic bacteria, fungi, viruses or viroids) (table 1). They have developed ingenious molecular strategies to defend themselves against such biotic and abiotic stresses, most often combined with an alteration of growth and development patterns. This explains why the concept of stress is intimately associated with the external conditions that adversely affect growth, development or productivity (Lutts and Kinet 1998). Although the term stress may be used for indicating the STRESSOR agent (that is the unusual factor, or a usual factor modified in such a way, excess or deficit, that it has the capability of causing bodily injury or disease), or for the alarm RESPONSE to extraordinary unfavorable condition (for instance wilting), it primarily concerns the altered PHYSIOLOGICAL STATE, impairing the performance of a vital function.

Stress, in any form, exerts strong evolutionary pressure on all organisms. To survive, any organism must develop tolerance, resistance or avoidance mechanisms. Tolerance allows the organism to withstand the assault unharmed. Resistance involves active countermeasures, while avoidance prevents exposure to the stress. Partly due to their sessile nature, plants have developed sophisticated metabolic responses to cope and survive, rather than avoiding stressful conditions as mobile organisms can. Stress can be defined by its negative effects on growth and development of the individual and can be external or internal. Internal stresses, such as spontaneous gene mutations or aberrant cell division might cause adverse effects on metabolic or genetic regulation. External stresses on plants can be divided into those of biotic or abiotic nature. Biotic stresses include pathogen attack (bacteria, fungi, virus) herbivory and competition. Abiotic stress arises from unfavorable environmental conditions, such as sub optimal temperature, water and nutrient availability, or light conditions. Stressful conditions can be a permanent state for the plant or they can be acute. Plants have adapted to permanent stress by altering their morphological features, such as succulence of their leaves, placement of their stomata, and specialization of tissues. To cope with acute stress plants have evolved responses that recognize the condition and subsequently set counteractive metabolic pathways in motion, such as in Systemic Acquired Resistance (SAR) against biotic stress or in the activation of heat shock proteins. Considerable
knowledge has been gained over the last decade on physiological stress responses in plants involving individual proteins and genes.

The duration, severity and rate at which stress is imposed all influence how a plant responds. Several conditions in combination may elicit a response differing from that for a single type of stress. Features of the plant, including organ or tissue identity, developmental age, and genotype also influence plant response to stress (Bray et al. 2000). At specific developmental stages, plants are either more or less sensitive to particular stressors. The sensitivity stages of development are called windows of sensitivity. A response may be triggered directly by a stress, such as drought, or may result from a stress-induced injury, such as loss of membrane integrity. Some responses clearly enable a plant to resist to stress, whereas the functional role of others is not apparent (figure 1).

Mechanism that permit stress survival are termed RESISTANCE mechanism and can allow an organism to tolerate or avoid stress. Thus, physiological responses to stressors can be divided into three possibilities. In one case, TOLERANCE plants have mechanism that maintain high metabolic activity (similar to that in the absence to stress) under mild stress and reduced activity under severe stress. In contrast, mechanisms of AVOIDANCE involve a reduction of metabolic activity, resulting in a dormant state, upon exposure to extreme stress (Osmond et al. 1987). Commonly, a plant species may have several tolerance or avoidance mechanisms, or a combination of both. For instance, drought stress may induce drought tolerance that can be followed by desiccation tolerance: in the later “dormant” state, the organism can survive the dry state for longer periods, i.e., years. Notice that the ability to rehydrate without damage can be considered as a part of the desiccation tolerance. The other issue is immediate or delayed DAMAGES through somaclonal variation, mutation, neoplastic progression, ultimate death via necrosis and / or apoptosis (figure 1).

Earth is a planet, with most of its water containing about 30 g of sodium chloride per litre. Although, amount of salt-affected land (about 900 X 10^6 ha) is imprecisely known, its extent is sufficient to pose a threat to agriculture (Abrol 1986, Flowers and Yeo 1986, Abrol 1986, Munns 1993, 2002, Munns and James 2003, Flowers 1977,
Although there is currently food enough for the world population, more than 800 million people are chronically undernourished (Conway 1997). Growth of human population by 50% from 6.1 billion in mid 2001 to 9.3 billion by 2050, means that crop production must increase if food security is to be insured. About 20% of the cultivated, and nearly half of all irrigated, lands in the world are affected by salinity and/or drought (Oldeman 1991, Tanji 1995). Soil salinization is one of the major factors of soil degradation. It has reached 19.5% of the irrigated land and 2.1% of the dry-land agriculture existing on the globe (FAO 2000, Wild 2003, Rengasamy 2006). Salinity effects are more conspicuous in arid and semiarid areas where 25% of the irrigated land is affected by salts.

Figure 1. Plant responses to environmental stress in correspondence with stress and plant characteristics (reproduced from Gaspar et al. 2002).

Plants in fields often experience multiple environmental stresses simultaneously. Both glycophytic and halophytic species are known to experience osmotic as well as ionic stress and secondary oxidative stress during high salinity (Allakhverdiev et al. 2000, Hasegawa et al. 2000). Plants with high levels of antioxidants have been reported to provide sufficient resistance against oxidative damage. Salt stress induced generation of reactive oxygen species (ROS) and their scavenging metabolism in cellular organelles has been studied in a halophyte, *Mesembryanthemum crystallinum* (Miszalski et al. 1998). Cheeseman et al. (1997) reported the role of antioxidative
enzymes as well as antioxidants on photoprotection in *Rhizophora stylosa* and *Rhizophora mangle* under field conditions. However, there is no report of gradient effects of NaCl on antioxidative enzymes in mangroves under *in vivo* conditions. *B. paviflora*, which occurs predominantly in the Bhitarkanika mangrove forests of eastern India, experiences a wide range of fluctuations in salinity.

Salinity is one of the major environmental factors limiting global crop production. Salt damage to plants is produced by a combination of several causes, including mainly osmotic injury and specific ion toxicity (Munns and Termat 1986, Munns and James 1993, Nandwal *et al.* 2000, Munns 2002) that affect a wide variety of physiological and metabolic processes in plants (Silveira *et al.* 2001, Demiral and Turkan 2005). Salt and water stresses have common osmotic effects that induce the plants to decrease their internal water potential to avoid desiccation. Nevertheless, salinity provokes ionic stresses other than osmotic stress, therefore, physiological mechanisms that plants use to respond to salinity or drought may partly differ on a case-by-case basis (Erdei *et al.* 1990, Lefevre *et al.* 2001).

**PRIMARY EFFECTS OF HIGH SALINITY**

1. Leads to low soil water potential
2. Increased accumulation of toxic Na$^+$ and Cl$^-$ ions
3. Give rise to ion imbalance

**SECONDARY EFFECTS OF HIGH SALINITY**

1. Nutritional disorders
2. Oxidative stress

Oxidative stress results from conditions promoting the formation of reactive oxygen species (ROS). Environmental factors that cause oxidative stress (*figure* 2) include air pollution (increased amounts of ozone or sulphur dioxide), oxidant-forming herbicides such as paraquat dichloride (methyl viologen, 1,1’-dimethyl 1-4,4’-bipyridinium), heavy metals, salinity, flooding, drought, heat and cold stress, wounding, UV light, highly intense light conditions that stimulate photoinhibition.
Plant cells challenged by viral, bacterial, or fungal pathogens, by elicitor molecules derived from pathogens, or by certain physical stresses also undergo an oxidative burst, that is a rapid accumulation of ROS. Oxidative burst also occurs during senescence.

ROS formed during certain redox reactions and during incomplete reduction of oxygen or oxidation of water by mitochondria or chloroplast electron transfer chains. In certain systems, there is evidence for a superoxide-producing NADPH oxidase complex formed from cytoplasmic and plasma membrane components. In other systems, other mechanisms are apparently effective with peroxidase as candidate for participation in apoplastic H$_2$O$_2$ synthesis. It is likely that different plant / elicitor systems utilize different mechanisms for synthesizing ROS in the plant cell oxidative burst (Murphy et al. 1998, Caspar et al. 2000).

**Figure 2.** Oxidative stress (oxidative burst if temporary), i.e. increase in the concentrations of reactive oxygen species (ROS) under the effect of environmental stresses, causing hypersensitive” reactions. The roles or consequences of the increase in ROS are indicated (reproduced from Gaspar et al. 2002).
Figure 3. Reactions producing Reactive Oxygen Species (ROS) and organic free radicals (reproduced from Gaspar et al. 2002).

Formally, the ROS represent the products of successive single-electron reductions of oxygen (Eqn. 1; see figure 3). Ground state O₂ is a triplet state molecule and is relatively unreactive, but it can accept a single electron from a variety of reducing agents, particularly if it has been converted to the single state (for instance, by the
transfer or energy from photochemically excited chlorophyll) if the reducing agent has been photochemically excited, or if the oxygen and reducing agents are in the presence of an appropriate enzyme. The product of this reduction is superoxide \((O_2^-)\), which under acidic conditions protonates to the hydroperoxyl radical (Eqn. 2 in figure 3). Subsequent reactions will form hydrogen peroxide \((H_2O_2)\), hydroxyl radical \((OH)\), and water. In cells the appearance of \(H_2O_2\) and \(OH\) generally does not occur through the sequence of reactions shown in Eqn. 2. Instead, once superoxide has been synthesized, interconversions of the various ROS may account for the appearance of the more reduced forms. For instance, disproportionation of the hydroperoxyl radical, or of superoxide in the presence of superoxide dismutase (SOD), forms \(H_2O_2\) (Eqn. 3 a, b in figure 3). The combination of hydrogen peroxide and superoxide in the presence of iron or other transition metals forms hydroxyl radical (the Fenton reaction – Eqn. 4 a, b in figure 3). Thus, in the oxidative burst, an enzymatic synthesis of superoxide may lead to the appearance of the other ROS.

ROS, especially \(OH\), are highly destructive to lipids, proteins and nucleic acids. Peroxidation of membrane lipids and other critical cell components can also occur through the production of organic free radicals. Hydroxyl radical is extremely reactive, with a life-time in the cell of nanoseconds. In cells it can oxidize-abstract H from almost any available biological molecule, forming an organic radical. Normally, the production of hydroxyl radical is limited by the presence of SOD, which keeps superoxide concentration low. Catalase or peroxidase, which remove hydrogen peroxide, also restrict the production of hydroxyl radical. On the other hand, free radicals may be formed also by peroxidases, using \(H_2O_2\) as initial oxidant (Eqn. 5 a, b, c in figure 3 and 4). Cassells and Curry (2001) have summarized the harmful effects of ROS when the pro- and anti-oxidant balance is perturbed in oxidative stress (figure 5).
Figure 4. Defence systems (enzymes, antioxidants) against reactive oxygen species (ROS).

The antioxidant defence systems include non-enzymatic and enzymatic antioxidants (figure 3 and 4). These compounds and enzymes are not distributed uniformly, so defence systems vary among specific subcellular compartments. The major antioxidant species in plants are ascorbate (Vitamin C), reduced glutathione (GSH), α-tocopherol (Vitamin E), carotenoids. Polyamines and flavonoids also may provide some protection from free radical injury. The ascorbate-glutathione cycle is the major antioxidant pathway in plastids where ROS are generated during normal biochemical processes that include photosynthetic electron transfer of electrons. The photosynthetic apparatus receives additional protection from oxidative damage by the exothermic production of the xanthophylls and zeaxanthin. Regulation of the concentrations of antioxidants and antioxidant enzymes, necessarily in a coordinated manner constitutes an important mechanism for avoiding oxidative stress. Under non-stress environmental conditions, ROS can modulate usual events such as DNA
synthesis, enzyme activation, selective gene expression and regulation of the cell cycle, hence the individual control of cellular differentiation, growth, development, and death (Franck et al. 2000) (figure 5).

Figure 5. The upper section shows the ROS produced constitutively in the cell, and the natural antioxidants and enzymes used to minimize their toxic effect. The lower section gives selected examples of the harmful effects of ROS when the pro- and anti-oxidant balance is perturbed in oxidative stress (Cassells and Curry 2001).
Stresses exert evolutionary pressures on all organisms, which have developed sophisticated responses to cope and survive. These responses involve cellular physiology, gene regulation and genome remodeling. Like biotic stress, abiotic stress can lead to a host of genetically programmed responses resulting, if successful, in stress avoidance or stress tolerance.

Figure 6. Molecular mechanisms for salinity tolerance.

In a few cases transposon activation in response to abiotic stress has been reported. The best studied example is the transpositional activity of Tam elements (transposable element of *Antirrhinum majus*), a temperature-sensitive class II transposon from snapdragon. Coen *et al.* (1986) characterized three snapdragon elements, Tam1, Tam2 and Tam3. All of these elements were first isolated in flower colour mutants. Three mutant alleles of the Nivea gene, encoding a chalcone synthase, were isolated (Coen *et al.* 1986, Almeida *et al.* 1989) and one mutant allele of the Pallida gene, encoding an enzyme involved in the cyanidine pathway resulting in red flowers. Each of these mutant alleles contained Tam element insertions in either their promoter sequences (Tam1 and Tam3) or in their coding sequence (Tam2). These transposon-induced mutations are unstable and lead to flower colour variegation. Interestingly, it was
shown that the rate of excision was greatly dependent on lower than normal temperatures (15 °C) resulting in 1000-fold higher transposition than in higher temperatures (25 °C). Of the three Tam elements only Tam3 shows this kind of temperature sensitivity but until today the molecular mechanism underlying this phenomenon has remained unclear. Kitamura et al. (2001) showed that low-temperature-induced Tam3 activation was also dependent on the position of the Tam3 copy in the genome, while silencing of transposition at higher temperatures appeared to occur simultaneously in all sampled loci. The position effect might be a function of binding affinity of the transposase to the TIRs of the element (Hashida et al. 2003). Hashida et al. (2003) sampled the methylation state of snapdragon DNA extracted from plants grown at 15 °C or 25 °C and found higher temperatures to result in hypermethylation of DNA and lower temperatures to result in demethylation. Remarkably, the methylation state was reversible within one generation. Yamashita et al. (1999) analysed the 500-bp region surrounding the Tam3 elements and noticed several hairpin structures, which might be targeted by methylation. Hashida et al. (2003) suggested that the temperature sensitivity of transposition activity of Tam3 might be correlated with the methylation state via a temperature-sensitive DNA methyltransferase or other proteins, whose expression is temperature-sensitive associate with regions of the element recognized by a methyltransferase and block access to the DNA.

Jiang et al. (2003) reported the characterization of the first active MITE from rice. This element is a Tourist-like MITE and was named mPing (for miniature Ping). A second element, Pong, was also found to be active in rice cell cultures. Since active transposition appeared to be preferentially occurring in rice cultivars that had been adapted from their original tropical and subtropical locations for cultivation in cool climates, the authors speculated that this might be another example of temperature-induced genomic shock that might have helped the diversification of rice cultivars. Interestingly, one mPing transposed into a rice homologue of the flowering time gene CONSTANS (Jiang et al. 2003), exemplifying the effect transposition of stress-activated elements can have on the adaptation of a shocked genome to a change in the environmental conditions the organism is exposed to. In Medicago sativa cold-induced transcriptional activation of multiple copies of a retrotransposon was observed. Interestingly, the cold-induced response was not concomitant with DNA
demethylation (Ivashuta et al. 2002). Microclimatic changes were also implicated in retrotransposon activity of the barley BARE1 retrotransposon (Kalander et al. 2000). Kimura et al. (1999) reported transcriptional activation of a SINE RNA from silk worm to heat shock, cycloheximide treatment and viral infection.

A mysterious but fascinating case of abiotic stress-induced genome remodelling has been observed in flax. When certain varieties are exposed to varying environmental conditions they produce progeny that has different, but stably inherited characteristics (Cullis 1973). These derivative strains, called genotrophs, appear to have numerous changes in DNA structure (Oh and Cullis 2003). Abiotic stress can result not only in well-programmed physiological stress responses but also in genome-wide changes. Stress-induced genomic responses include transposon activation, transposition, and structural genome changes. Like other stress responses transposon-mediated alterations in transcriptional activity of affected genes might lead to avoidance or tolerance of the stress. Unlike many other stress responses, however, transpositional activation appears to be a reaction not directly targeting an evolutionarily developed physiological pathway but is a hit-or-miss approach to finding an appropriate way of handling an unusual challenge. A common response to stresses may be the relaxation of epigenetic regulation, leading to activation of suppressed sequences and secondary effects as regulatory systems attempt to re-establish genomic order (Madlung and Comai 2004).

A stress response, with changes in metabolism and development, is initiated when the plant recognizes a stress at the cellular level. Even if disintegrated cell walls can be indirect source of elicitors, intact, disrupted or damaged (plasma) membranes are sensors of environmental change. Phytohormones and second messengers (Ca\(^{2+}\) for instance) are the transducers of information from membranes to metabolism. Carbon balance is the master integrator of plant response (Nilsen and Orcutt 1996). Betwixt and between, some genes are expressed more strongly, whereas others are repressed. The protein products of stress-induced genes often accumulate in response to unfavorable conditions (figure 7). Although most studies have focused on transcriptional activation of gene expression, growing evidence suggests that the accumulation of gene products is also influenced by posttranscriptional regulatory mechanisms that increase the amounts of specific mRNAs, enhance translation,
stabilize proteins, alter protein activity, or some combinations of these (Bray et al. 2000). After stress recognition, the signal is communicated within cells and throughout the plants. All parameters that regulate cell growth must act through a small number of physical processes. These are cell wall rheology, water transport and solute transport. Auxins, gibberellins, abscisic acid (ABA), cytokinins and ethylene have all been implicated in influencing one or more of these processes (Tomos 1990).

It is important to notice that plant responses to different stresses may use common ways. It is also true that apparently different stresses such as drought, heat, cold, salt, etc may result in the same ultimate stress, water deficit. On the other hand, mostly depending on the plant genera or families, a diversity of responses to the same stress can exist because the same function can be fulfilled by different compounds. For example, starch and fructan may be equally effective as carbon storage compounds, while sucrose and polyols such as mannitol and sorbitol could be equally effective as carbon transport compound in the phloem.

**Figure 7.** Cell signaling mediators in metabolic and structural adaptation to stress. Membranes are the sensors; phytohormones and second messengers are the transducers; carbon balance is the master integrator; in between, some genes are expressed, others are repressed (Gaspar et al. 2002).
Components of the signaling pathways thus are mostly similar to those implicated in other signaling cascades in eukaryotes, and include reversible protein phosphorylation steps, calcium/calmodulin-regulated events, and the production of reactive oxygen species (Leon et al. 2001). The interaction between different factors can be another difficulty in the understanding of plant responses. For example, low air humidity creates an environment that enhances the potential for significant plant water loss. At the same time regulating water loss through stomatal closure causes a reduction in carbon dioxide (CO₂) diffusion that limits growth. High air humidity, on the other hand, promotes environmental conditions favourable for decrease development (Danneberger 2000). The different possible signaling pathways, as well as consequences of altered gene expression that alter biochemical reaction downstream of stress sensing, are discussed in several recent books and reviews (Basra 1994, Smirnoff 1995, Nilsen and Orcutt 1996, Asard et al. 1998, Lutts and Kinet 1998, Grover et al. 1998, Bray et al. 2000, Hasegawa et al. 2000, Wilkinson 2000, Zhu 2001, Gaspar et al. 2002, Mansour and Salama 2004). Several imaging techniques have been used to detect the early signs of stress by monitoring changes in water status, photosynthetic efficiency, accumulation of secondary metabolites or structural modifications. Thermography, reflectance detection and fluorescence imaging are currently the most highly evolved of these techniques (Cassells et al. 1999, Chaerle and Van Der Straeten 2000).

Rice (Oryza sativa L.), greengram (Vigna radiata L. var. Wilckzek) and mustard (Brassica compestris L.) are among the most important dietary crop plants growing in sub-tropical climates of Indian sub-continent and increasing salinity problem worldwide including present modern agricultural practices has put them to a serious degree of salinity stress problem and decreases the crop productivity. Moreover, this region Barak Valley, South Assam harbors more than 2,000 traditional cultivars of rice and is the most important cereal crop of this region. Though sodium chloride (NaCl) stress imposes water deficit, ion imbalance, affects various other plant processes, very little is known about the biochemical responses of Indian rice and other crop varieties. The present investigation aims at abridging the gap on the possible physiological and biochemical mechanism of NaCl-salinity injury in rice (sensitive and tolerant varieties), greengram and mustard plants.